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The ichthyotoxic alga *Chattonella marina* induces Na^+, K^+-ATPase, and CFTR proteins expression in fish gill chloride cells *in vivo*

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Abstract

Our previous studies demonstrated that the ichthyotoxic *Chattonella marina* stimulated proliferation of branchial chloride cell (CC) and induced osmotic distress akin to hyperactive elimination of ions in fish (*Rhabdosargus sarba*). To ascertain the *in vivo* effects of *C. marina* on key CC ion transporters, the localization and expression of Na^+, K^+-ATPase (NKA) and cystic fibrosis transmembrane conductance regulator (CFTR) proteins in response to *C. marina* exposure were investigated, using a quantitative immunocytochemical approach. The polarized distributions of NKA (α subunit) and CFTR proteins in branchial CCs of *R. sarba* remained unchanged under *C. marina* exposure. However, significant inductions of these two ion-transporters were detected in CCs of fish after 6 h exposure. By real-time PCR, no significant changes in gill NKA and CFTR mRNA expressions were detected, suggesting a post-transcriptional pathway is likely involved in regulating the ion transporters abundance. This study is the first to demonstrate the *in vivo* effects of harmful algal toxin on NKA and CFTR protein expressions in gill transepithelial cells. Taken together, an augmentation of branchial CCs together with hyper-stimulation of NKA and CFTR in CCs attribute to the rapid development of osmotic distress in *C. marina* susceptible fish.

Keywords: Na^+, K^+-ATPase; Cystic fibrosis transmembrane conductance regulator (CFTR); Gill chloride cells; Marine fish; Osmoregulation; Harmful algal bloom (HAB); *In vivo*

Chattonella marina, a notorious harmful algal bloom (HAB) species, has caused massive fish kills and serious economic losses worldwide. However, the underlying ichthyotoxic actions remain unresolved (see review of Landsberg [1]). Our previous studies showed that *C. marina* was able to cause severe osmoregulatory distress in the marine teleost goldlined seabream (*Rhabdosargus sarba*) [2,3]. The distressed fish showed a significant decline of blood osmolality shortly after exposure to *C. marina* and upon moribund (ca. 70%). Interestingly, concomitant increases of branchial chloride cell (CC) density, CC apical fractional area, volume densities of CC, and mitochondria within CC were also observed [2,3]. Despite we demonstrated that *C. marina* was able to induce ultrastructural changes of branchial CC and osmotic impairment akin to hyper-excretion of ions in the goldlined seabream, the harmful effects of *C. marina* on key transporters in CC for ion regulation remain virtually unknown.

Marine teleosts are hypotonic to the seawater environment, extra body ions (Na^+ and Cl^-) are mainly eliminated through chloride cells of gill epithelia [4–6]. The current proposed model for marine teleost chloride cell function is based on the findings of seawater euryhaline teleosts, which involves the basolateral NKA enzyme, that provides a primary driving force to establish transmembrane Na^+ gradient to facilitate the subsequent transport of Cl^- into

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CCs via the Na^+, K^+, 2Cl co-transporters. The intracellular Cl^- ions are then excreted via the apical anion channel homologous to CFTR (see reviews by Evans et al. [7]; Wilson and Laurent [8]; Marshall [9]; McCormick et al. [10]). Induction of CFTR mRNA expression and immunoreactivities were evident in gill CCs of varied euryhaline species under seawater adaptation e.g., the Atlantic salmon, killifish, mudskipper and Hawaiian goby [10–15]. However, CFTR has not been demonstrated in CCs of marine teleosts that are not truly euryhaline.

In *R. sarba*, induction of gill NKA α-, β-subunit transcripts proteins and activity suggest an activation of Na^+ excretion in gill CCs [13,17]. Earlier *in vitro* studies using the cyanobacterium (*Microcystis aeruginosa*) and the dinoflagellate (*Gymnodinium breve*) showed that the harmful algae could inhibit NKA activities in cultured gill cells of rainbow trout [18] and sea bass [19], respectively. Thus far, there is no information available on the *in vivo* effects of harmful alga on NKA and CFTR, and no attempts have been made to link abnormal expressions of these key ion-transporters to osmotic impairment in *C. marina* susceptible fish. The present study investigates the distribution of NKA and CFTR in branchial CCs of *R. sarba* (using quantitative immunocytochemistry), and measures concomitant changes of these CC ion transporters (protein and mRNA levels) in response to *C. marina* exposure. Such information is essential to ascertain the *in vivo* effect of *C. marina* on osmoregulation of marine teleost.

Materials and methods

Fish maintenance. Goldlined seabream (*Rhabdosargus sarba* = *Sparus sarba*) [16] (fork length: 20 ± 2 cm), purchased from a local fish farm, were acclimated in the laboratory with running seawater for at least 7 days prior to experiments. Dissolved oxygen (5 ± 0.5 mg/L), temperature (22 ± 1 °C), salinity (31 ± 1‰) and pH (8 ± 0.2) were monitored and maintained in all tanks. Gentle aeration was provided in all aquaria.

Algal exposure experiment. Gill tissues in this study were sampled from the same fish used for gill ultrastructural and blood osmolality studies in Tang and Au [2], and details on the preparation of *C. marina* culture (2000 cells/ml) and the system for the algal exposure experiment were described. A non-toxic algal control, *Dunaliella tertiolecta* (American Type Culture Collection No. 30929) at the same biomass density was also used in parallel for the exposure studies to ensure that any noticeable cytological effects were not solely due to direct physical interactions caused by algal cells. A total of 18 fish (6 replicate tanks × 3 fish/tank) were used for each treatment. Five distressed alive fish were sampled from the *C. marina* treatment (2000 cells/ml density) at 6 h post-exposure (median lethal time, LT_{50}). At the same time point, five fish were sampled from the *D. tertiolecta* exposed group.

Fish were first anesthetized in aerated (0.1 g/L) tricaine methanesulfonate (MS-222) for about 45 s and the gill tissues were immediately removed. The second gill arch from the left side of each anesthetized fish was excised and was cut into 2–3 mm length pieces in fish saline (saccharose 250 mM, heparin 20 U/ml, NaCl 12.4 mM, and Hepes 5 mM, pH 7.6) [20]. The cut gill pieces were immediately fixed in 0.1 M cacodylate buffer (pH 7.6) containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.05% $CaCl_2$ and kept overnight at 4 °C for immunocytochemistry. For mRNA expression and Western blot analyses, gills were scraped to remove bony tissues and quick-frozen in liquid nitrogen and stored at −80 °C prior to the extraction and analyses.

Antibodies. Gill NKA was immunolocalized using a monoclonal antibody specific for the α-subunit of chicken NKA [21] purchased from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242, USA, under contract NO1-HD-7-3263 from the National Institute of Child Health and Development (NICHD), USA. This antibody (α5) has been widely used for fish [12,22,23]

Cystic fibrosis transmembrane regulatory (CFTR). Two monoclonal antibodies for human CFTR were tested in this study for the localization of CFTR immunoreactivities in branchial CCs of goldlined seabream: (i) a monoclonal antibody against the full-length human CFTR (NeoMarkers Inc. CA, USA) employed by Wilson et al. [12] for the immunohistochemical localization of CFTR in gills of mudskippers, and (ii) a monoclonal antibody against 104 amino acids at the carboxyl terminus of the human CFTR (R&D Systems, Boston, MA, USA), used by McCormick et al. [10] to localize CFTR in CCs of seawater-adapted Hawaiian goby.

Western blot analysis. The gill scrapings were washed with 2–3 changes of cold PBS and then homogenized in 1:4 w/v of lysis buffer (250 mM Tris–HCl, pH 8, 1% NP-40, and 150 mM NaCl). The homogenates were pelleted (10,000*g*), supernatants were assayed for protein concentration (DC Protein Assay Kit II, Bio-Rad Pacific Ltd.) and finally mixed with LDS sample buffer that was then subjected to electrophoresis in NuPage 4–12% Bis-Tris gradient gel (Invitrogen). The gel was blotted onto a PVDF membrane. The membrane was then incubated with a blocking solution containing mouse anti-CFTR (clone 24-1, R&D Systems) or mouse anti-NKA (α5, Developmental Studies Hybridoma Bank) followed by HRP-conjugated goat anti-mouse antibody. The membrane was developed with chemiluminescence reagent.

Quantitative immunocytochemical analysis. A low temperature gill tissue preparation protocol was used and followed the procedures described in Tang [24]. Briefly, chemically fixed gill tissue was cryoprotected in 0.8 M sucrose in 0.1 M cacodylate buffer and subjected to plunge freezing in liquid propane at −187 °C in a multifunctional cryopreparation system (EM CPC, Leica). Cryofixed frozen samples were freeze-substituted in an automatic system (EM AFS, Leica) using absolutely dried 100% methanol containing 0.1% uranyl acetate as a substitution medium. The freeze substitution procedures for gill tissues were programmed as follows: (i) substituted for 8 h at −90 °C, warming up at a rate of 5 °C/h to −50 °C, and another 8 h at −50 °C; (ii) embedded stepwise in Lowicryl HM20 at −50 °C (30%, 50%, and 100% resin) over 2 h for the first two embedding steps, and 16 h for the pure resin. Polymerization was carried out with fresh pure resin under ultraviolet light (wavelength = 360 nm) at −50 °C for 2 d, and the polymerized block was further hardened under UV light for 2 d at room temperature. Ultrathin sections (90 nm) of Lowicryl HM20 embedded gill tissues were prepared on a Leica UCT ultramicrotome and collected onto Formvar/carbon-coated copper grids.

Optimization of immunocytochemical conditions, using a range exposure concentrations and incubation times, were first performed for each antibody as well as the secondary antibody [24]. In brief, prior to immunolabelling, sections were rehydrated by floating the grids on drops of PBS. Grids were then transferred to drops of diluted primary antiserum (1:10 for α5; 1:100 for CFTR) and incubated at room temperature (20 min for α5; 40 min for CFTR). They were then rinsed and transferred to drops of goat anti-mouse secondary antibody conjugated to 10 nm colloidal gold (Sigma, USA) (diluted at 1:10 for α5; 1:100 for CFTR), and incubated for 20 and 40 min, for α5 and CFTR, respectively. To ensure there was no non-specific gold labeling on gill sections, omission of the primary antibody, or replacement with bovine serum albumin and/or omission of the secondary antibody were performed, and in no case was antibody binding observed. The immunolabelled sections were rinsed with PBS and fixed for 5 min in 1% glutaraldehyde/PBS. After a brief rinse in double-distilled water, the sections were stained with Reynold's lead citrate and 2% uranyl acetate. Sections were viewed under a Philips Tecnai 12 BioTWIN transmission electron microscope (FEI, Netherlands) at 80 kV.

Five fish were studied for each treatment group. For each fish, three embedded tissue blocks were randomly chosen for ultrathin sectioning. One intact gill section from each block was selected for TEM examination. Ten chloride cells were uniform randomly sampled as described in

Howard and Reed [25]. Each chloride cell consisted of 25 montage pictures captured at 43,000× magnification and recorded by a CCD camera (Gatan 792 BioScan camera, USA). A total of 30 chloride cells (3 blocks × 10 chloride cells/section) were analysed for each gill sample. Quantification of gold particles per surface unit (μm^2) of each chloride cell was measured by using the imaging software AnalySIS 3.2 (Soft Imaging System, Germany). The density of gold particles binding on gill chloride cells represent the abundance of NKA/CFTR protein present in the cells.

mRNA expression of gill NKA and CFTR. NKA α-subunit, CFTR and actin PCR products were generated by PCR of total RNA derived from the gill samples. The primers were designed on the basis of the published sequence of NKA [TCTGATGTCTCCAAGCAGGC-forward and CTG GTCAGGGTGTAGGC-reverse] [26]; CFTR [ACTTCCTAGCTCTGG GTCTCTG-forward and CTGACCAGTGCTGATCTTATCCAA-reverse], and actin [CTGGTATCGTGATGGACTCT-forward and AGC TCATAGCTCTTCTCCAG-reverse]. The PCR fragments for NKA (150 bp), CFTR (176 bp), and actin (300 bp) were purified and subjected to dideoxy sequencing for verification.

Real-time PCR. The tissue samples were homogenized in TRIZOL Reagent (Gibco/BRL) and total RNA was extracted according to the manufacturer's instructions. Purified RNA with a ratio of 1.6–1.8 at A_{260}/A_{280} ratio was used in this study. Real time PCR was conducted for mRNA quantification. Briefly, 1 μg of total cellular RNA was reverse transcribed using iScript® cDNA synthesis kit (Bio-Rad). Sample cDNAs were analyzed by iCycler iQ real-time PCR detection system using iQ™ SYBR® Green Supermix (Bio-Rad). The arbitrary copy number for each sample was calculated and the data were normalized using the expression level of actin mRNA. The PCR conditions were 95 °C for 3 min and 40 cycles of 95 °C for 30 s, 56 °C for 30 s and 72 °C for 1 min. Fluorescent signals were captured at 82 °C, the occurrence of primer–dimers and secondary products were inspected using melting curve analysis. Control amplifications were done either without RT or without RNA. Following PCR amplification, the reaction products were run at 100 V on a 1% agarose gel with 0.5 μg/ml ethidium bromide to determine products specificity. All glass- and plastic-ware were treated with diethyl pyrocarbonate and autoclaved.

Statistics. Student's *t*-test was used to test the null hypotheses that there were no quantitative changes in the abundance of NKA/CFTR protein as well as mRNA in chloride cells between the treatment and control. Data were normalized by $\log_{10}$ transformation prior to *t*-test to achieve equality of variance.

Results

Quantitative immunocytochemical analysis

Antibody specificities were determined by Western blotting. Specific bands of molecular sizes of ~100 kDa for NKA α-subunit, ~165 kDa for CFTR were detected by anti-NKA α5 and anti-CFTR (clone:24-1) antibodies, respectively (Fig. 1). The results indicated that these antibodies exhibit high specificity to the proteins of interest in this study.

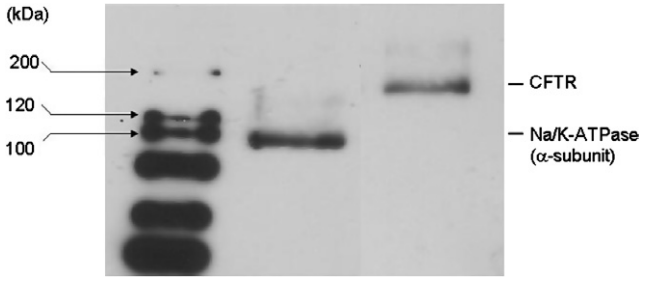

Fig. 1. Western blot analyses of the gill NKA α-subunit (middle lane) and CFTR protein (right-lane) of *R. sarba*.

In the non-toxic *D. tertiolecta* control fish, NKA immunoreactivity was highly abundant and largely localized on the tubule-vesicular system and throughout the basolateral region of the CCs (Fig. 2). No NKA labeling was found in the cell nucleus, mitochondria and the most apical region of the cells (Fig. 3). Upon exposure to 2000 cells/ml of *C. marina* (Fig. 4), NKA immunoreactivities were increased in gill CCs of distressed fish, while the localization pattern of NKA-gold labels remained unchanged (Figs. 2 and 3). Quantification of NKA-gold labels in CCs of *C. marina* exposed fish revealed an 30% increase of NKA immunoreactivities as compared to *D. tertiolecta* control ($p < 0.05$) (Fig. 5A).

The monoclonal antibody against 104 amino acids at the carboxyl terminus of the human CFTR used by McCormick et al. [10] on goby was proved to be successful for the localization of CFTR in the goldlined seabream. The distribution pattern and abundance of CFTR immunogold labeling in CCs differed substantially from that of NKA. The CFTR-gold labels were exclusively located at the apical regions of CCs in the non-toxic algal control fish (Fig. 6A). Moreover, the immunogold labeling density of CFTR was much lower as compared to that of NKA-gold labels (Figs. 2 and 3). When fish were exposed to 2000 cells/ml *C. marina* (Fig. 6B), the abundance of CFTR-gold labels was increased by about 30% as compared to the *D. tertiolecta* control ($p < 0.05$) (Fig. 5B). In this study, we have also tested the specificity of another antibody that directs against the full-length of human CFTR (used for mudskipper in Wilson et al. [12]). Our data indicated that

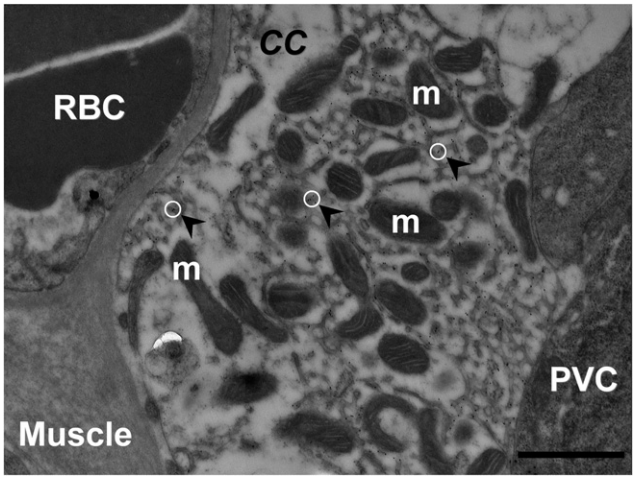

Fig. 2. Immunolocalization of NKA (α5 antibody labeled with 10 nm colloidal gold) on a submerged gill chloride cell (CC) of *R. sarba* exposed to *D. tertiolecta,* a non-toxic alga for 6 h. The electron-dense gold labels are highly abundant and located exclusively on the tubule-vesicular system in the cytoplasm. A few gold labels are highlighted (circled and black arrowheads) for easy reference. Non-specificity of labeling was not observed on neighboring muscular tissue (muscle), red blood cell (RBC), and pavement cell (PVC). Scale bar = 1 μm.

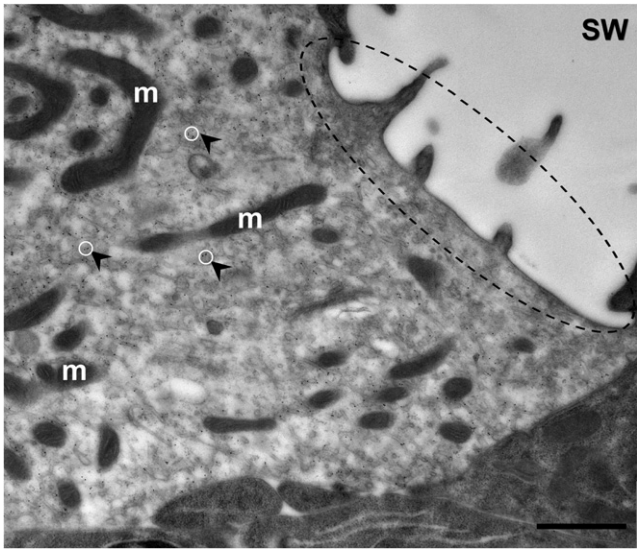

Fig. 3. Immunolocalization of NKA (α5 antibody labeled with 10 nm colloidal gold) on an opened gill chloride cell (CC) of *R. sarba* exposed to *D. tertiolecta,* a non-toxic alga for 6 h. Absence of electron-dense gold labels is found at the CC apical opening region (black eclipse) facing the external milieu, seawater (SW). The electron-dense gold labels are highly abundant at the basolateral region of CC. A few gold labels on the tubule-vesicular system are highlighted (circled and black arrowheads) for easy reference. Non-specific labeling was not observed outside CC. Scale bar = 1 μm.

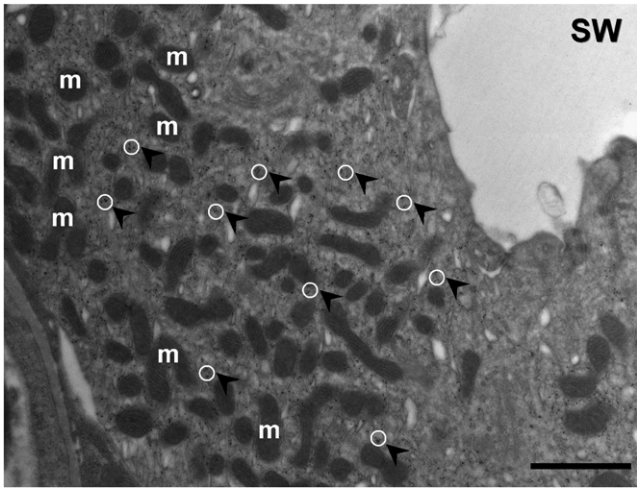

Fig. 4. Immunolocalization of NKA (α5 antibody labeled with 10 nm colloidal gold) on an opened gill chloride cell (CC) of *R. sarba* exposed to the toxic *C. marina* for 6 h. The electron-dense gold labels are more abundant on the tubule-vesicular system, whereas the distribution pattern was similar to the non-toxic *D. tertiolecta* control (Figs. 2 and 3). A few gold labels are highlighted (circled and black arrowheads) for easy reference. Scale bar = 1 μm.

the antibody did not show any immunoreactivities to our gill samples (data not shown).

Expression levels of NKA and CFTR mRNA

To determine if there were inductions of NKA and CFTR transcript levels in the gill epithelia of *C. marina*

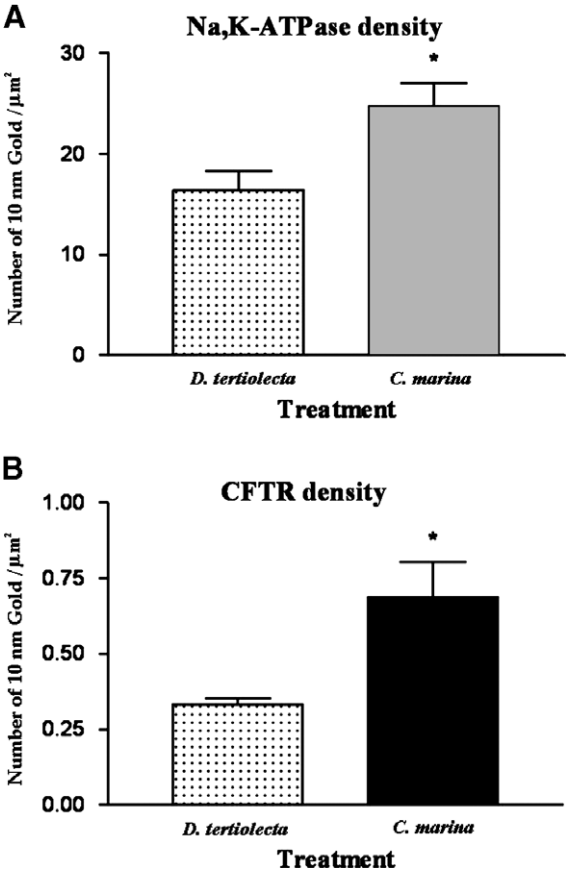

Fig. 5. Gold labeling densities for (A) NKA and (B) CFTR in chloride cells of *R. sarba* exposed to (i) non-toxic algal control *D. tertiolecta*, and (ii) toxic raphidophyte *C. marina* for 6 h.

exposured fish, we measured the respective mRNA levels using real-time PCR method. In the *C. marina* treatment, the expressions of Na^+, K^+-ATPase, and CFTR mRNA were comparable to that of the *D. tertiolecta* control group (Fig. 7).

Discussion

Our quantitative immunocytochemistry (q-ICC) results clearly demonstrate the presence of CFTR proteins at the apical plasma membrane and NKA on the basolateral region and tubule-vesicular system in branchial chloride cells of *R. sarba*. The polarized distribution of these two ion transporters in CCs of *R. sarba*, a "true" marine teleost, agree with the current model of seawater euryhaline CCs function in the seawater environment.

Importantly, we demonstrate for the first time that the ichthyotoxic *C. marina* induces *in vivo* expressions of NKA (α5 subunit) and CFTR protein in ion-transport cells of *C. marina* susceptible fish. Induced immunoreactivity of NKA α5 subunit protein in CCs associated with a parallel increase of branchial NKA activity has been demonstrated in gills of seawater-acclimated spotted green pufferfish [27]. Extra ATP supply for active ion transport in CCs could be evident by a parallel increase of mitochondria volume

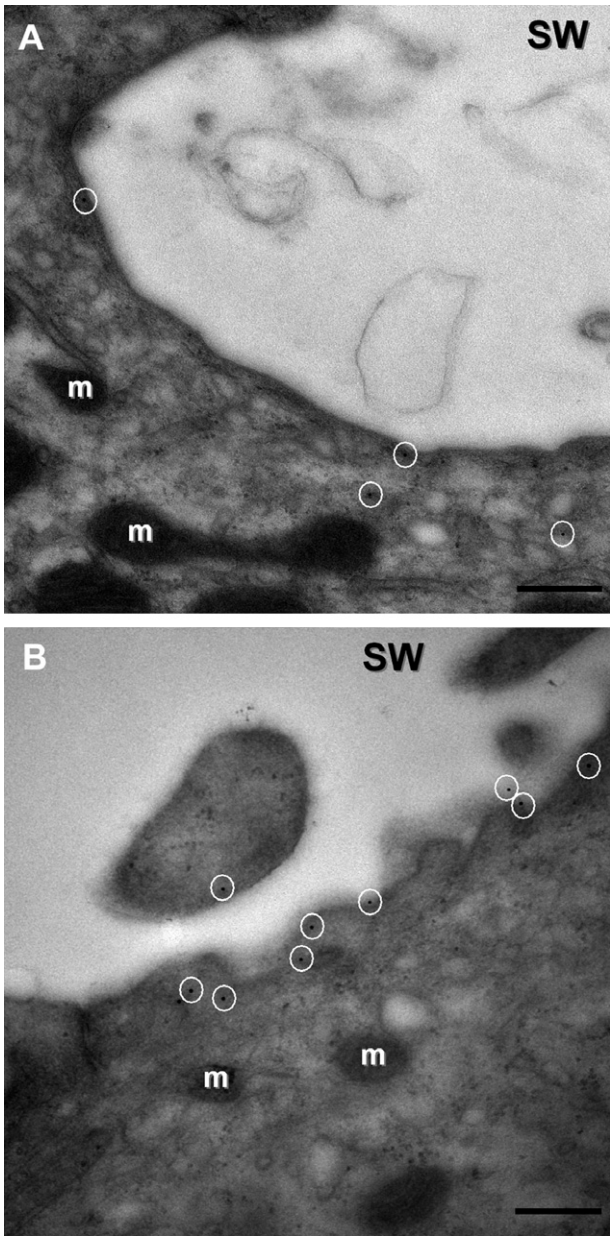

Fig. 6. Immunolocalization of CFTR on opened gill chloride cells (CC) of *R. sarba* exposed to (A) Non-toxic *D. tertiolecta* for 6 h. Electron-dense gold labels (circles) are sparsely found and confined at the apical region of CC facing the external milieu (SW). (B) Toxic *C. marina* for 6 h. Significant increase in gold labeling (circles) was observed at the apical region of CC. Scale bars = 0.2 μm.

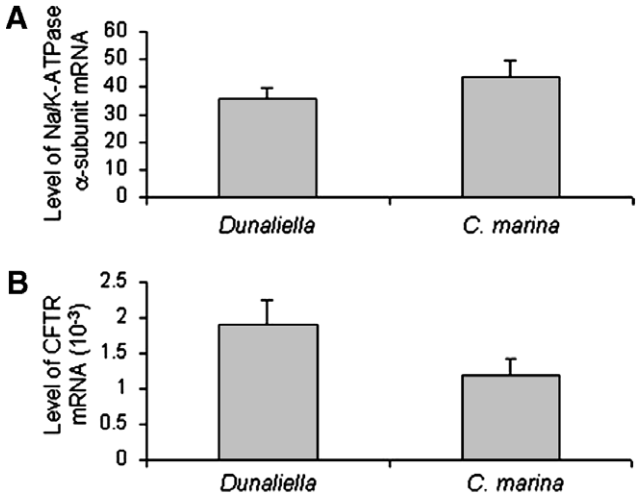

Fig. 7. The expression on gill (A) NKA α-subunit and (B) CFTR mRNA in moribund *R. sarba* upon exposure to the non-toxic algal control *D. tertiolecta* and toxic raphidophyte *C. marina* for 6 h ($n = 5 \pm$ SEM). Noted that the mRNA levels were normalized by actin expression level.

density in CCs of *R. sarba* exposed to *C. marina* [3,4]. Since concomitant elevation of branchial CC density, fractional area, and volume density were detected in *C. marina* distressed fish [3,4], we reason that the observed osmotic distress in *R. sarba* induced by *C. marina* was mediated via an induction of functional NKA and CFTR proteins in CCs together with an augmentation of branchial CCs.

When *R. sarba* were exposed to bloom concentration of *C. marina* (8000 cells/ml density), fish mortality started to occur from 1 h onwards (LT_{10}) and similar CC induction and blood osmolality decline were detected in distressed *R. sarba* [24], indicating that *C. marine* mediated induction of ion-transporter proteins in fish branchial CCs could happen as fast as in an hour. Our q-ICC study showed that the increased cellular density of NKA and CFTR proteins in CCs are unlikely just recruitment from defined intracellular compartments [28]. Using RT-PCR, we did not identify stimulation of branchial NKA and CFTR mRNAs transcription in *R. sarba* at 6 h post-exposure. Arguably, transient induction of these ion-transporters gene expressions might have already occurred within 6 h. Based on the recent studies of Scott et al. [29,30], increase of CFTR mRNA expression in killifish gills under abrupt salinity stress could occur in 3 h and sustain for weeks; whereas NKA α_{1a} mRNA expression was inducible after 1 day and declined rapidly. These data substantiate that NKA and CFTR mRNA expressions in branchial CC of *C. marina* exposed *R. sarba* could not have occurred and subsided as rapid as within 6 h. Conceivably, the induction of NKA and CFTR abundance in *C. marine* distressed fish is regulated at the post-transcriptional level.

The observed induction of these key ion transporters in *C. marina* stressed gill CCs could be the cause of osmotic disturbance, or they are a response to osmotic disturbance in the distressed fish. Further studies on ionic fluxes across gill epithelium are necessary to decipher the mode of toxic actions for *C. marina* in fish. In conclusion, our present findings ascertain that the toxic *C. marina* is capable to affect fish osmoregulatory homeostasis *in vivo*, possibly by triggering hyperactive excretion of Na^+ and Cl^- in gill chloride cells through an induction of the key osmoregulatory proteins, NKA and CFTR. Given these two ion-transporters are highly conserved and present in ion-regulatory cells of vertebrates, the *C. marina* biotoxin(s) may probably induce similar cytopathological effects on ion-regulatory tissues of other vertebrates, including humans.

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