Acclimation responses of gill ionocytes of red tilapia (*Oreochromis mossambicus* × *O. niloticus*) to water salinity and alkalinity

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Abstract

To understand the acclimation strategies of red tilapia to different environments, this study aimed to evaluate different responses of red tilapia (*O. mossambicus* × *O. niloticus*) to salinity (10-30‰), alkalinity (1-3 gL^{-1} $NaHCO_3$) and salinity and alkalinity (10/1-30/3 ‰/$gL^{-1}NaHCO_3$) environments. Localization, type, size, and numeration of gill ionocytes were investigated on the same specimens by scanning electron microscopy (SEM) and immunohistochemistry (IHC) with antibodies of Na^+/K^+-ATPase (NKA), $Na^+/K^+/2Cl^-$contransporter (NKCC), cystic fibrosis transmembrane conductance regulator (CFTR) and carbonic anhydrase (CA). Ionocytes were only located on filaments conducted by SEM. Four types of ionocytes namely pit, convex, concave and transitory types were determined morphologically by their apical openings of which concave and transitory type were not present in freshwater (FW) and saltwater (SW) fish (10). Both ionocytes size and number increased with elevated stress levels. In comparison to FW, density of ionotypes increased to about 4.75, 3.00 and 3.44 fold in SW (30), AW (3) and S&AW (30/3) respectively. Immunoreactive cells on gill filaments confirmed branchial distribution of ionocytes. Immunoreaction of NKA, NKCC and CA appeared in FW except for CFTR while they all appeared in SW (30), AW (3) and S&AW (30/3).

Keywords: Acclimation response, Ionocytes, Salinity, Alkalinity, Red tilapia.

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Introduction

Osmoregulation is one of the most vital functions to all euryhaline fish (Evans, 2008), which provide hyperosmo-regulatory and hypoosmo-regulatory abilities to keep osmolality of body fluids within a narrow physiological range in freshwater (FW) and seawater (SW) fish, respectively (Kang *et al.,* 2013). Osmoregulation is mainly achieved by the gills, kidney and intestine. Among these osmoregulatory organs, the gills are the unique to aquatic animals (Evans *et al*., 2005; Gilmour and Perry, 2009).

The transporting epithelia contains a subset of specialized cells called ionocytes (formally called mitochondria-rich cells or chloride cells; Keys and Willmer, 1932), the major cells in fish gills that transport ions actively (Perry *et al.,* 2003; Evans *et al*., 2005; Hwang, 2009), these ionocytes could secrete and absorb ions in SW and FW environments, respectively (Hirose *et al*., 2003; Hwang and Lee, 2007), in addition to carrying out acid-base regulation and other functions (Perry and Gilmour, 2006; Gilmour and Perry, 2009).

The basolateral membranes of ionocytes form extensive tubular systems (Karnaky *et al*., 1976; Sardet *et al*., 1978), whereas the primary site of ion transportation between ionocytes and the external environment is the apical membrane (Christensen *et al*., 2012). On the apical and basolateral membranes, ionocytes possess a specific complement of transporters or channels that allow for directional movement of ions (Dymowska *et al*., 2012). The apical surface morphology of the gill ionocytes are usually found to vary across salinity environments (Uliano *et al*., 2010). The cellular, biochemical and molecular mechanism of fish ionocytes in acclimation to salinity have been well developed (Hwang *et al*., 2011). Meanwhile, in some inland waters, alkalinity is another form of environmental factors that fish always encounter, which also has adverse effects on fish survival and growth (Fielder *et al*., 2001). However, the physiological mechanism of fish in adaption to water alkalinity or salinity & alkalinity is less understood.

Tilapia is a group of euryhaline fish and has been often served as model for ionoregualtion studies. Work on tilapia has been mostly performed on Mozambique tilapia *O. mossambicus*, and different types of ionocytes gills have been revealed in tilapia based on their apical openings (Lee *et al.,* 1996; Chang *et al.,* 2001; Inokuchi *et al.,* 2009, Choi *et al.,* 2011). Also, there is an increase in size and number of ionocytes in response to increasing salinity (Ayson *et al.,* 1994; Kültz *et al.,* 1995; Van Der Heijden *et al.,* 1997; Hiroi *et al.,* 2005). Due to their specific abilities to grow and reproduce in hypersaline environments, the black-chin tilapia *Sarotherodon melanothern* was used as a model for hypersaline adaption studies (Lorin-Nebel, 2012). The small cichlid fish, *O. alcalicus graham* are found in hot, alkaline lagoons surrounding Lake Magadi in Kenya (Walsh *et al.,* 2001). An early work on morphology of ultra-structure of the chloride cells and

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their modification on extreme alkalinity revealed that the ionocytes decreased in number and size in lower alkalinity environments (Maina, 1990).

Red tilapia was a cross hybrid of a mutant reddish-orange female *O. mossambicus* and a normal male *O. niloticus* (Galman, 1983). It has been developed as a popular commercial cultured species in Southeast Asia, as well as the Middle East, the Caribbean, Central and South America (Watanabe *et al*., 2002). Since their fast growth and high tolerance, they are of great potential to be developed and cultured in broad environments. The purpose of this study was therefore to investigate the localization, morphological and numeration change of gill ionocytes of Red tilapia in response to different environmental treatments, including salinity, alkalinity, and salinity & alkalinity by scanning electron microscopy (SEM). In the meantime, immunohistochemistry (IHC) of some important ion transporters were also carried out to verify the external observation. Taken together, these data would contribute to better understanding of osmoregulation strategies of Red tilapia gill ionocytes to these different environments.

Materials and methods

Experimental fish

Red tilapia (*O. mossambicus* × *O. niloticus*) fish, averaged 5.5± 0.3 cm in total length, were obtained from Fish Germplasm Station, Shanghai Ocean University, taken to laboratory and maintained in recirculated aquariums with freshwater for two weeks adjustment. Salt waters (SW) were prepared using local fresh water with sodium chloride (NaCl). Alkaline waters (AW) were prepared using local fresh water with sodium bicarbonate ($NaHCO_3$). Both NaCl and $NaHCO_3$ were used in preparing salt & alkaline waters (S&AW). Three treatment levels were also designed for each kind of environment: (1) SW: 10‰, 20‰, and 30 ‰ salinity, (2) AW: 1 gL^{-1}, 2 gL^{-1}, and 3 gL^{-1} $NaHCO_3$, (3): S&AW: 10/1, 20/2, and 30/3 ‰/gL^{-1} $NaHCO_3$, using Mettler Toledo (SG7-ELK, USA) for the concentration of salinity and Hanna instruments (HI755, USA) for the concentration of alkalinity.

All fish were gradually acclimated from freshwater to 5, 10, 15, 20, 25, 30 (‰) salt waters, 1, 2, 3 (gL^{-1} $NaHCO_3$) alkaline waters, and 5/1, 10/1, 15/2, 20/2, 25/3, 30/3 (‰ /gL^{-1} $NaHCO_3$) salt & alkaline waters, and stayed at each treatment for 3 days before next transfer. During the experiment, fish were fed two times per day and water temperature was maintained at 25-27 °C, half of the water was changed every three days to preserve optimal water quality. After having acclimated to different treatments for one week, 10 fish were randomly sampled in each treatment level, the outmost pair of gills were removed for scanning electron microscopy analysis. In the meantime, 5 individuals at FW, SW (30), AW (3), and S&AW (30/3) treatments were also sampled for immunohistochemistry analysis.

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Scanning electron microscopy (SEM)

The gills were fixed in a fixative, which consisted of 5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2), at 4°C for 12 hours. Fixed gills were rinsed with 0.1 M PB for 15 min three times, and then dehydrated in a series of ascending concentrations of ethanol from 30%. The samples were dried at room temperature for 1 hour, and then mounted on specimen stubs, and coated with platinum-palladium in an ion sputter (Hitachi E-1010, Tokyo, Japan). Ionocytes were examined and photographed with a Hitachi S-3400N Scanning Electron Microscopy (Tokyo, Japan). For each individual, ionocytes were counted and measured within 10 continuous filaments by image analysis software (NIS-Elements F program).

Antibody

Primary antibodies include: (1) a mouse monoclonal antibody (α5; Developmental Studies Hybridoma Bank), raised against the α-subunit of the avian Na^+/K^+-ATPase (NKA); (2) a mouse monoclonal antiserum (T4; Developmental Studies Hybridoma Bank) raised against the C-terminus of human $Na^+/K^+/2Cl^-$ contransporter (NKCC); (3) a mouse monoclonal antibody (R&D Systems, Boston, MA, USA) directed against 104 amino acids at the C-terminus of the human cystic fibrosis transmembrane conductance regulator (CFTR); (4) a rabbit polyclonal antibody against a synthetic peptide corresponding to the N-terminal end of human Carbonic anhydrase 1 (CA; Boster company, China). Samples were stained with Cell and Tissue Staining Kit (Mouse kit; HRP-DAB system, R&D Systems®, Boston, MA, USA).

Immunohistochemistry (IHC)

The gills were fixed in 4% paraformaldehyde for 12-16 hours, and then washed in 0.01 M phosphate-buffered saline (PBS) twice for 40 min each. The samples were dehydrated through a graded ethanol series, infiltrated with xylene, and embedded in paraffin. Cross sections of the gills were provided at 8 μm thickness and mounted on Poly-D-lysine and 3-Aminopropyl-Triethoxysilane (APES) coated glass slides. The deparaffinized sections were incubated with xylene and rehydrated sections were incubated with a graded ethanol series and dH_2O, respectively. The antigen retrieval (AR) technique, which is predominantly based on high-temperature heating of tissues, is used as a non-enzymatic pretreatment for immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections for 5 min at 100°C and then the sides were cooled at room temperature for 30 min and washed in PBS for 5 min twice. They were immunohistochemically stained with primary antibody, a monoclonal antibody (α5) to Na^+/K^+-ATPase α-subunit and Cell & Tissue Staining Kit (Mouse kit; HRP-DAB system, R&D Systems®, Boston, MA, USA).

Statistical analysis

Data were presented as means ± standard error of the mean. The significance of

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differences at $p<0.05$ was examined using one-way analysis of variance (ANOVA), followed by Tukey–Kramer *post-hoc* test.

Results

Scanning electron microscopy

Red tilapia has four gill arches at each side. Each gill arch supports one set of paired gill filaments. Each gill filament in turn supports numerous secondary lamellae, which extend out from both sides of the filament body (Fig 1a.). Under observation in scanning electron microscopy, each ionocytes were characterized with an apparent apical opening, and they were only located in the gill filaments of FW, SW, AW and S&AW acclimated fishes (Fig 1b.)

Four types of ionocytes with different apical openings were observed during the acclimation experiments: pit, concave, convex and a transitory apical surface (Fig 2.), these apical structures also showed some variation within the same types. The apical opening of pit type was ellipse-shaped with a narrow and deep hole; the internal structure could not be observed (Fig 2a.). The concave type was somewhat ellipse, its surfaces was slightly dented or flat with a mesh-like structure (Fig 2b.). The convex type was a rough surface, curved or bowed outward like the outside of a bowl or sphere or circle (Fig 2c.). The transitory apical surface was somewhat similar to both concave surface and enlarged pit and its opening was more deeply dented and appeared as a large crater (Fig 2d.). The convex and transitory types were not observed in FW and SW (10) treatments, however, these four types were presented in SW (20), SW (30) treatments and all AW and S&AW treatments (Fig 3.).

The mean size (area) of different ionocytes in acclimation to SW, AW and S&AW are shown in Table 1 and Fig 3. The size of pit type averaged 1.14±1 μm^2 in FW and increased about 11.70 fold in SW, whereas it was relatively constant in AW and S&AW. The mean size of concave type increased significantly about 5.74, 8.39, and 6.11 fold in SW, AW and S&AW, respectively, with an increasing trend. The convex and transitory types were absent in FW and SW (10), convex type increased about 1.84 fold from SW (20) to SW(30), 3.29 fold from AW (1) to AW (3), and 3.62 fold from S&AW (10/1) to S&AW (30/3), the transitory type increased about 2.85 fold from SW (20) to (30), 3.94 fold from AW (1) to AW (3), and 4.53 fold from S&AW (10/1) to S&AW (30/3). In comparison to FW, the mean size of ionotypes increased to about 13.66, 14.19, and 14.07 fold in SW (30), AW (3) and S&AW (30/3), respectively.

The mean densities (number) of different ionocytes in acclimation to SW, AW and S&AW treatments are shown in Table 2 and Fig 4. In FW fish, the pit type was dominant with mean density of 5.54×10^3 cells/mm^2, and the concave type accounted for 0.7×10^3 cell/mm^2. In acclimation to SW, the mean density of ionocytes increased significantly and became about 4.75-fold in SW (30) (pit type 37%, concave type 23%, convex type 19%, and transitory type 21%).

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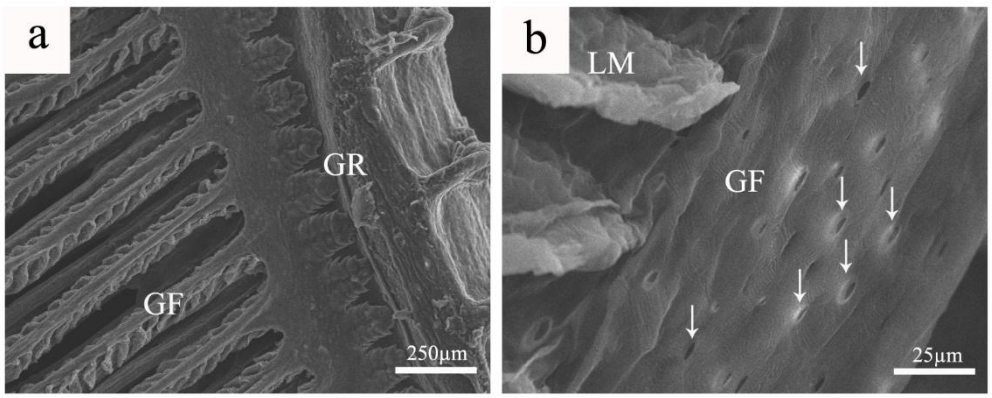

Figure 1: Structure of the gill of red tilapia (A) and location of ionocytes (B) identified by scanning electron microscopy. Gill arch (GR), gill filaments (GF), lamella (LM), ionocytes were showed by arrow.

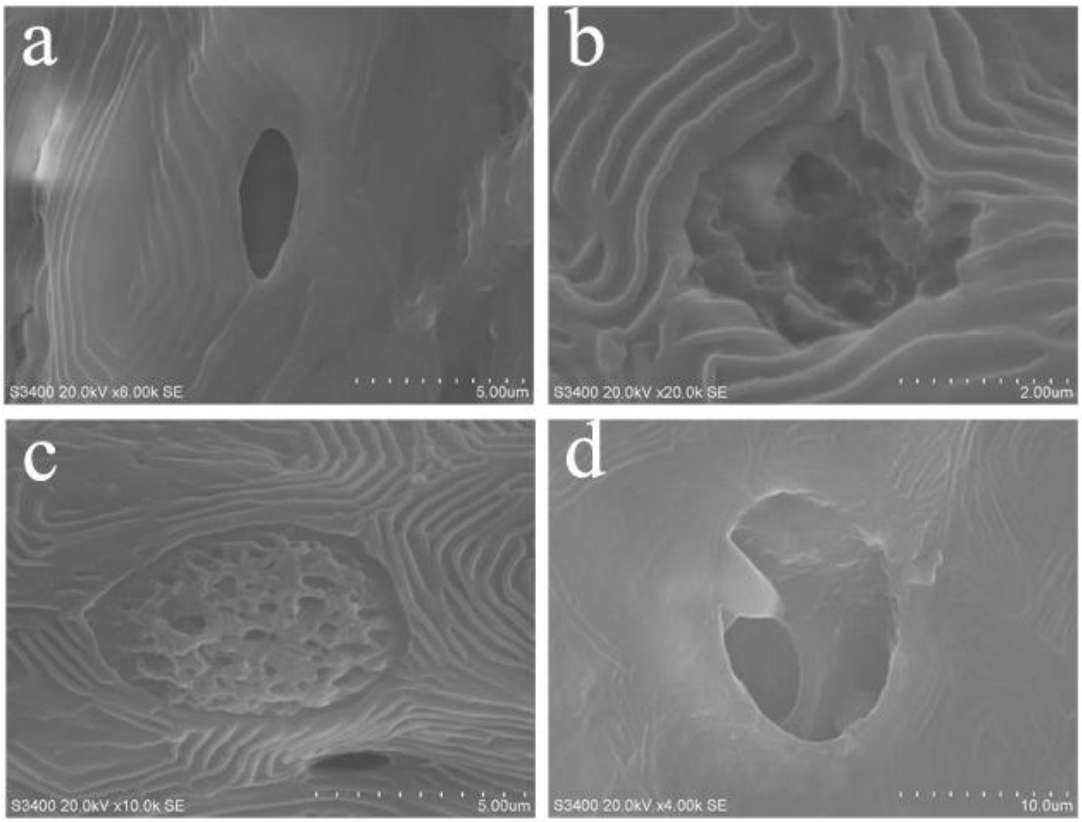

Figure 2: Four types of ionocytes in gill filaments of red tilapia identified by scanning electron microscopy (SEM). (A) pit, (B) concave, (C) convex, (D) transitory.

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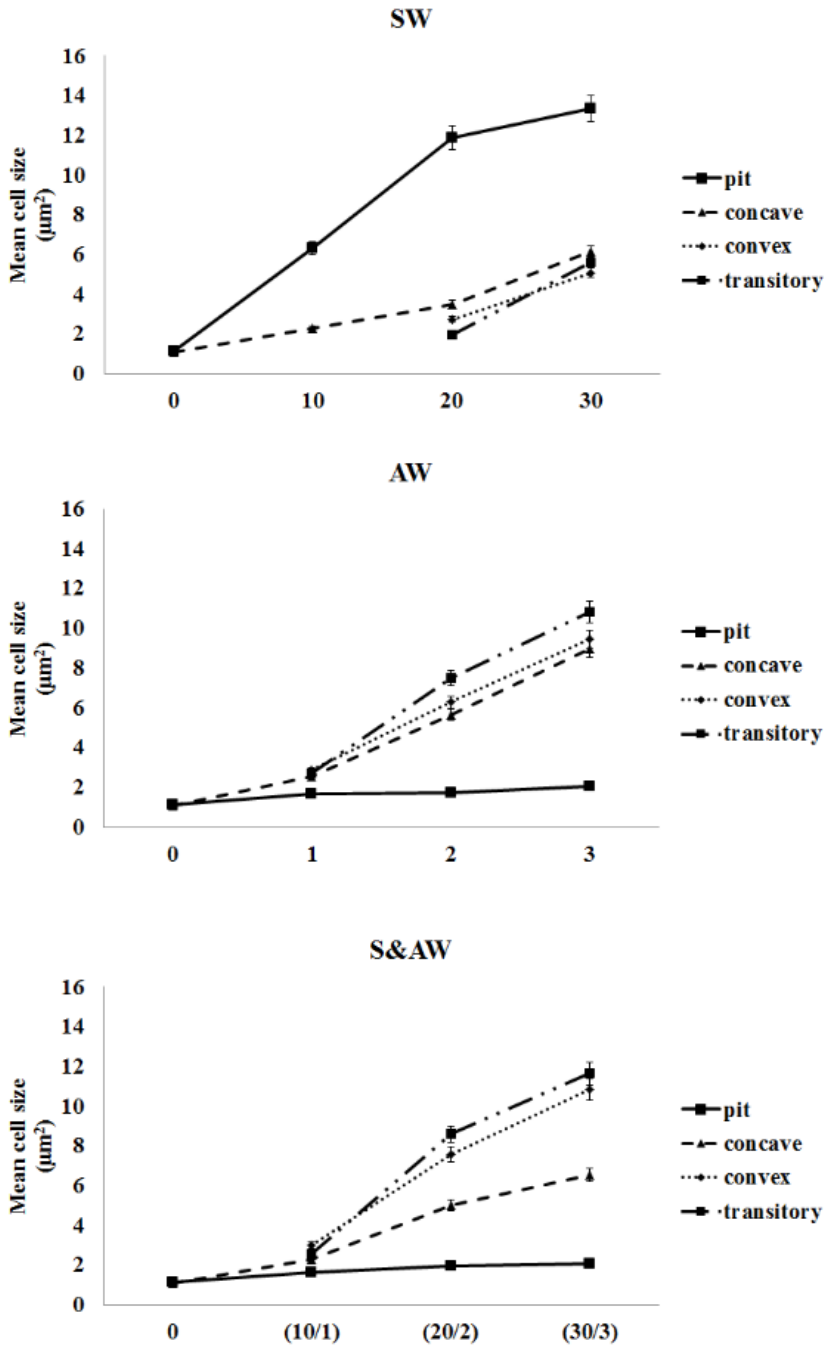

Figure 3: Change of the mean size of four types of gill ionocytes of red tilapia in acclimation to freshwater (FW), salinity water (SW), alkalinity water (AW), salinity & alkalinity water (S&AW).

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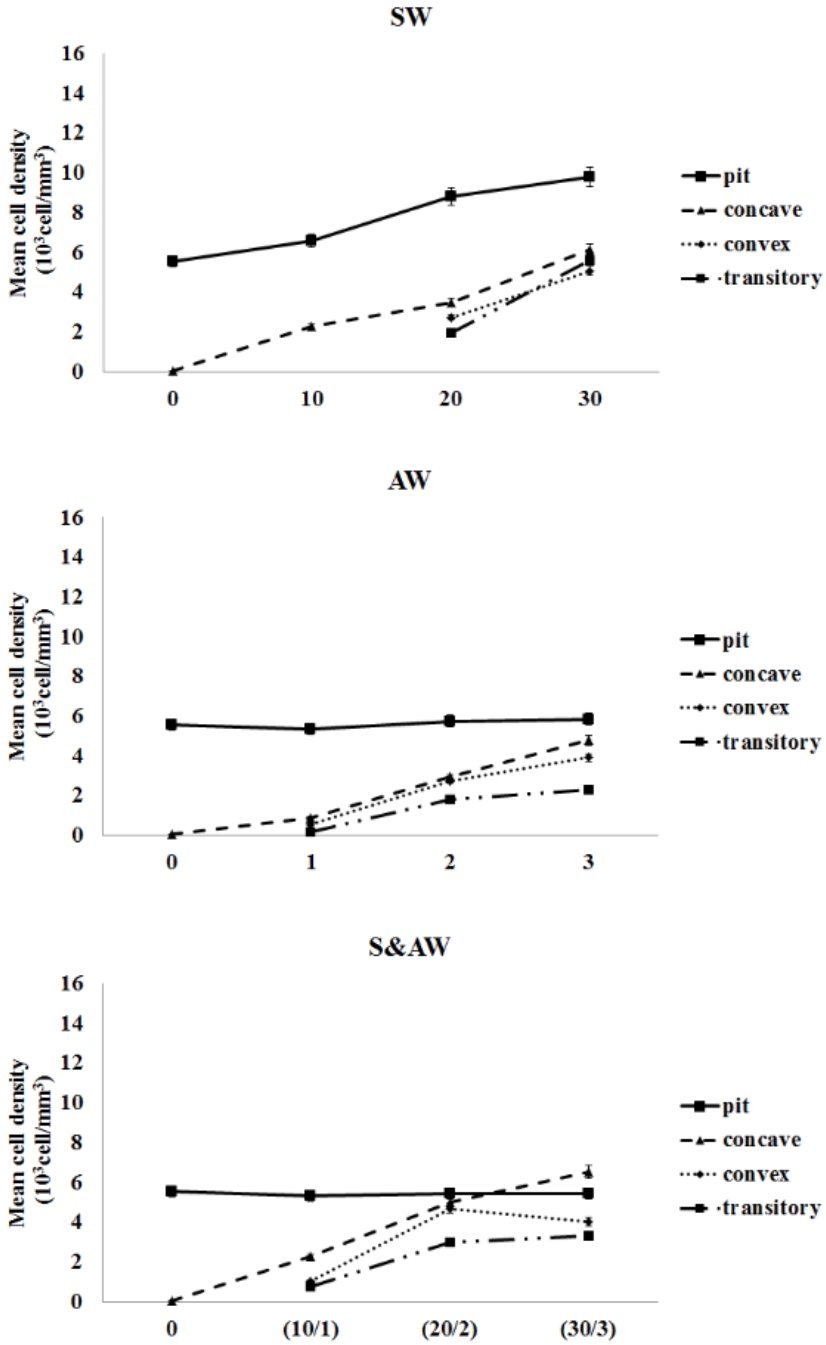

Figure 4: Change of the mean density of four types of gill ionocytes of red tilapia in acclimation to freshwater (FW), salinity water (SW), alkalinity water (AW), salinity & alkalinity water (S&AW).

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Table 1: The mean size of four types of gill ionocytes of red tilapia in different environmental treatments (μm^2).

Type	FW	SW(‰)			AW(gL^{-1})			S&AW(‰/gL^{-1})		
	0	10	20	30	1	2	3	10/1	20/2	30/3
pit	1.14±1	6.34±0.3	11.88±1.2	13.34±0.3	1.6±1.3	1.72±0.4	2.07±0.33	1.64±0.44	1.97±1	2.06±0.4
concave	1.07±0.8	2.28±2	3.51±1	6.15±0.55	2.55±0.78	5.67±1	8.98±0.3	2.3±0.5	5.02±0.3	6.54±0.5
convex			2.75±2.3	5.08±0.75	2.87±2.1	6.3±2	9.46±0.5	2.99±1	7.58±0.8	10.85±1
transitory			1.97±0.8	5.62±23	2.75±0.3	7.543±0.8	10.85±1	2.57±1	8.59±1	11.65±2

Table 2: The mean density of four types of ionocytes of Red tilapia in o different environmental treatments (10^3 cell /mm^2)

Type	FW	SW(‰)			AW(gL^{-1})			S&AW(‰/gL^{-1})		
	0	10	20	30	1	2	3	10/1	20/2	30/3
pit	5.54±0.23	6.59±0.7	8.83±0.12	9.8±0.04	5.34±0.15	5.75±0.77	5.85±0.76	5.34±0.09	5.44±0.93	5.45±0.04
concave	0.07±0.02	2.28±0.39	3.51±0.44	6.15±0.08	0.89±0.59	2.93±0.89	4.78±0.33	2.3±0.94	5.02±0.5	6.54±0.35
convex			2.75±0.05	5.08±0.3	0.54±09	2.74±0.45	3.9±0.44	1.04±0.16	4.7±0.2	4.04±0.78
transitory			1.97±0.32	5.62±0.49	0.18±28	1.8±0.22	2.3±0.02	0.76±0.65	2.99±0.66	3.3±0.03

The mean density of ionocytes increased about 3.00-fold in AW (3) (pit type 35%, concave type 28%, convex type 23%, and transitory type 14%), and about 3.44-fold in S&AW (30/3) (pit type 28%, concave type 34%, convex type 21%, and transitory type 17%). In comparison to FW, the densities of ionotypes increased to about 4.75, 3.00, and 3.44 fold in SW (30), AW (3) and S&AW (30/3), respectively.

Immunohistochemistry (IHC)

Whole-mount immunohistochemistry with anti-NKA, anti-NKCC, anti-CFTR, and anti-CA of gill filaments were investigated in FW, SW (30), AW (3), and S&AW (30/3) treatments. In comparison with the control (without the first antibody), immunoreactions of NKA, NKCC, CFTR, and CA were found only at the inter-lamellar region of the gill filaments (Fig 5.). In FW treatment, immunoreactions of NKA, NKCC and CA appeared, while CFTR disappeared. In SW (30), AW (3) and S&AW (30/3) treatments, immunoreactions of NKA, NKCC, CA and CFTR were spotted.

Discussion

Distribution of ionocytes in FW, SW, AW and S&AW

The present study explored the chronic responses of gill ionocytes of Red tilapia in acclimation to salinity, alkalinity and salinity & alkalinity in waters, the ionocytes were only located on the filaments in FW, SW, AW and S&AW adapted fishes under SEM observation, which was also confirmed by immuoreaction detection in IHC.

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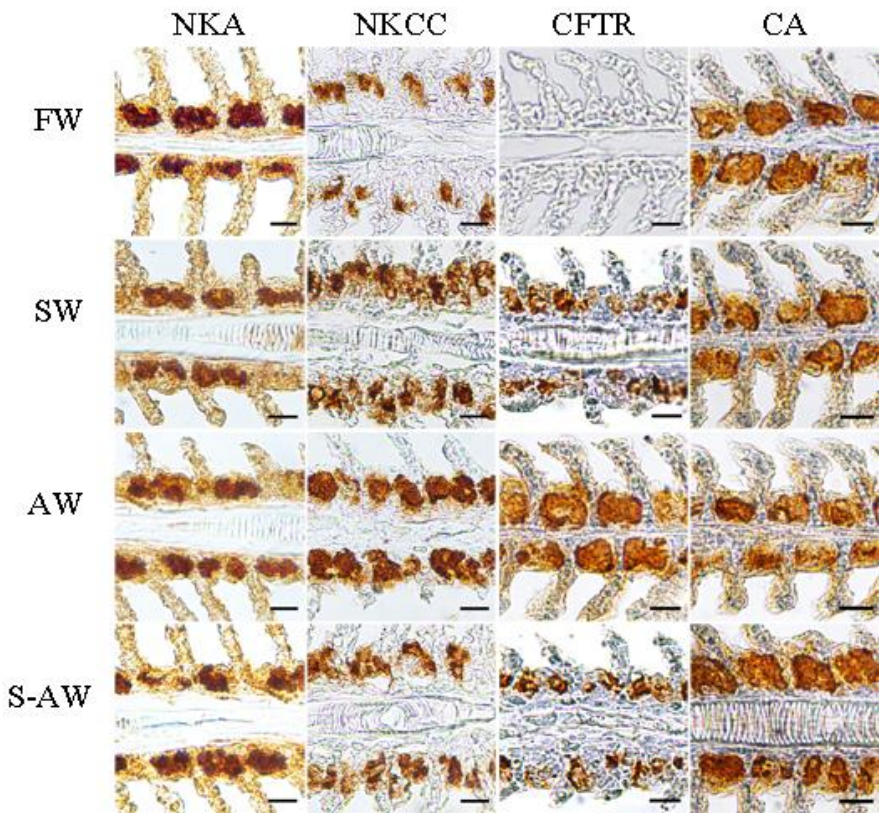

Figure 5: Whole-mount immunohistochemistry with anti-Na^+/K^+-ATPase (NKA), anti-$Na^+/K^+/2Cl^-$ cotransporter (NKCC), anti- cystic fibrosis transmembrane conductance regulator (CFTR), and anti- carbonic anhydrase (CA) of gill filaments of Red tilapia in freshwater (FW), salt water (SW 30), alkaline water (AW 3) and salinity & alkalinity in water (S&AW 30/3) Scale bar, 10µm.

This observation was similar to that described for Mozambique tilapia (*O. mossambicus*), where ionocytes in SW had always been located in the filaments (Kültz *et al*., 1995; Lin *et al.,* 2004a; Sardella *et al.,* 2004; Inokuchi *et al*., 2008; Choi *et al*., 2011). In contrast, the ionocytes of the black-chin tilapia (*Sarotherodon melanotheron*) were localized on the filaments and expanded extensively to the lamellae in acclimatizing to seawater or hyper saline waters (Ouattaraa *et al*., 2009).

Different patterns of ionocytes in the gills have been revealed in fish: (1) ionocytes were found on filaments and lamellae in freshwater and seawater (Zydlewski and McCormick, 2001; Lin *et al.,* 2006; Christensen *et al.,* 2012), (2) ionocytes were distributed on both filaments and lamellae in freshwater, where lamellae ionocytes disappeared following seawater transfer (Varsamos *et al*., 2002; Nebel *et al.,* 2005; Hiroi and McCormick, 2007), (3) ionocytes were found only in the filament and rarely in the lamellae in both freshwater and seawater (Uchida *et al.,* 2000; Lin *et al*., 2004b; Yang *et al*., 2009). The function of gill lamellae is to improve the gill function. Thin plates of tissue on gill filaments that contain the capillary across

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which gases are exchanged, increase the total area of the gills (Ito *et al*., 2005; Jeffery *et al*., 2007). The different distributional patterns of ionocytes might reflect a more suitable mechanism for different euryhaline teleosts to cope with environmental fluctuations, or an adapted mechanism acquired during the teleosts evolution.

Response of types, size and densities of ionocytes to SW, AW and S&AW

Different types of ionotypes have been described in tilapia fish encountering various environments, and involved in different ionic transportation processes. Pisam *et al*. (1995) firstly reported that there were two types of MR cell (α and β cells) in the gill epithelium of freshwater-adapted tilapia, the apical surface of α cells were slightly depressed to form shallow apical cavities while the apical surface of β cells were flattened and hardly depressed. During seawater acclimation, α cells were transformed into seawater-type MR cells, whereas β cells degenerated and disappeared. Later, three types of MR cells, wavy convex, shallow basin and deep holes, were identified by scanning electron micrographs, wavy-convex and shallow-basined MR cells were evident in FW adapted fish, whereas the deep hole type was dominant in SW (5) -adapted fish (Lee *et al*., 2000), and SW (30)-adapted fish (Lee *et al*., 1996). Recently, Choi *et al.*, (2011) proposed that the ionocytes could be classified into four types: an apical pit, a convex apical surface, a concave apical surface and a transitory apical surface. In fish acclimated to freshwater, three types of apical opening were observed, the pit, the convex and the concave. Following transfer to 70% seawater, the convex was absent completely; and the transitory appeared (Choi *et al*., 2011). Based on the newly classification method, our SEM observations also revealed there were four types of ionocytes with different apical openings in red tilapia in acclimation to SW, AW, and S&AW, pit, a convex, concave, and transitory type. Only the pit type and concave type were observed in FW and low SW (10) treatments. Since SW (10) is about the iso-osmotic for many tilapias, these two type ionocytes might be enough to maintain homeostasis at hypo- and iso-osmotic environments, where there was no or little load for ionic excretion. In response to higher salinities (20 and 30), alkalinities, salinity and alkalinities treatments, all four kinds of ionocytes emerged. Therefore, the convex type and transitory type were of great necessity to Red tilapia in adaption to (higher) salinity, alkalinity and salinity-alkalinity in waters to cope effectively with higher load of ionic excretion.

The size change of different ionocytes types in acclimation to SW, AW and S&AW showed different responses. In SW, the mean size of pit type increased greatly than that of the concave, convex, and transitory type with increasing salinity. On the contrary, the mean size of the concave, convex, and transitory type increased highly than that of the pit type in AW and S&AW, respectively. Moreover, the size of convex and transitory types increased greatly than that of concave type in

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S&AW (Fig 3). In acclimation to different environments, enlarged ionocytes could be a fast and effective way to improve their transportation capacities and responsible for more ionic transportation.

The ionocytes number of red tilapia also changed highly in acclimation to SW, AW, and S&AW, the density of ionocytes in SW (30), AW (3), and S&AW (30/3) treatments was about 4.75, 3.00, and 3.44 fold in comparison to that in FW, respectively, which was consistent with some previous work, supporting their significance of ionocytes number in hyperosmotic conditions in the cichlid fish **(**Mozambique tilapia *O. mossambicus*, Pratapa and Wendelaar, 1993; Hiroi *et al.*, 2005; Inokuchi *et al.*, 2008; Choi *et al.*, 2011; *Sarotherodon melanotheron*, Ouattara *et al.*, 2009). The elevated number of ionocytes was probably a higher and long-termed response, which is a result of the proliferation and differentiation of newly recruited ionocytes. Increase of ionocytes number also improved transportation capacities. For comparison of different environments, the density in AW (3) was smaller than that in SW (30), which probably due to less stress strength. An interesting finding was the density in salinity and alkalinity treatment (30/3) was not, but less than the sum of the single salinity (30) and single alkalinity (3). There might be some interaction among different ions in salt and alkaline water, that decrease the total stress strength, or ionocytes might use the same transports or exchangers simultaneously to regulate both salinity and alkalinity.

The different functions of inocytes

The ionocytes were first identified as possessing chloride secreting function (Keys and Willmer, 1932), currently, ionocytes have been known to be involved in multiple functions (Marshall, 2003; Evans *et al*., 2005; Wang *et al*., 2009; Hwang *et al*., 2011). Thus, they were also functionally classified by immunoreactive of various transport proteins (Hiroi *et al*., 2005; Ouattara *et al.*, 2009).

IHC revealed that immunoreaction of NKA, NKCC, CFTR, and CA were found only at the inter-lamellar region of the gill filaments of red tilapia in FW, SW, AW, S&AW, except for CFTR which was not detected in FW. NKA was sodium pump and responsible for the active Na^+ transportation, it also provided a driving force for other transporting systems (Tang *et al*., 2012). NKCC was a membrane transport protein that contransported Na^+, K^+, and Cl^- into or out of cells (Haas, 1994). CFTR functioned as a cAMP-activated ATP-gated anion channel (e.g. Cl^-) (David *et al*., 2006). CA catalyzed the rapid dehydration of HCO_3^- or hydration of CO_2 ($HCO_3^- + H^+ \leftrightarrow CO_2 + H_2O$) in acid-base regulation (Badger and Price, 1994). In contrast to SW, AW, and S&AW, the absence of CFTR in FW tilapia suggested there was no or little anions transportation in FW for Red tilapia. In different ionic transportation conditions in SW, AW, and S&AW, various transporters and exchangers were located in different kinds of ionocytes needed for effective transportation, as a result, NKA, NKCC, CFTR, and CA all present in SW,

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AW, and S&AW without obvious difference. Since the functions of different types of ionocytes are still uncertain, we could not clearly explain which specific transporters in each ionocytes types and what specific transportation functions involved in osmoregulation. Therefore, more ionic transporters/channels and integrated work should be implemented to better understand the molecular mechanism of fish in acclimation to different environments.

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