Differential Expression of Hypothalamic and Gill-crh System With Osmotic Stress in the Euryhaline Black Porgy, Acanthopagrus schlegelii

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The local gill production of corticotropin releasing hormone (crh) and crh-receptor (crhr) is hypothesized to play important roles during seawater (SW) and freshwater (FW) acclimation in euryhaline black porgy (Acanthopagrus schlegelii). The mRNA expression of crh, crhr, and Na⁺/K⁺-ATPase (α-nka) was examined in SW and FW diencephalon (Dien) and in the gills at different exposure time by Q-PCR analysis. The in situ hybridization results indicate that crh mRNA hybridization signals were more abundant in FW fish in the gigantocellular (PMgc) and parvocellular (PMpc) part of the magnocellular preoptic nucleus versus SW fish. The crh and crhr-expressing cells were located in basal cells of gill filament. Furthermore, in vitro dexamethasone (DEX) treatment could increase the crh-system in the gill. Increased transcripts of the crh-system in the gill via in vitro and in vivo CRH treatments suggest that CRH may regulate the system in a local manner. The α-Nka cells were localized in the filament and secondary lamellae mitochondria rich cells (MRCs) of FW fish at 8 h and 1 day. α-Nka cells were seen in both filament and lamellae in the FW but much less in SW fish indicating that gills play key roles in black porgy osmoregulation. Gill crh and crhr play important roles in the response to salinity stress.

Keywords: crh, crhr, α-nka, gill, fish, brain, osmotic stress

INTRODUCTION

Stress stimulates the corticotrophin releasing hormone (crh) and crh-receptor (crhr) from the hypothalamus and pituitary by activation of the hypothalamic pituitary interrenal (HPI/fish) and/or hypothalamic pituitary adrenal (HPA/mammals)-axis (Dautzenberg and Hauger, 2002). In vertebrates, crh is the dominant hypothalamic neuropeptide controlling the HPI-axis (Vale et al., 1981; Rivier and Vale, 1983). Stress acts in the nervous system and converges at the hypothalamus, and the final products are corticosteroid hormones, particularly, cortisol, which is believed to regulate homeostasis. Furthermore, this same corticosteroid terminates the stress...
response by interacting directly with the hypothalamus or anterior pituitary to attenuate crh and crhr production (Slominski et al., 2000).

In mammals, the skin is a barrier between the external environment and the internal milieu and plays a critical role in maintaining internal homeostasis. Slominski and Wortsman (2000) suggest that the skin has a high sensory capability for stress stimuli and is tightly coupled to a local response system. During stress, the mammalian skin expressed crh and crhr and is highly reactive to common stressors such as immune cytokines, ultraviolet radiation, cutaneous pathology, or even the physiological changes associated with the hair cycle phase (Slominski et al., 2001). However, the body surfaces of teleosts are continuously exposed to an aquatic environment and are in intimately physiological contact with fish via the body fluid compartments and the epithelium of the gill, kidney, and intestine (Flik et al., 2006). Physiological processes, such as gas exchange, osmoregulation, excretion of nitrogenous waste products and acid-base balance, take place in the osmoregulatory organs (Evans et al., 2005). Thus, damage to these organs could result in the compromise of fish survival.

The gill is the only organ that is diffused by the entire cardiac output and has an extensive vascular surface area in contact with the plasma (Olson, 1998). The gills also play a dominant role in endocrine regulation. They are an endocrine target and are metabolically active tissue (Evans et al., 2005). The teleost gill possesses two morphologically distinct epithelia. One is a multilayered filament epithelium with pavement, mucous, and mitochondria rich cells (MRCs) which is largely involved in ion exchange. Another is a bi-layered lamellar epithelium made of undifferentiated, myoepithelial-like, granular, and neuroendocrine cells involved in gas exchange between the blood and the ambient water (Li et al., 1995). Versus terrestrial vertebrates, aquatic fish face a more challenging task to achieve an internal homeostasis due to the presence of ionic and/or osmotic gradients that are hostile to the body fluids of fish (Kaneko et al., 2002; Kaneko and Hiroi, 2008).

Black porgy, Acanthopagrus schlegelii, is an euryhaline teleost that maintains ion and water balances in a wide variety of environmental salinity via osmoregulatory mechanisms (Tomy et al., 2009). Thus, in fish, the gill is an important osmoregulatory organ that creates ionic and osmotic gradients between the body fluid and external environments (Marshall and Grosell, 2006). In the gill, MRCs or chloride cells present in gill epithelia are a major site of ion secretion and absorption. They are important in SW and FW adaptation, respectively (Kaneko et al., 2008). The chloride cells or MRCs are structurally characterized by many mitochondria including an abundant tubular system where ion-transporting enzymes such as Na+/K+-ATPase, Ca2+-ATPase, and Ca2+/Na+ exchanger is located (Wendelaar Bonga et al., 1990; Flik and Verbost, 1993; Marshall, 2002). The MRCs are the site of the active Ca2+ transport that underlies transepithelial Ca2+ uptake in both freshwater (FW) and seawater (SW) fish (Marshall et al., 1992; McCormick et al., 1992; Verbost et al., 1994).

Here, we hypothesize that the gill is an essential vital organ that directly faces the exterior and has an endocrine-like function to acclimate and adapt to environmental changes. To maintain homeostasis, a gill crh system (crh/crhr) may be activated by external stressors including in vivo and/or in vitro conditions. Locally produced crh and crhr in the black porgy gill can respond to ambient salinity stress versus the hypothalamic crh-system. Based on limited information available on the expression of crh and crhr in the gill of common carp, Cyprinus carpio, we study here the expression of crh, crhr, and Na+/K+-ATPase (a-nka) in the black porgy diencephalon (Dien) and gill by Q-PCR analysis (Mazon et al., 2006). This is also the first study to localize the transcripts of crh and crhr in SW and FW-acclimated black porgy gill at day 1 and day 30 by in situ hybridization. We further performed in vitro and in vivo dexamethasone (DEX) and CRH treatment to analyze the mRNA expression pattern of crh-system in Dien and gill. Immunohistochemical studies with a-Nka antibody have identified the a-Nka immunoreactivity (ir) cells in the gill at day 1 and day 30 during SW and FW acclimation.

MATERIALS AND METHODS

Experimental Fish

Black porgy (6–7 months old, n = 190) (body weight = 17.69 ± 0.59 g, body length = 9.96 ± 0.37 cm) were procured from an aquaculture farm of southern Taiwan (Chiayi County) and allowed to acclimated for 1 month at the National Taiwan Ocean University aquarium in seawater (33 ppt) with a natural light system. The water temperature, pH and dissolved oxygen (DO) values of which ranged from 19 to 24°C, 8.11 to 8.18, and 7.7 to 7.8 mg/L, respectively. Seawater was obtained from the open sea and filtered through sand. Freshwater was obtained from the tap and was put in the tank for 1 week with aeration for dechlorination (pH and DO values were 7.91–8.08 and 7.8–8.0 mg/L, respectively). The fish tanks were aerated to maintain adequate dissolved oxygen. The water was continuously aerated through air stones. The level of the aeration was sufficient to sustain dissolved oxygen levels for all experiments. The sponge filters and gravel were used to filter the solids from fish waste and uneaten feeds. Fish were fed pelleted dry food ad libitum at a daily ration of 1% of their estimated body weight. For each experiment, the fish were anesthetized with 1% glyкопhenol monophenyl ether and decapitated. The diencephalon and gill samples were collected and snap frozen in liquid nitrogen at −80°C. All experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, National Taiwan Ocean University, Taiwan (# 99026).

Experimental Design-Experiment 1

Seawater to Freshwater Transfers at Different Time Exposure

To characterize the endocrine changes in osmoregulation in response to acute salinity stress, an experiment was conducted where fish were randomly divided into two groups and maintained in SW (33 ppt). After an initial acclimation period (30 days), fish were maintained in SW (n = 40) and transferred to FW (0 ppt). Respective control groups (n = 40) received...
the SW with no salinity changes. The diencephalon and gill samples \((n = 10)\) were collected after 8 h, 1 day, 4 days, and 30 days of transfer.

**Experimental Design-Experiment 2**

**In vivo** Treatment of **crh**

The fish \((n = 24)\) were cultured in 2,000 L tanks for 2 weeks. After an initial acclimation, the fish were randomly divided into three groups \((n = 8 \text{ per group})\). The different doses of mammalian **crh** (Sigma, St. Louis, MO, United States) \((10 \text{ and } 40 \mu g/kg)\) were prepared by dissolving **crh** with phosphate buffered saline (PBS) \((pH 7.4)\). Fish were injected intraperitoneally with **crh** twice on day 1 and day 4. Control fish were injected with coconut oil and treated as a control. We collected the samples on day 5.

**Experimental Design-Experiment 3**

**In vitro** Gill Culture With **crh** and Dexamethasone

The fish \((n = 64)\) were cultured in 2,000 L tanks for 2 weeks. After an initial acclimation, the fish were randomly divided into eight groups \((n = 8 \text{ per group})\). The serial dilutions of mammalian DEX \((\text{low } 10^{-8} \text{ M}, \text{ medium } 10^{-6} \text{ M}, \text{ and high } 10^{-4} \text{ M})\) and **crh** \((\text{low } 10^{-9}, \text{ medium } 10^{-7}, \text{ and high } 10^{-5} \text{ M})\) were prepared and the gill filaments were incubated for 2 and 4 days with the respective concentrations. A fish group was incubated with L-15 medium as a control. After **in vitro** culture, gill filaments were removed with forceps and then snap frozen in liquid nitrogen at \(-80^\circ\text{C}\) until RNA isolation.

**Primary Gill Culture**

Primary gill filaments were excised from the arches and separated from one another. Five to six primary gill filaments were placed in each petri dish \((n = 8 \text{ fish, each fish per petri dish})\) containing 1% penicillin-streptomycin in Leibovitz L-15 medium (Gibco, United States), this was incubated in ice for an hour. After incubation, the filaments were washed with 1X PBS to remove blood. The gill filaments were again rinsed with 5% penicillin-streptomycin in Leibovitz L-15 medium \((\text{55}^\circ\text{C for 1 min, and 72}^\circ\text{C for 2 min for 30 cycles})\) and the gill filaments were incubated for 2 and 4 days with the respective concentrations. A fish group was incubated with L-15 medium as a control. After **in vitro** culture, gill filaments were removed with forceps and then snap frozen in liquid nitrogen at \(-80^\circ\text{C}\) until RNA isolation.

**RNA Extraction, First-Strand cDNA Synthesis and Cloning**

RNA was isolated from the Dien and gill by using TRiZol® reagent and reverse transcribed (Gibco BRL, Grand Island, NY, United States) according to the protocol of the manufacturer. The resulting cDNA was used as a template for the subsequent PCR amplification of the genes used here.

Genes involved in the stress response were cloned from cDNA of the black porgy gill. Multiple alignments of previously published sequence of the respective genes were constructed using CLUSTAL X program (version 1.81) to find the conserved region. Primers were designed from here (Table 1). PCR reactions were performed with 2.5 µl of 10X reaction buffer \([200 \text{ mM Tris–HCl (pH 8.4)}, 500 \text{ mM KCl)}, 1 \mu l \text{ of 10 mM dNTP}, 1 \mu l \text{ of 2 mM MgCl}_2, 0.5 \mu l \text{ each of 10 } \mu \text{M sense and antisense primers, } 1 \mu l \text{ cDNA, and 0.2 } \mu \text{l superscript enzyme (Invitrogen, Carlsbad, CA, United States) in a final volume of 25 } \mu \text{l. The PCR conditions were set as follows: 94^\circ\text{C for } 5 \text{ min, } 94^\circ\text{C for } 30 \text{ s, } 50^\circ\text{C for } 30 \text{ s, } 72^\circ\text{C for } 30 \text{ s for } 35 \text{ cycles, and } 72^\circ\text{C for 10 min. The PCR products were verified by electrophoresis on a 1.5% agarose gel and visualized using ethidium bromide staining DNA fragments were excised using a Gel-MTM Gel Extraction system Kit (Bio 101) (Viogene, La Jolla, CA, United States) and cloned into pGEM® – T Easy vector (Promega, Madison, WI, United States). Plasmids containing the insert were sequenced using a dye terminator cycle sequencing kit (Perkin Elmer, Foster City, CA, United States) and submitted to the Basic Local Alignment Search Tool (BLAST) for making comparison with the known sequences accessible in NCBI database.

**Tissue Distribution mRNA Expression of **crh, **crhr, and **α-nka in Dien and Gill**

Total RNA was extracted from the Dien and gill of black porgy \((n = 3)\). The complementary DNA (cDNA) strand was synthesized with 1 µg of total RNA using SuperScript II with the oligo(dT)\(_{12-18}\) primers. Gene specific primers were employed for RT-PCR analysis (Table 1). The **glyceraldehyde 3-phosphate dehydrogenase (gapdh)** was used as an internal control. The **gapdh** transcripts did not significantly change between or among the treatments. The PCR conditions were as follows: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min for 30 cycles and the PCR products were separated on 1.5% agarose gel. Reactions in which reverse transcriptase was neglected were used as negative controls for SW and FW fish.

**Q-PCR Analysis**

Q-PCR analysis was performed to analyze the gene expression of **crh, crhr1, and α-nka** in the Dien and gill during FW and SW acclimation with iQ™ Multicolor Real-Time PCR Detection system (Bio-Rad). Primers were designed using primer expression software (Applied Biosystems, Foster City, CA, United States) (Table 1). Gene quantification of standards, samples, and controls was conducted simultaneously in a Q-PCR machine (iQ™ Multicolor Real-Time PCR Detection System; Bio-Rad Co., Hercules, CA, United States) with iQ™ SYBR green (Bio-Rad) as a dsDNA minor-groove binding agent, forward and reverse primers, and water according to our previous study (Aruna et al., 2012a). Calculation of PCR efficiency was based on the slope of the relationship between log input cDNA (transcript concentrations) vs. cycle threshold (Ct). We used an efficiency-corrected method to calculate the relative expression level of **crh, crhr**, and **α-nka** in the hypothalamus and the gill. The correlation of the standard curve was \(-0.999\). The **gapdh** was used as a...
control gene to calibrate the mRNA expression level, because there were no significant changes in the expression levels between all controls and experimental groups.

**In situ Hybridization**

The in situ hybridization was carried out to localize the crh and crhr transcripts in the hypothalamus and gill of black porgy. The tissues were fixed in a 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C overnight and embedded in paraffin. Paraffin sections (5 µm) were collected on TESPA-treated slides and with commercial primary antibody (a-Nka-IgG) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) overnight and embedded in paraffin. The sections were then incubated with 1.5% normal goat serum for 30 min at 4°C. This was followed by incubation with anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, United States) overnight. DAB (3,3′-diaminobenzidine, Sigma).

**Statistical Analysis**

Data are expressed as means ± standard error of the mean. The values were analyzed by one-way ANOVA, followed by Student–Newman–Keuls multiple tests. Value with *p* < 0.05 indicating a significant difference and are denoted by “a” and “b.” Student’s t-test was also conducted to determine significant differences (*p* < 0.05) between SW and FW fish denoted by asterisks (*).

**RESULTS**

**Tissue Distribution of crh, crhr, and a-nka**

The expected single band of crh, crhr, and a-nka was obtained in all the examined tissues except a-nka in Dien by RT-PCR, though

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**TABLE 1 | List of primers used for RT-PCR, Q-PCR, and in situ hybridization analysis.**

| Gene       | Orientation | Nucleotide sequence (5′ – 3′) | Usage  | Accession no. |
|------------|-------------|-------------------------------|--------|---------------|
| Cth        | F           | 5′-GGCAGGAATCACTCTGCGATCT-3′  | RT-PCR | FJ445422      |
|            | R           | 5′-GACCTGCCACTCCACCTGAGT-3′   | RT-PCR |               |
|            | F           | 5′-CAAGGGCTCTCTCCAGAGAT-3′    | Q-PCR  |               |
|            | R           | 5′-CCAAACGCCCCTCCAGAGAT-3′    | Q-PCR  |               |
| S          |              | 5′-CCGCTACGATTGCTGGGCTATGGAG-3′ | in situ |               |
| AS         |              | 5′-CTTCCCTCCCTCCTCATCGAGTC-3′ | in situ |               |
| crh-r      | F           | 5′-CCACCCAGTGAAGCGAGCAT-3′    | RT-PCR | FJ445423      |
|            | R           | 5′-GTGTGTGGAGTCTCCTGGAGGA-3′  | RT-PCR |               |
|            | F           | 5′-AGAGGTGGTGGGATGGAAATAGTT-3′ | Q-PCR  |               |
|            | R           | 5′-GGAGGTGCACAAAGCAACGT-3′    | Q-PCR  |               |
| S          |              | 5′-GGACACTCTACGCCCTCTCATC-3′  | in situ |               |
| AS         |              | 5′-CCCAAGGAGGAGAAGAACAC-3′    | in situ |               |
| a-nka      | F           | 5′-ACGGTGAGCCCATGT-3′         | RT-PCR | EF621407      |
|            | R           | 5′-GGTCTCGCTCTGTGGTCTGTA-3′   | RT-PCR |               |
|            | F           | 5′-ACGGTGAGCCCATGT-3′         | Q-PCR  |               |
|            | R           | 5′-GGTCTCGCTCTGTGGTCTGTA-3′   | Q-PCR  |               |
| gapdh      | F           | 5′-AGGCTTACCTCTACCTCAGATAGAT-3′ | RT-PCR | DQ399798      |
|            | R           | 5′-GGTGCCTGTGGTGATGT-3′       | RT-PCR |               |
|            | F           | 5′-GCATTTGCAAGGCTAACT-3′      | Q-PCR  |               |
|            | R           | 5′-GGCGCGCCGACATCAGAT-3′      | Q-PCR  |               |

F: Forward primer; R: Reverse primer; in situ, in situ hybridization.

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The serial sections proceeded to rehydration, deparaffinization, and hybridization according to our previous study (Aruna et al., 2012a). The sections were incubated with a-abc by RT-PCR, though

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Immunohistochemistry

Immunohistochemical analysis of the a-Nka was performed in the gill of black porgy. A few deparaffinized serial sections (5 µm) were incubated with 3% H2O2 in PBS. The sections were then incubated with 1.5% normal goat serum for 30 min and with commercial primary antibody (a-Nka-IgG) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, United States) overnight at 4°C. This was followed by incubation with anti-rabbit IgG (Vector Laboratories Inc., Burlingame, CA, United States) for 1 h at room temperature. The sections were then visualized by an ABC kit (avidin-biotin, Vector Laboratories Inc.) and DAB (3,3′-diaminobenzidine, Sigma).
with varying intensity in expression. A strong band of crh and crhr was found in the Dien and gill. In the gill, the expression of a-nka was strong, but no expression was observed in Dien (Figure 1A). crh, crhr, and a-nka mRNA levels were determined by RT-PCR in Dien and gill of SW and FW black porgy. gapdh was used as an internal control (Figure 1A).

Q-PCR Analysis of crh and crhr mRNA Expression in Dien During Freshwater Transfer

The expression of crh in FW was highly expressed in Dien at day 4 and day 30 versus the control (SW) fish (Figure 1B). In contrast, the expression of crhr (Figure 2A) was significantly (p < 0.05) downregulated in the Dien at day 4 FW when compared to their respective controls. No significant difference between SW and FW was observed in the expression of crhr in Dien at 8 h, day 4, and day 30 (Figure 2A).

Q-PCR Analysis of crh, crhr, and a-nka mRNA Expression in Gill During Freshwater Transfer

A significant (p < 0.05) reduction was found in the gill crh at day 1 and day 4 in FW versus SW (Figure 1D). In contrast, the crhr mRNA levels were significantly (p < 0.05) increased in gill at day 1 in FW versus SW (Figure 2B). The expression levels of a-nka were 5, 6, 4.5, and 4.4-fold higher in FW at 8 h, day 1, day 4, and day 30, respectively, versus controls (Figure 3A). No significant difference was observed in the expression of crh and crhr in gill between SW and FW fish at 8 h and day 30 (Figures 1D, 2B).

Localization of crh Transcripts in Hypothalamus at Day 30 by in situ Hybridization

The localization of crh transcripts was performed in the hypothalamus at day 30 SW (Figures 1Ca–c) and FW (Figures 1Cd–f) by in situ hybridization. The crh transcript hybridization signals were detected in both SW (Figure 1Ca) and FW fish (Figure 1Cd). The crh mRNA hybridization signals were more abundant in FW fish in the gigantocellular part of the magnocellular preoptic nucleus (PMgc) (Figure 1Ce) and parvocellular part of the magnocellular preoptic nucleus (PMpc) (Figure 1 Cf) versus the SW fish (Figures 1Cb,c).

Localization of crh and crhr Transcripts in Gill at Day 1 and Day 30 by in situ Hybridization

The expression of crh (Figure 1E) and crhr (Figure 2C) gene was examined by in situ hybridization in gill at day 1 and day 30. The hybridization signals of crh and crhr mRNA in the gill were located near and around the afferent filamental artery (Figures 1Ea–h, 2Ca–j). The transcripts of crh hybridization signals were decreased in FW acclimated fish in the branchial epithelium (Figures 1Ec,d) versus the SW acclimated fish (Figures 1Ea,b) at day 1. In contrast, the mRNA expression of crhr signals was more abundant throughout the gill in the FW acclimated fish (Figures 2Cd–f) versus the SW fish (Figures 2Ca–c) at day 1. The hybridization signals of crh and crhr were similar between SW (Figures 1Ef,f, 2Cg,h) and FW (Figures 1Eg,h, 2Ci,j) fish at day 30 in the branchial epithelium of the gill.

Immunohistochemistry Analysis of a-Nka Antibody in Gill Tissue

The expression of a-Nka was examined in the gill (Figures 3Ba–d). The a-Nka cells were mainly expressed in the MRCs. The expression of a-Nka genes in the gill was increased in FW at 8 h, day 1, day 4, and day 30. However, we performed the immunohistochemistry at 8 h and day 1 in both SW (Figures 3Ba,b) and FW (Figures 3Bc,d). The a-Nka cells were expressed in the filaments and lamellae in the FW fish at 8 h and day 1 (Figures 3Bb,d) but the a-Nka cells were expressed only in the gill filament but not lamellae in SW fish (Figures 3Ba,c). The fluorescent hybridization showed the pattern of a-Nka cells in SW and FW fish (Figures 3Ca,b). The a-Nka cells were localized in the filament at SW (Figure 3Ca). However, FW fish had a-Nka cells localized in both filament and lamellae (Figure 3Cb).

Non-colocalization of crh/crhr Transcript Cells With a-nka Cell in Gill Filament

We further examined the co-localization of crh transcript cells vs. a-Nka cells and crhr transcript cells vs. a-Nka cells in the gill filament or lamellae of FW and SW fish by ISH and IHC (Figure 4). Our data found that crh and crhr transcript cells were not co-localized with a-Nka cells in either FW or SW fish (Figure 4). The crh and crhr cells are suggested to localize in the basal cells of the filament and a-Nka cells are localized in the filament epithelium (Figure 4).

Q-PCR Analysis of crh, crhr, and a-nka mRNA Expression in D ien and Gill During in vivo CRH Treatment

The transcripts of crh were significantly (p < 0.05) increased in the gill in vivo at low (10 µg/kg) and high doses (40 µg/kg) of crh (Figure 5A). No significant (p > 0.05) difference was observed in the mRNA expression of crhr (Figure 5B) and a-nka (Figure 5C) in the gill in vitro at both low and high doses of CRH. No significant (p > 0.05) difference was found in the transcripts of crh and crhr in the Dien at low and high doses of in vivo crh treatment (Figures 5D,E).

Q-PCR Analysis of crh and crhr mRNA Expression in Gill During in vitro CRH Treatment

The transcripts of crh were significantly increased at medium (10⁻⁷ M) and high (10⁻⁵ M) doses of CRH (Figure 6A). The crh was significantly (p < 0.05) increased only at high dose in the gill on day 2 (Figure 6B). The long duration of gill culture with CRH did not change the mRNA levels of CRH on day 4 (Figure 6C). However, high dose of crh significantly (p < 0.05) downregulated the crhr mRNA expression in the gill on day 4 (Figure 6D). In
We show that the mRNA expression of DISCUSSION Dien and gill of black porgy during a SW to FW acclimation at crhr (their controls at day 2. In addition, there was no significant − at low (10 p No significant ( was significantly (crh The mRNA expression of Dexamethasone Treatment in vitro Expression in Gill During Q-PCR Analysis of crhr mRNA Expression in Gill from SW to FW at 8 h, day 1, day 4, and day 30. Each data had 10 fish. Localization of crh in the hypothalamus gigantocellular (PMgc) and parvocellular part of the magnocellular preoptic nucleus (PMpc). Figure 6E The in situ hybridization was performed to localize crh transcripts in black porgy gill during SW and FW acclimation at day 1 (c,d) and day 30 (g,h). The transcripts of crh hybridization signal were weak in FW acclimated fish (c,d) in the branchial epithelium when compared to the SW acclimated fish (a,b) at day 1. And, the hybridization signals of crh were similar between SW (e,f) and FW (g,h) fish at day 30 in the branchial epithelium of the gill. The representative fish is shown from one of 3 fish. There was no notable signal found in the representative sense pictures (not shown). aa, afferent artery; be, branchial epithelium; ea, efferent artery; fi, filament; la, lamellae.

addition, the low dose (10−9 M) of CRH did not change the crh and crhr transcripts in the gill on day 2 and day 4 (Figures 6A–D).

Q-PCR Analysis of crh and crhr mRNA Expression in Gill During in vitro Dexamethasone Treatment

The mRNA expression of crh (Figure 6E) and crhr (Figure 6F) was significantly (p < 0.05) increased at high dose (10−4 M). No significant (p > 0.05) difference was observed in the gill at low (10−8 M) and medium (10−6 M) doses of DEX versus their controls at day 2. In addition, there was no significant (p > 0.05) difference in the transcripts of crh (Figure 6G) and crhr (Figure 6H) at low, medium, and high dose of DEX at day 4.

DISCUSSION

We show that the mRNA expression of crh, crhr, and a-nka in the Dien and gill of black porgy in SW and gill of black porgy in situ hybridization at day 1 and day 30. The crh (in vitro, in vivo) and DEX (in vitro) treatments with different doses were performed to analyze the differential mRNA expression pattern of crh and crhr in Dien and gill. Furthermore, we localized the a-Nka cells in FW and SW-acclimated black porgy gill at day 1 and day 30 by immunohistochemistry. The mRNA expression of crh, crhr, and a-nka transcripts during FW transfer and in vivo and in vitro crh and DEX treatment at different days with different doses were elaborately framed in the current study.

The serum osmolality levels were significantly decreased in the FW black porgy at day 1 and 30 (unpublished data). In the SW fish, serum osmolality was significantly higher compared to the FW fish, as indicated in our previous study (Tomy et al., 2009). Serum cortisol was significantly increased at day 1 and returned to normal levels at 30 (unpublished data). According to this, it seems that black porgy may require only a short period for acclimation to osmotic stress. The total concentration of solutes such as the inorganic ions present in the fluid is the osmolality (Soegianto et al., 2017). However, at the time of initial acclimation
the serum osmolality levels were significantly different between SW and FW fish; this reflects the acclimation. The euryhaline teleost reduced their ion secretion rapidly when quickly moved to a FW environment. Overall, the black porgy maintained its teleost reduced their ion secretion rapidly when quickly moved to a FW environment. The euryhaline teleost maintained its osmolality within the range of 270–420 mOsmol kg⁻¹. This suggests efficient hyper or hypo osmoregulation in black porgy (Tomy et al., 2009).

Generally, the teleost crh system was activated through the HPI-axis during the initial stress (Huising et al., 2004; Doyon et al., 2005). The expression of crh could regulate itself in response to the stress induced by the elevation of glucocorticoids and neurogenic signals (Lachize et al., 2009). The increased cortisol levels could affect gill function via regulating gill ion transporters (Küblerich et al., 2007). The elevated hypothalamic crh markedly downregulated the pituitary crhr expression because continuous release of the hypothalamic crh desensitized the pituitary corticotrope cells (Huising et al., 2004). Furthermore, repeated or chronic stress can increase the crh mRNA levels in mammalian hippocampus, paraventricular nucleus, and tilapia forebrain and hypothalamus (Makino et al., 1995; Aruna et al., 2015). This suggests that the intensity and duration of the salinity stress are important external factors regulating the brain crh system (Doyon et al., 2005; Aruna et al., 2012a).

Seawater and FW acclimation activated crh, crhr, and antka transcripts in the black porgy hypothalamus and gill. In agreement with Doyon et al. (2005), we show here that the expression of crh mRNA was significantly increased at day 4 and day 30 whereas the crhr transcripts were significantly downregulated at day 4 in the Dien of the black porgy. These data suggest that the transiently elevated levels of plasma cortisol may exert negative-feedback effects on the level hypothalamus (hypothalamic crh) as suggested in carp pituitary (Huising et al., 2004). In addition, we detected the crh transcript hybridization signals in both SW and FW fish. Similar to Q-PCR data, the crh mRNA hybridization signals were more abundant in FW fish at PMgc and PMpc versus SW fish at day 30. The data suggest that the expression of crh mRNA is essential to the salinity stress.

Increased expression of crhr mRNA in the gill suggests that the gills are responsible for the stressors and regulate the ventilation rate and blood oxygen transport (Wendelaar Bonga, 1997; Mazon et al., 2006). Furthermore, the Q-PCR and in situ hybridization results show that the crh transcripts were significantly reduced in the black porgy gill at day 1 and day 4. Interestingly, the crhr was significantly increased in the gill at day 1. The local production of crh and/or crhr in the gill may be directly activated by external stressors.

There are very few studies on the expression and localization of crh and crhr in the gill during SW and FW acclimation. This is the first study in black porgy to localize the crh and crhr transcripts in SW and FW-acclimated gill at day 1 and day 30 by in situ hybridization. Similar to the Q-PCR results, the cellular transcripts of the crh and crhr were lower and higher in FW-acclimated fish, respectively. The transcripts of

**FIGURE 2** (A) Comparative analysis of crh, gene expressions as determined by quantitative real-time PCR in diencephalon (Dien) of black porgy from SW to FW at 8 h, day 1, day 4, and day 30. Each data had 10 fish. (B) Comparative analysis of crh, gene expressions as determined by quantitative real-time PCR in the gill of black porgy from a SW to FW transfer at 8 h, day 1, day 4, and day 30. Each data had 10 fish. (C) The in situ hybridization was performed to localize crhr transcripts in black porgy gill during SW and FW acclimation on day 1 (a–f) and day 30 (g–j). The mRNA expression of crh signals was more abundant throughout the gill in FW-acclimated fish (d–f) compared to the SW fish (a–c) on day 1. On day 30, the hybridization signals of crh were almost similar between SW (g,h) and FW (i,j) fish in the branchial epithelium of the gill. The representative fish is shown from one of 3 fish. There was no notable signal found in the representative sense pictures (not shown). aa, afferent artery; be, branchial epithelium; ea, efferent artery; fi, filament; la, lamellae. Student’s t-test was conducted to determine significant differences (p < 0.05) between SW and FW denoted by asterisks (*).
FIGURE 3 | (A) Comparative analysis of a-nka, gene expressions as determined by quantitative real-time PCR in diencephalon (Dien) of black porgy from SW to FW at 8 h, day 1, day 4, and day 30. Each data had 10 fish. (B) The immunohistochemistry was performed to localize the a-Nka cells in black porgy gill during SW and FW acclimation on day 1 and day 30. The expression of a-Nka in the gill was increased in FW at 8 hrs (b) and day 1 (d) compared to the respective SW fish (a,c). The Nka was expressed in the filaments and lamellae in the FW fish (b,d) but the a-Nka was expressed only in the gill filament not lamellae in SW fish (a,c). The representative fish is shown from one of 3 fish. There were no notable signals found in the control (not shown). (C) Localization pattern of a-Nka cells during SW (a) and FW (b) of black porgy. The a-Nka cells were also appeared in the lamellae (pointed with arrow heads) in addition to filament in FW fish (b). MRC: mitochondria rich cell, fi: filament, la: lamellae. Student’s t-test was conducted to determine significant differences (p < 0.05) between SW and FW denoted by asterisks (*).

FIGURE 4 | There was no co-localization of crh transcript cells vs. a-Nka cells and crhr transcript cells vs. a-nka cells in the gill filament of FW and SW fish. Fi, filament; la, lamellae. (A) crh transcripts in the gill filament/lamellae of FW (a,b) and SW (c,d) fish. (B) crhr transcripts in filament/lamellae of FW (a,b) and SW (c,d) fish.

the crh and crhr were highly localized in the apical membrane of the branchial epithelium in both SW and FW black porgy. In addition, the crhr transcripts were localized in the basal cells of the gill filament as reported in common carp by immunohistochemistry (Mazon et al., 2006). Our previous studies showed glucocorticoid receptors and mineralocorticoid
FIGURE 5 | Q-PCR analysis was performed to examine the mRNA expression of crh, crhr, and a-nya in the gill (A–C) and diencephalon (Dien) (D,E) during in vivo CRH treatment with low (10 µg/kg) and high (40 µg/kg) doses. Data (n = 8 for each data) are expressed as means ± standard error of the mean. The values were analyzed by one-way ANOVA, followed by a Student–Newman–Keuls multiple test, with p < 0.05 indicating a significant difference, are denoted by “a” and “b.”

receptors in tilapia gill (Aruna et al., 2012b). Taken together, the expression and localization of crh and crhr in black porgy gill highlight that positive involvement of the crh system can balance the homeostasis during salinity stress.

The DEX (5 mg/kg)-treated pigs had no effect on the mRNA expression of crh in the hypothalamus after 3 h (Vellucci and Parrott, 2000). In mice, in vitro DEX treatment for 24–96 h caused a specific decrease in crh mRNA (Adler et al., 1988). Here, we elaborately framed out the mRNA expression of crh and crhr in the Dien and gill by in vitro and in vivo crh and DEX treatment. In contrast to Adler et al. (1988), a high in vitro dose of DEX increased the transcripts of the crh and crhr in black porgy gill on day 2. Pierson et al. (2004) showed that the high dose of DEX treatment leads to a chronic stress, which may activate the local crh-system in the gill.

We also found that the transcripts of crh were significantly increased in gill by in vitro and in vivo CRH treatment. In vivo CRH treatment did not cause any effects on the expression of crh and crhr transcripts in the black porgy Dien with different doses. Consistent with our findings, the in vitro and/or in vivo CRH treatment decreased and/or did not change the mRNA expression of CRHR in the mammalian anterior pituitary after acute or repeated immobilization stress (Hauger et al., 1977; Luo et al., 1995; Makino et al., 1995; Sakai et al., 1996; Roseboom et al., 2001). Sumitomo et al. (1987) and Yokoe et al. (1988) strongly suggested that the main source of plasma CRH was the hypothalamic paraventricular nucleus neurons with their terminals in the median eminence of mammals. In contrast to the mammals, the descending spinal crh-ir projections appear to be absent in teleosts (Lovejoy and Balment, 1999; Pepels et al., 2002). Taken together, the local gill crh system may respond more rapidly than the hypothalamic crh in black porgy.

The crh system is a powerful stress response tool in teleosts (HPI-axis/local crh). It can trigger cortisol release from the cascade signals of brain-pituitary to inter-renal gland to respond to the various stressors (Flik et al., 2006). Generally, the presence of crh in the brain (nucleus of preopticus) is associated with fear and anxiety (Majzoub, 2006). Pepels et al. (2002) found a crh in the heads of larvae of Mozambique tilapia 5 days post hatching, suggesting the importance of the crh system in teleosts. The cutaneous crh system may respond to external stressors in mammals (Slominski et al., 2001). We also found the changes in the expression of crh-crhr in the brain-pituitary and corticosteroid receptors in the gill during the stress response in tilapia (Aruna et al., 2012a,b, 2015). Gill tissue separates the internal and external environment and is constantly activated by external stressors or internal factors. The presence of local crh, crhr, and corticosteroid receptors in the gill may be important to respond to stress in fish. Thus, the activation of the local gill crh-crhr system may be regulated by the circulation (crh-acth system...
In vitro treatment – gill culture with CRH and dexamethasone

FIGURE 6 | (A–D) Q-PCR analysis was performed to examine the mRNA expression of *crh* and *chr* in gill on day 2 (A,B) and day 4 (C,D) during in vitro gill culture with CRH low (10^{-9} M), medium (10^{-7} M) and high (10^{-5} M) doses. Data (n = 8 for each data) are expressed as means ± standard error of the mean. The values were analyzed by one-way ANOVA, followed by a Student–Newman–Keuls multiple test, with *p* < 0.05 indicating a significant difference, and are denoted by “a” and “b.”

(E–H) Q-PCR analysis was examined the mRNA expression of *crh* and *chr* in gill on day 2 (E,F) and day 4 (G,H) during in vitro gill culture with DEX low (10^{-8} M), medium (10^{-6} M), and high (10^{-4} M) doses. Data (n = 8 for each data) are expressed as means ± standard error of the mean. The values were subjected to analysis by one-way ANOVA, followed by a Student–Newman–Keuls multiple test, with *p* < 0.05 indicating a significant difference, are denoted by “a” and “b.”

from the brain-pituitary, and cortisol from interrenal gland) or local *crh* during stress.

Consistent with the physiological model for ion secretion by gill epithelia, the FW transfer significantly increased the mRNA expression of a-Nka at 8 h, day 1, day 4, and day 30 in black porgy gill. These findings suggest that gills actively adapt to FW with possible functional plasticity along with more rapid responses to environmental changes. Similar elevation in branchial a-Nka mRNA transcripts has been reported in *Anguilla anguilla* (Cutler et al., 1995) and *Salmo trutta* (Madsen et al., 1995) for the transfer from FW to SW. These data suggest that the changes in the ionic composition of hypotonic media can induce dramatic modifications in the functions of the gills and alter the morphology of MRCs (Laurent et al., 1985; Perry and Laurent, 1989; Perry et al., 1992; Lee et al., 1996). Thus, there may be two types of MRC. A type whose function rules out ions in a marine environment and another type that
absorbs Na\(^+\), Cl\(^-\), Ca\(^{2+}\) in a FW environment (Perry, 1997; Goss et al., 2001; Marshall, 2002). These MRCs were located along the filamental epithelium for many species and along the secondary lamellae (Wendelaar Bonga and van der Meij, 1989; Katoh and Kaneko, 2003; Carmona et al., 2004; Lima and Kültz, 2004; Allen and Cech, 2007). Consistent with these results, MRCs have been shown to localize on both the lamellae and filaments in hypo-osmotic environment (Cataldi et al., 1995; McKenzie et al., 1999). Furthermore, the expression and localization of crh, crhr and α-Nka cells in the gill showed that the brain-gill crh system might be involved in the osmoregulation.

**CONCLUSION**

This study demonstrated crh and crhr in the gill by *in situ* hybridization during SW and FW in black porgy. The differential expression pattern of crh mRNA was found in Dien and gill during FW transfer and *in vivo* treatment of CRH in black porgy suggesting that the crh mRNA in the gill is independently functioned and regulated during environmental stress. Increased transcripts of crh mRNA in the gill by *in vitro* and *in vivo* crh treatment provide evidence that the crh may be regulated by itself in an autocrine manner. In addition, the increased expression of a-nka mRNA with differential localization of a-Nka cells in the filament and lamellae at 8 h and day 1 in FW fish suggests that a-Nka cells play a key role in ionic homeostasis during FW acclimation. The changes in the localization of the a-Nka cells in the black porgy gill adaptively respond to changing levels of environmental ions. Thus, the presence of crh and crhr in fish gill is essential to respond to the ambient salinity and/or stress to maintain the ion/base regulation as well as homeostasis.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**ETHICS STATEMENT**

The animal study was reviewed and approved by the National Taiwan Ocean University (#99026).

**AUTHOR CONTRIBUTIONS**

C-FC conceived and designed the project, and supervised, wrote, reviewed, and edited the manuscript. AA and C-FC wrote the manuscript. AA, T-PW, J-CC, D-SL, and GN collected, measured, and analyzed the sample. AA and GN wrote the first draft of the manuscript. AA, GN, and C-FC worked for the revision. All authors contributed to the article and approved the submitted version.

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**REFERENCES**

Adler, G. K., Smas, C. M., and Majzoub, J. A. (1988). Expression and dexamethasone regulation of the human corticotropin-releasing hormone gene in a mouse anterior pituitary cell line. *J. Biol. Chem.* 263, 5846–5852. doi: 10.1016/0021-9258(88)60643-x

Allen, P. J., and Cech, J. J. (2007). Age/size effects on juvenile green sturgeon (*Acipenser medirostris*): ultrastructural modifications in seawater acclimated specimens. *J. Fish Biol.* 79, 211–229.

Aruna, A., Nagarajan, G., and Chang, C. F. (2012a). Involvement of corticotropin-releasing hormone and corticosteroid receptors in the brain–pituitary–gill of tilapia during the course of seawater acclimation. *J. Neuroendocrinol.* 24, 818–830. doi: 10.1111/j.1365-2826.2012.02282.x

Aruna, A., Nagarajan, G., and Chang, C. F. (2012b). Differential expression patterns and location of glucocorticoids and mineralocorticoid receptor transcripts in the osmoregulatory organs of tilapia during salinity stress. *Gen. Comp. Endocrinol.* 179, 465–476. doi: 10.1016/j.ygcen.2012.08.028

Aruna, A., Nagarajan, G., and Chang, C. F. (2015). The acute salinity changes activate the dual pathways of endocrine responses in the brain and pituitary of tilapia. *Gen. Comp. Endocrinol.* 211, 154–164. doi: 10.1016/j.ygcen.2014.12.005

Carmona, R., García-Gallego, M., Sanz, A., Domezaín, A., and Osost-Garrido, M. N. (2004). Chloride cells and pavement cells in gill epithelia of *Acipenser naccarii*: ultrastructural modifications in seawater acclimated specimens. *J. Fish Biol.* 64, 553–566.

Cataldi, E., Ciccotti, E., Di Marco, P., Di Santo, O., Bronzi, P., and Cataudella, S. (1995). Accumulation trials of juvenile Italian sturgeon to different salinities: morpho-physiological descriptors. *J. Fish Biol.* 47, 609–618. doi: 10.1111/j.1095-8649.1995.tb01927.x
these proteins in the acute stress response. J. Mol. Endocrinol. 32, 627–648. 
doi: 10.1677/jme.0.0330267

Kaneko, T., and Hiroi, J. (2008). “Osmo- and ionic regulation,” in Fish Larval 
Physiology, eds R. N. Fin and B. G. Kapoor (Enfield: Science Publishers), 
163–183.

Kaneko, T., Shiraiishi, K., Katoh, F., Hasegawa, S., and Hiroi, J. (2002). Chloride 
cells in early life stages of fish and their functional differentiation. Fish. Sci. 68, 
1–9. doi: 10.1046/j.1444-2906.2002.00382.x

Kaneko, T., Watanabe, S., and Lee, K. M. (2008). Functional morphology of 
mitochondrion-rich cells in euryhaline and stenohaline teleosts. Aquat. Biosci. 
Monogr. 1, 1–62. doi: 10.1007/BF00221380

Katoh, F., and Kaneko, T. (2003). Short-term and long-term replacement of 
branchial chloride cells in killifish transferred from seawater to freshwater, 
revealed by morphofunctional observations and a newly established ‘time-
differential double fluorescent staining’ technique. J. Exp. Biol. 206, 4113–4123. 
doi: 10.1242/jeb.00659

Killerich, P., Kristiansen, K., and Madsen, S. S. (2007). Cortisol regulation of 
ion transporter mRNA in Atlantic salmon gill and the effect of salinity on 
the signaling pathway. J. Endocrinol. 194, 417–427. doi: 10.1677/JOE-07-0185

Lachize, S., Apostolakis, E. M., van der Laan, S., Tijsjen, A. M. I., Xu, J., Kloet, 
E. R., et al. (2009). Steroid receptor coactivator-1 is necessary for regulation of 
corticotropic-releasing hormone by chronic stress and glucocorticoids. Proc. 
Natl. Acad. Sci. U.S.A. 98, 8038–8042.

Laurent, P., Hobe, P., and Dunel-Erb, H. S. (1985). The role of environmental 
sodium chloride relative to calcium in gill morphology of freshwater salmonid 
fish. Cell Tissue Res. 240, 675–692. doi: 10.1007/bf02163566

Lee, T. H., Hwang, P. P., and Feng, S. H. (1996). Morphological studies of gill and 
mitochondria-rich cells in the stenohaline Cyprinid teleosts, Cyprinus carpio 
and Carassius auratus, adapted to various hypotonic environments. Zool. Stud. 
35, 272–278.

Li, J., Eygensteyn, J., Lock, R. A. C., Verbost, P. M., Van Der Heijdden, A. J. H., 
Majzoub, J. A. (2006). Corticotropin-releasing hormone physiology. 

McCormick, S. D., Hasegawa, S., and Hirano, T. (1992). Calcium uptake in the 
skin of a freshwater teleost. Proc. Natl. Acad. Sci. U.S.A. 89, 3635–3638. 
doi: 10.1073/pnas.89.8.3635

McKenzie, D. J., Cataldi, E., Di Marco, P., Mandich, A., Romano, P., Ansfelri, S., 
et al. (1999). Some aspects of osmotic and ionic regulation in Adriatic sturgeon 
Asteracanthus maroccan. II: morpho-physiological adjustments to hyperosmotic 
environments. J. Appl. Ichthyol. 15, 61–66. doi: 10.1111/j.1439-0426.1999. 
th00208.x

Olson, K. R. (1998). Hormone metabolism by the fish gill. Comp. Biochem. Physiol. 
A 119, 55–65.

Pepels, P., Meek, J., Wendelaar Bonga, S. E., and Balm, P. H. M. (2002). 
Distribution and quantification of corticotropin-releasing hormone (CRH) in 
the brain of the teleost fish Oreochromis mossambicus (tilapia). J. Comp. Neurol. 
453, 247–268.

Perry, S. F. (1997). The chloride cell: structure and function in the gills of freshwater 
fishes. Ann. Rev. Physiol. 59, 325–347. doi: 10.1146/annurev.physiol.59.1.325

Perry, S. F., Goss, F., and Fenwick, C. (1992). Interrelationships between gill 
chlolesterol cell morphology and calcium uptake in freshwater fishes. Fish 
Physiol. Biochem. 10, 327–337. doi: 10.1007/BF00004482

Perry, S. F., and Laurent, P. (1989). Adaptational responses of trout gill to 
lowered external NaCl concentration: contribution of the branchial chloride 
cell. J. Exp. Biol. 147, 147–168. doi: 10.1242/jeb.147.1.147

Pierson, P. M., Lamers, A., Flik, G., and Mayer-Gostanc, N. (2004). The stress axis, 
stanniocalcin, and ion balance in rainbow trout. Gen. Comp. Endocrinol. 137, 
263–271. doi: 10.1016/j.ygcen.2004.03.010

Rivier, C., and Vale, W. (1983). Modulation of stress-induced ACTH release by 
corticotropic-releasing factor, catecholamines and vasopressin. Nature 305, 
325–327. doi: 10.1038/305325a0

Roseboom, P. H., Unben, C. M., and Kalin, N. H. (2001). Persistent corticotropin-
releasing factor 1 receptor desensitization in the human retinoblastoma cell 
line IMR-32. Mol. Brain Res. 92, 115–127. doi: 10.1016/s0169-328x(01) 
00162-0

Sakai, K., Horiba, N., Sakai, Y., Tozawa, F., Demura, H., and Suda, T. (1996). 
Regulation of corticotropin releasing factor receptor messenger ribonucleic 
acid in rat anterior pituitary. Endocrinology 137, 1758–1763.

Slominski, A., and Wortsman, J. (2000). Neuroendocrinology of the skin. Endocr. 
Rev. 21, 457–487.

Slominski, A., Wortsman, J., Ruger, T., Paus, R., and Solomon, S. (2000). 
Corticotropin releasing hormone and proopiomelanocortin involvement in the 
cutaneous response to stress. Physiol. Rev. 80, 979–1020. doi: 10.1152/physrev. 
2000.80.3.979

Slominski, A., Wortsman, J., Pisarchik, A., Zbytek, B., Linton, E. A., Mazurkiewicz, 
J. E., et al. (2001). Cutaneous expression of corticotropin-releasing hormone 
(CRH), urocortin, and CRH receptors. FASEB J. 15, 1678–1693. doi: 10.1096/
00162-0

Soegianto, A., Adhim, M. H., Zainuddin, A., Putranto, T. W. C., and Irawan, 
B. (2017). Effect of different salinity on serum osmolality, ion levels and 
hematological parameters of East Java strain tilapia Oreochromis niloticus. Mar. 
Fresh. Behav. Physiol. 50, 105–113. doi: 10.1080/10236244.2017.1333391

Sumioto, T., Suda, T., Tomori, N., Yajima, F., Nakagami, Y., Ushiyama, T., 
et al. (1987). Immunoactive corticotropin-releasing factor in rat plasma. 
Endocrinology 120, 1391–1396. doi: 10.1210/endo-120-4-1391

Thise, C., and Thise, B. (2008). High-resolution in situ hybridization to whole 
organometallic embryos. Nat. Protocols 3, 59–69. doi: 10.1038/nprot.2007.
07.514

Tomy, S., Chang, Y. M., Chen, Y. H., Cao, J. C., Wang, T. P., and Chang, 
C. F. (2009). Salinity effects on the expression of osmoregulatory genes in 
the euryhaline black porgy Acanthopagrus schlegeli. Gen. Comp. Endocrinol. 
161, 123–132. doi: 10.1016/j.ygcen.2008.12.003

Vale, W., Spiess, J., Rivier, C., and Rivier, J. (1981). Characterization of a 41-
residue ovine hypothalamic peptide that stimulates secretion of corticotropin 
and beta-adrenochrome. Science 213, 1394–1397. doi: 10.1126/science.626799

Vellucci, S. V., and Parrott, R. F. (2000). Gene expression in the forebrain of 
dexamethasone-treated piglet: effect on stress neuropeptides in the 
hypothalamus and hippocampus and glutamate receptor subunits in the 
hippocampus. Res. Vet. Sci. 69, 25–31. doi: 10.1053/rvsc.2000.0380

Verboest, P. M., Schoenmakers, T. J., Flik, G., and Wendelaar Bonga, S. E. (1994). 
Kinetics of ATP- and Na+-driven gradient Ca2+ transport in basolateral
membranes from gills of freshwater- and seawater-adapted tilapia. *J. Exp. Biol.* 186, 95–108. doi: 10.1242/jeb.186.1.95

Wendelaar Bonga, S. E. (1997). The stress response in fish. *Physiol. Rev.* 77, 591–625.

Wendelaar Bonga, S. E., Flik, G., Balm, P. H. M., and Van Der Meij, J. C. A. (1990). The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. *Cell Tissue Res.* 259, 575–585. doi: 10.1007/bf01740786

Wendelaar Bonga, S. E., and van der Meij, C. J. M. (1989). Degeneration and death, by apoptosis and necrosis, of the pavement and chloride cells in the gills of the teleost *Oreochromis mossambicus*. *Cell Tissue Res.* 55, 235–243. doi: 10.1007/bf00229086

Yokoe, T., Audhya, T., Brown, C., Hutchinson, B., Passarelli, J., and Hollander, C. S. (1988). Corticotropin-releasing factor levels in the peripheral plasma and hypothalamus of the rat vary in parallel with changes in the pituitary-adrenal axis. *Endocrinology* 123, 1348–1354. doi: 10.1210/endo-123-3-1348

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