ORIGINAL PAPER

Pituitary control of branchial NCC, NKCC and Na$^+$, K$^+$-ATPase α-subunit gene expression in Nile tilapia, *Oreochromis niloticus*

Jason P. Breves · Andre P. Seale · Benjamin P. Moorman · Darren T. Lerner · Shunsuke Moriyama · Kevin D. Hopkins · E. Gordon Grau

Abstract This study investigated endocrine control of branchial ionoregulatory function in Nile tilapia (*Oreochromis niloticus*) by prolactin (Prl$_{188}$ and Prl$_{177}$), growth hormone (Gh) and cortisol. Branchial expression of Na$^+$/Cl$^-$ cotransporter (*ncc*) and Na$^+$/K$^+$/2Cl$^-$ cotransporter (*nkcc*) genes were employed as specific markers for freshwater- and seawater-type ionocytes, respectively. We further investigated whether Prl, Gh and cortisol direct expression of two Na$^+$, K$^+$-ATPase (*nka*)-α1 subunit genes, denoted *nka-α1a* and *nka-α1b*. Tilapia transferred to fresh water following hypophysectomy failed to adequately activate gill *ncc* expression; *ncc* expression was subsequently restored by Prl replacement. Prl$_{188}$ and Prl$_{177}$ stimulated *ncc* expression in cultured gill filaments in a concentration-related manner, suggesting that *ncc* is regulated by Prl in a gill-autonomous fashion. Tilapia transferred to brackish water (23 ‰) following hypophysectomy exhibited a reduced capacity to up-regulate *nka-α1b* expression. However, Gh and cortisol failed to affect *nka-α1b* expression in vivo. Similarly, we found no clear effects of Gh or cortisol on *nkcc* expression both in vivo and in vitro. When considered with patterns previously described in euryhaline Mozambique tilapia (*O. mossambicus*), the current study suggests that *ncc* is a conserved target of Prl in tilapiine cichlids. In addition, we revealed contrasting dependencies upon the pituitary to direct *nka-α1b* expression in hyperosmotic environments between Nile and Mozambique tilapia.

Keywords Prolactin · Growth hormone · Cortisol · Tilapia · Gill · Hypophysectomy

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J. P. Breves (✉) · A. P. Seale · B. P. Moorman · D. T. Lerner · E. G. Grau
Hawai'i Institute of Marine Biology, University of Hawai'i, Kaneohe, HI 96744, USA
e-mail: breves@bio.umass.edu

J. P. Breves
Department of Biology and Center for Neuroendocrine Studies, University of Massachusetts, Amherst, MA 01003, USA

B. P. Moorman
Department of Molecular Biosciences and Bioengineering, University of Hawai'i at Mānoa, Honolulu, HI 96822, USA

D. T. Lerner · E. G. Grau
Sea Grant College Program, University of Hawai'i at Mānoa, Honolulu, HI 96822, USA

S. Moriyama
School of Marine Biosciences, Kitasato University, Sagamihara, Kanagawa 252-0373, Japan

K. D. Hopkins
Pacific Aquaculture and Coastal Resources Center, University of Hawai'i at Hilo, Hilo, HI 96720, USA

Introduction

Salinity is regarded to be among the most important physical characteristics of the aquatic environment that governs the distribution of species in nature (Whitehead et al. 2011). African cichlids, including tilapias, are widely distributed across habitat types with contrasting environmental salinities. For example, Mozambique tilapia (*Oreochromis mossambicus*) are native to estuaries and lower reaches of rivers from the Zambezi River to the southeast coast of South Africa and generally do not range more than a mile from the tidal ebb and flow. Nile tilapia (*O. niloticus*), on the other hand, are found in river basins and lakes of Israel and northern to central Africa (Trewavas 1983). Reflective

of these contrasting distributions, Mozambique tilapia are strongly euryhaline and can acclimate to salinities in excess of 70 ‰ (Stickney 1986; Kültz et al. 1992; Uchida et al. 2000), while Nile tilapia are far less tolerant of high salinities and do not readily acclimate to salinities exceeding 25 ‰ (Watanabe et al. 1985). The distinct hyposmoregulatory capacities of Nile and Mozambique tilapia are likely related to the divergent life-history patterns of these two congeners.

Adaptive physiological responses to osmotic conditions in the external environment often entail the rapid and coordinated modulation of genes encoding effectors of ion transport (Fiol and Kültz 2007). Indeed, the contrasting seawater (SW)-adaptability of Mozambique and Nile tilapia is matched with variant plasticity in how gene expression is regulated in the gill following salinity challenges (Breves et al. 2010b). Even within a species, disparate transcriptional responses to salinity changes between populations/strains with contrasting salinity tolerances have been characterized in Atlantic salmon (*Salmo salar*) (Nilsen et al. 2007), Arctic char (*Salvelinus alpinus*) (Bystriansky et al. 2007) and Atlantic killifish (*Fundulus heteroclitus*) (Whitehead et al. 2011). Collectively, these studies raise a fundamental question: what are the regulatory systems that underlie these divergent patterns of gene expression in response to salinity challenges?

In teleosts, the neuroendocrine system plays critical roles in the homeostatic regulation of salt and water balance. The pituitary in particular coordinates osmoregulatory responses to changes in environmental salinity via the release of hormones in direct response to perturbations of extracellular osmolality (Seale et al. 2012a). It is well established that prolactin (Prl) promotes acclimation to fresh water (FW) by acting on target tissues such as the gill, kidney and gastrointestinal tract to up-regulate ion conserving and water excreting processes (Hirano 1986). Consistent with these activities, Prl release from tilapia pituitary increases in response to reductions in extracellular osmolality associated with FW transfer both in vivo and in vitro (Yada et al. 1994; Seale et al. 2002). On the other hand, growth hormone (Gh) and cortisol support SW acclimation, at least in part, through the promotion of ion extrusion pathways in the gill (McCormick 2001; Pelis and McCormick 2001; Tipsmark and Madsen 2009). The capacity for Gh to promote SW tolerance in salmonids is well established (Sakamoto et al. 1993); however, the degree to which Gh promotes SW tolerance in tilapias and other non-salmonids has not been fully resolved (Auperin et al. 1995; Sakamoto et al. 1997; Mancera and McCormick 1998; Breves et al. 2010d). Moreover, Prl and Gh may act synergistically with cortisol to elicit adaptive physiological changes during FW or SW acclimation, respectively (McCormick 2001).

The gill is regarded as the primary site of net sodium and chloride transport through the activities of highly specialized 'ionocytes,' also termed 'mitochondrion-rich cells.' Thus, the capacity to rapidly initiate the recruitment of ionocytes with functions supporting ion absorptive processes in a hyposmotic environment, and ion secretive processes in a hyperosmotic environment, is an essential aspect of euryhalinity (Kaneko et al. 2008). In tilapia, a Na^+/Cl^- cotransporter (Ncc) is specifically expressed in the apical membrane of FW-type ionocytes while a $Na^+/K^+/2Cl^-$ cotransporter (Nkcc) is expressed in the basolateral membrane of SW-type ionocytes (Hiroi et al. 2008; Inokuchi et al. 2008). Importantly, *ncc* and *nkcc* gene expression levels closely match the morphological and functional changes of ionocyte populations that accompany salinity acclimation (Hiroi et al. 2008; Inokuchi et al. 2008). Located in the basolateral membrane of both FW- and SW-type ionocytes, Na^+, K^+-ATPase plays a critical role in energizing ion transport (Kaneko et al. 2008). We have recently reported that two Na^+, K^+-ATPase (*nka*)-α1 subunit genes, *nka-α1a* and *nka-α1b*, exhibit salinity-dependent expression in Mozambique tilapia (Tipsmark et al. 2011), a pattern similarly observed in salmonid ionocytes (Richards et al. 2003; Bystriansky et al. 2006; Madsen et al. 2009; McCormick et al. 2009). Collectively, the expression patterns of *ncc*, *nkcc*, *nka-α1a* and *nka-α1b* provide a means to probe how a suite of ionoregulatory pathways are potentially regulated by pituitary hormones.

Methodologically, tilapias are well-established models for endocrine research with the capacity to reveal the actions of hormones through hypophysectomy and hormone replacement techniques, a classic endocrine paradigm modified by Nishioka (1994) for application in tilapia. In the current study, we combined this in vivo paradigm with in vitro gill filament culture to examine whether Prl_{188}, Prl_{177}, Gh and/or cortisol regulate *ncc*, *nkcc*, *nka-α1a* and *nka-α1b* expression in Nile tilapia. By employing Nile tilapia as a model, we now have the opportunity to directly compare the control of ionoregulation in this species with the patterns of hormone action in Mozambique tilapia (Breves et al. 2010d; Tipsmark et al. 2011). We hypothesized that the contrasting life-history patterns (and accompanying salinity tolerances) of these two tilapias are linked to divergent patterns in how pituitary hormones mediate adaptive responses to changes in environmental salinity.

Materials and methods

Fish and surgical protocols

Mature Nile tilapia (*O. niloticus*; 70–200 g) of both sexes were selected from a stock maintained at the University of

Hawai'i Hilo Farm Laboratory (Panaewa, Hawaii). This stock was obtained through the University of Arizona, and with all experiments with tilapias, care must be taken when comparing closely related species because of potential, undocumented interbreeding at some point in the past. To verify that animals were not tolerant of full-strength SW, a preliminary experiment established that this stock could not acclimate to salinities exceeding 25 ‰. We also confirmed that animals ranging from 70 to 200 g did not possess differing degrees of tolerance to hyperosmotic conditions. Fish were reared in outdoor tanks with a continuous flow of FW under natural photoperiod and fed a commercial diet (Rangen, Catfish EXTR 350, Buhl, ID). The Institutional Animal Care and Use Committee of the University of Hawai'i approved all housing, surgical and experimental protocols.

Hypophysectomy was performed by the transorbital technique (Nishioka 1994). Briefly, tilapia were anesthetized by immersion in buffered tricaine methanesulfonate (100 mg l^{-1}, Argent Chemical Laboratories, Redmond, WA) and 2-phenoxyethanol (2-PE; 0.3 ml l^{-1}, Sigma, St. Louis, MO) in FW. Following removal of the right eye and underlying tissue, a hole was drilled through the neurocranium, and the pituitary was aspirated with a modified Pasteur pipette. The orbit was then packed with microfibrillar collagen hemostat (Ethicon, Somerville, NJ) and fish were allowed to recover in brackish water (BW; 12 ‰) composed of SW (Hilo Bay, Hawaii) diluted with FW. Following recovery, fish were transferred to re-circulating experimental aquaria containing aerated BW and treated with kanamycin sulfate (National Fish Pharmaceuticals, Tucson, AZ). Sham operations were carried out in the same manner, but without aspiration of the pituitary. Intact animals were transferred to experimental aquaria without previous handling.

Effects of hypophysectomy

The first objective was to identify gene transcripts in the gill sensitive to hypophysectomy during acclimation to FW or BW (23 ‰). In a pair of experiments, intact, sham-operated and hypophysectomized tilapia ($n = 5$–12) were acclimated to FW or BW (23 ‰) for 3 days. Following a 5-day post-operative period in BW (12 ‰), salinity transfers were conducted by adding FW or SW directly to re-circulating aquaria. In the first group, FW conditions were reached after 90 min and maintained for the duration of the experiment. For the second group, SW was gradually added to the aquaria to raise the salinity to 23 ‰ after 60 min. This mode of hyperosmotic challenge was adopted to ensure adequate survival (Breves et al. 2010b). Fish were not fed for the duration of the recovery, and transfer periods and water temperatures were maintained between 21

and 22 °C. At sampling, all fish were anesthetized in 2-PE (0.3 ml l^{-1}), and blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin (200 U ml^{-1}, Sigma). Plasma was separated by centrifugation for measurement of plasma osmolality using a vapor pressure osmometer (Wescor 5100C, Logan, UT). Fish were rapidly decapitated and completeness of hypophysectomy was confirmed by post-mortem inspection of the hypothalamic region. Fifteen to twenty filaments were excised from the first gill arch (left side) of each fish and stored in RNA stabilization reagent (RNAlater; Life Technologies, Carlsbad, CA) at −80 °C until RNA isolation.
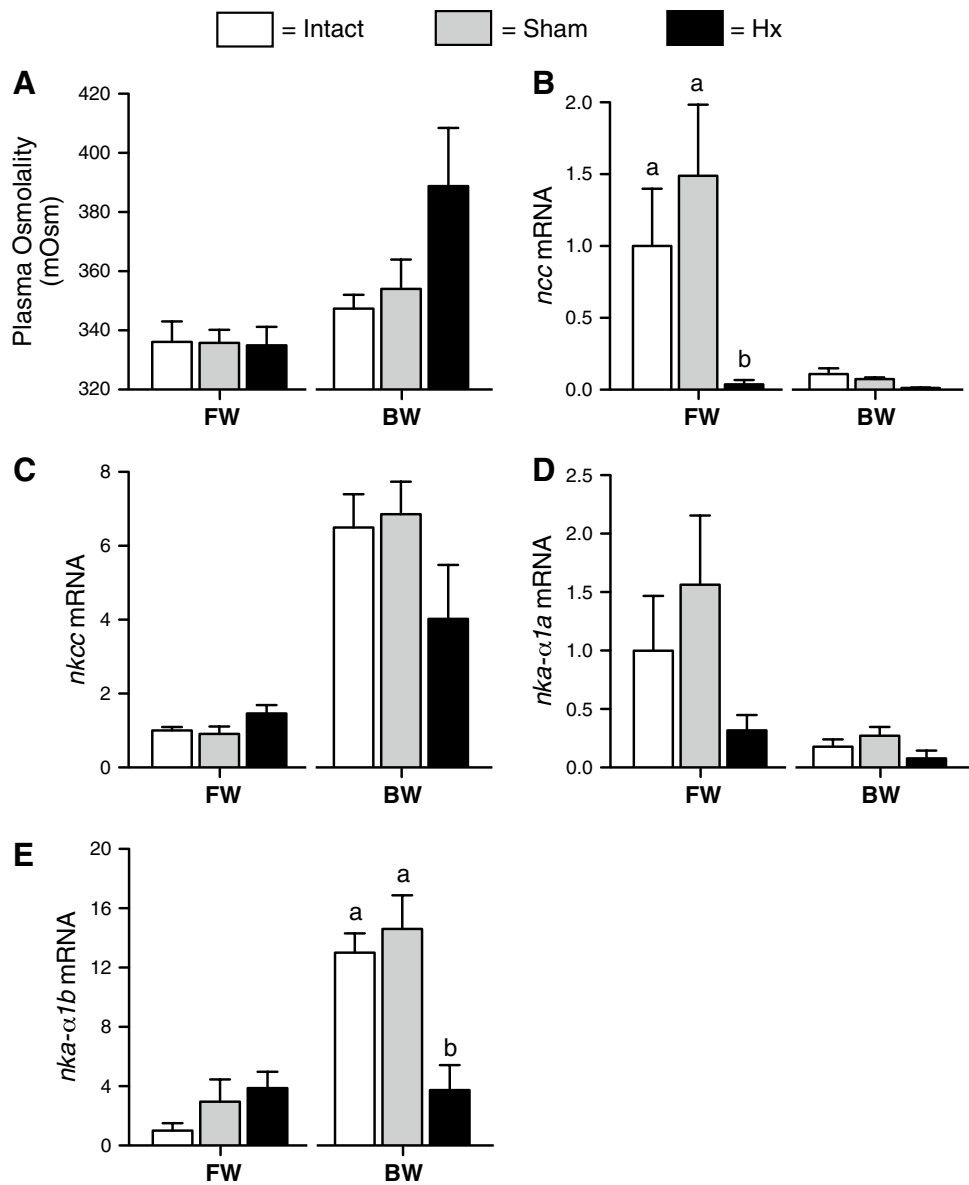
Effects of hormone replacement following hypophysectomy

In a second set of experiments, the effects of hormone replacement on ionoregulatory genes were identified in hypophysectomized fish in both FW and BW (23 ‰). Hypophysectomized fish kept in BW (12 ‰) for 3 days ($n = 6$–9) were administered tilapia Prl_{188} (5 µg g^{-1} body weight) or cortisol (Cort; 1 µg g^{-1}), alone or in combination, by intraperitoneal injection concurrently with FW acclimation. A second group of hypophysectomized fish kept in BW (12 ‰) ($n = 6$–9) was administered tilapia Gh (5 µg g^{-1}) or cortisol (Cort; 1 µg g^{-1}), alone or in combination, concurrently with BW-acclimation (23 ‰). The salinity transfers (FW- and BW-acclimation) were conducted in the same manner as in the preceding hypophysectomy experiment. Since Mozambique and Nile tilapia are so closely related, the amino acid and cDNA sequences of pituitary hormones and their receptors are almost identical (≥98 % nucleotide similarity) (Rentier-Delrue et al. 1989a, b; Yamaguchi et al. 1991, 1988); therefore, Mozambique tilapia Prl_{188} and Gh were purified from cultured pituitaries (described below) and used for injections. Hormones were delivered in saline vehicle (0.9 % NaCl; 1.0 µl g^{-1} body weight). The first injection occurred immediately prior to transfer to either FW or BW. Twenty-four hours later, fish were netted and given a second injection. Fish were then returned to aquaria and left undisturbed for 48 h, after which time they were sampled as previously described. Sham-operated fish were injected with saline vehicle only. Intact fish were not included in the hormone replacement experiments since there were no significant differences between intact and sham-operated fish in the first experiment (Fig. 1).

Purification of Prl_{188}, Prl_{177} and Gh

Tilapia Prl_{188}, Prl_{177} and Gh were purified from media following pituitary tissue culture (Seale et al. 2002; Uchida et al. 2009). Briefly, frozen media samples were thawed

overnight at 4 °C and 1 ml of 1 % acetic acid was added to ~30 ml media prior to centrifugation to remove precipitates. Media was then passed through a C-18 cartridge (5 μm particle size; TOSOH, Tokyo, Japan), equilibrated with 0.1 % trifluoroacetic acid (TFA) and eluted with 20 % acetonitrile (ACN), followed by 80 % ACN. The eluate was frozen at −80 °C for 40 min and lyophilized overnight. Lyophilized samples were dissolved in 0.1 % TFA and separated by HPLC (Gulliver, Jasco, Tokyo, Japan) using a 20–80 % ACN elution profile. Fractions were collected when the highest absorbance peaks were recorded. Lyophilized HPLC-purified fractions were run on an SDS-PAGE and blotted onto a PVDF membrane following Moriyama et al. (2008). Bands were cut from the membrane and applied to a protein sequence analyzer (Shimazu PPSQ-10, Kyoto, Japan). N-terminal amino acid sequences for

the identification of Prl_{188}, Prl_{177} and Gh were VPINDLL, VPINDLI and LFSIAV, respectively.

Effects of hormones on cultured gill filaments

To identify direct effects of Prl, Gh and cortisol on branchial gene transcript levels, we cultured filaments from the second and third gill arches of mature tilapia following Watanabe et al. (in preparation). Filaments were collected from animals maintained in BW (12 ‰). Excised gill arches were first washed in sterilized balanced salt solution (BSS: NaCl 120 mmol l^{-1}; KCl 4 mmol l^{-1}; $MgSO_4$ 0.8 mmol l^{-1}; $MgCl_2$ 1.0 mmol l^{-1}; $NaHCO_3$ 2 mmol l^{-1}; $CaCl_2$ 1.5 mmol l^{-1}; KH_2PO_4 0.4 mmol l^{-1}; Na_2HPO_4 1.3 mmol l^{-1}; $CaCl_2$ 2.1 mmol l^{-1}; Hepes 10 mmol l^{-1}; pH 7.4) and then incubated in 0.025 % $KMnO_4$ for 1 min.

After a second wash in BSS, individual gill filaments were cut from the arches, cut sagittally under a dissecting microscope, and then placed in 24-well plates (Becton–Dickinson, Franklin Lake, NJ) containing Leibovitz's L-15 culture medium (Life Technologies). Culture medium was supplemented with 5.99 mg l^{-1} penicillin and 100 mg l^{-1} streptomycin (Sigma), adjusted to 330 mOsm kg^{-1}, and sterilized with a 0.2-μm filter. Two gill filaments were placed in each well, which contained 500 μl culture medium supplemented with 0, 5, 25, 100 and 500 ng ml^{-1} of tilapia Prl_{188}, Prl_{177}, or Gh, or 0, 0.01, 0.1, 1.0 and 10 μg ml^{-1} cortisol (Sigma) ($n = 6$). After overnight incubation (15 h) at 26 °C, gill filaments were preserved in RNA later prior to RNA extraction and gene expression analyses.

RNA extraction, cDNA synthesis, quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from gill filaments by the TRI Reagent procedure (MRC, Cincinnati, OH) according to the manufacturer's protocol. RNA concentration and purity were assessed by spectrophotometric absorbance (Nanodrop 1000, Thermo Scientific, Wilmington, DE). First-strand cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative levels of mRNA were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). All primer pairs are previously described; *ncc* and *nkcc* (Inokuchi et al. 2008), *nka-α1a* and *nka-α1b* (Tipsmark et al. 2011) and *ef1α* (Breves et al. 2010c). The qRT-PCR reactions were setup as previously described (Pierce et al. 2007). Briefly, 200 nM of each primer, 3 μl cDNA and 12 μl of SYBR Green PCR Master Mix (Life Technologies) were added to a 15-μl final reaction volume. The following cycling parameters were employed: 2 min at 50 °C, 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. After verification that *ef1α* mRNA levels did not vary across treatments, *ef1α* levels were used to normalize target genes. Reference and target genes were calculated by the relative quantification method with PCR efficiency correction (Pfaffl 2001). Standard curves were prepared from serial dilutions of untreated gill cDNA and included on each plate to calculate the PCR efficiencies for target and normalization genes. Relative gene expression ratios between treated and control groups are reported as a fold-change from controls.

Statistics

Group comparisons were performed using one-way ANOVA, followed by Tukey HSD (GraphPad Prism 5.0, San Diego, CA). When data were not normally distributed, ranked data were used to determine differences between groups. Significance for all tests was set at $P < 0.05$.

Results

Effects of hypophysectomy in FW and BW

There were no significant differences in plasma osmolality among any of the groups transferred to either FW or BW. Hypophysectomized fish in BW exhibited a tendency for increased plasma osmolality that was not statistically significant (Fig. 1a). In FW, *ncc* expression was markedly reduced in hypophysectomized fish as compared with intact and sham-operated controls. Expression of *ncc* was considerably lower in all BW groups when compared with intact and sham-operated fish in FW; there was no effect of hypophysectomy on *ncc* expression in BW (Fig. 1b). On the other hand, *nkcc* expression was markedly higher in BW than in FW; no significant effect of hypophysectomy was observed in either salinity (Fig. 1c). Similar to the patterns observed for *ncc* expression, *nka-α1a* expression was considerably lower in BW when compared with FW. While *nka-α1a* expression in hypophysectomized fish in FW was 0.4-fold that of intact animals, this reduction did not reach statistical significance (Fig. 1d). *nka-α1b* expression was increased in intact and sham-operated fish in BW compared with all FW animals; *nka-α1b* expression in BW was sensitive to hypophysectomy, with levels being 0.3-fold those of intact animals (Fig. 1e). Expression levels of all gene targets (normalized to *ef1α* levels) did not differ significantly between male and female animals (data not shown) within any of the in vivo experiments (Figs. 1, 2, 3), thus data from both sexes are pooled.
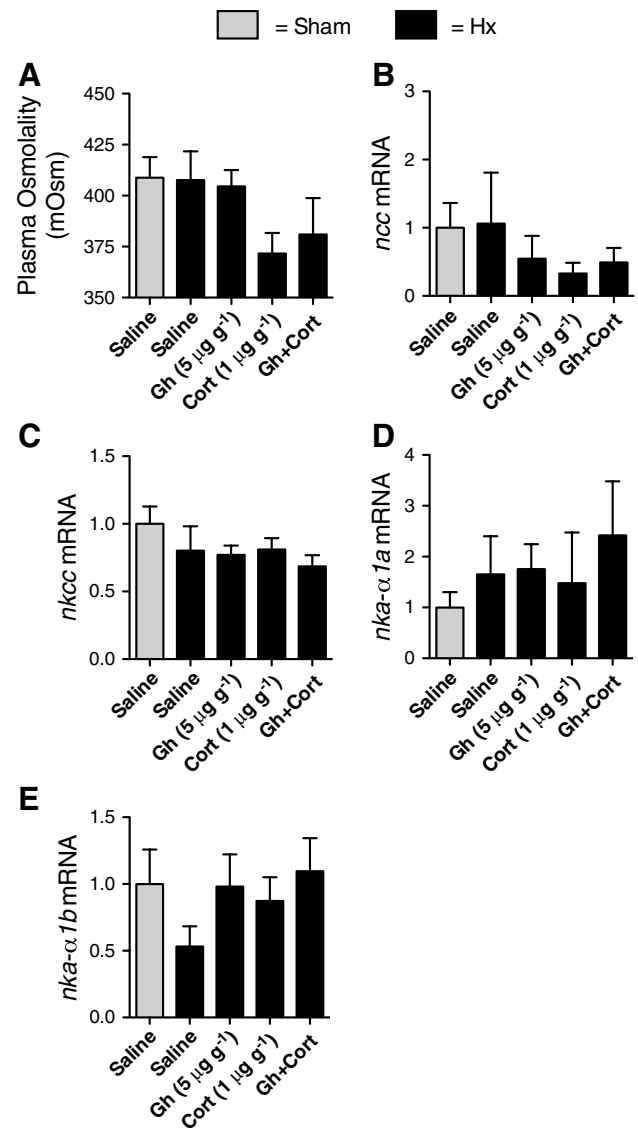
Effects of hormone replacement in FW

As observed in the first experiment (Fig. 1a), there was no perturbation of plasma osmolality in FW following hypophysectomy; there were no effects of hormone replacement (Fig. 2a). Also consistent with the first experiment (Fig. 1b), *ncc* expression was strongly diminished following hypophysectomy in FW as compared with sham-operated controls. Prl_{188} administered alone or in combination with cortisol restored *ncc* expression. There was no effect of cortisol alone on *ncc* expression (Fig. 2b). There were no effects of Prl_{188} or cortisol on *nkcc* expression in FW (Fig. 2c). Expression of *nka-α1a* was markedly reduced in saline-injected hypophysectomized fish as compared with sham-operated animals. Prl_{188} administered either alone or in combination with cortisol removed this significant drop in expression (Fig. 2d). There were no effects of Prl_{188} or cortisol on *nka-α1b* expression in FW (Fig. 2e).
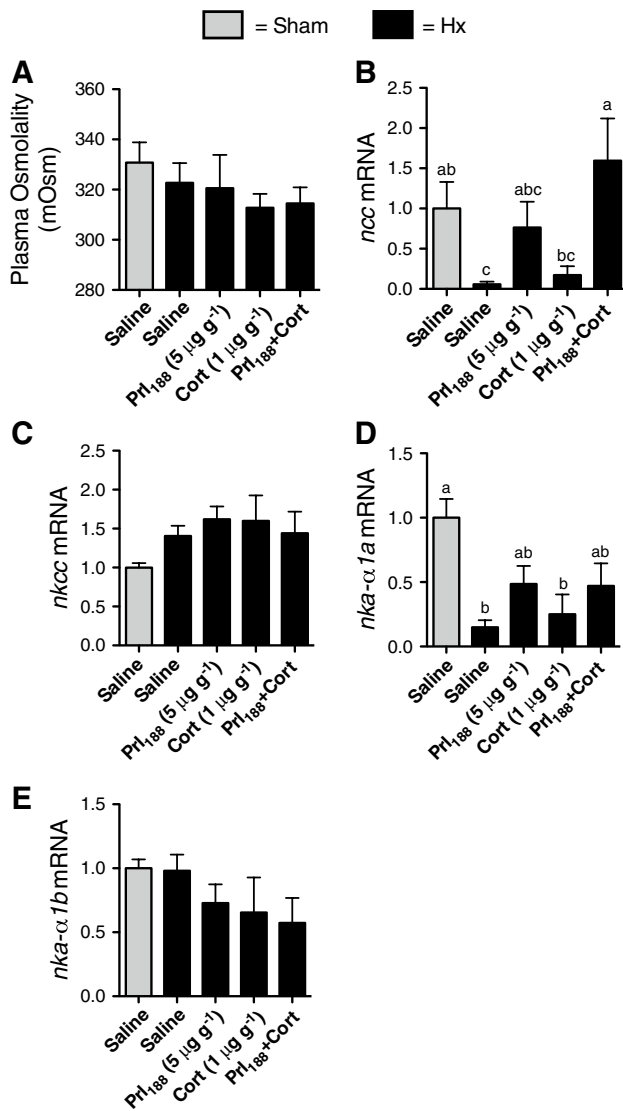
Fig. 2 Effects of hypophysectomy (Hx) and replacement therapy with Prl_{188} and cortisol on plasma osmolality (**a**) and branchial gene expression of *ncc* (**b**), *nkcc* (**c**), *nka-α1a* (**d**) and *nka-α1b* (**e**) in fresh water (FW). Mean ± SEM (*n* = 6–9). Gene expression is presented as a fold-change from the saline-injected sham group. Fish received two intraperitoneal injections of Prl_{188} (5 μg g^{-1}) alone or in combination with cortisol (1 μg g^{-1}). The first injection occurred immediately prior to transfer to FW from brackish water (BW; 12 ‰), the second occurred at 24 h. Fish were sampled 3 days after initial transfer to FW. Sham-operated (*shaded bars*) and hypophysectomized fish (Hx; *solid bars*) receiving saline injections served as controls. Means not sharing the same letter are significantly different (ANOVA, Tukey HSD tests, *P* < 0.05)

Fig. 3 Effects of hypophysectomy (Hx) and replacement therapy with Gh and cortisol on plasma osmolality (**a**) and branchial gene expression of *ncc* (**b**), *nkcc* (**c**), *nka-α1a* (**d**) and *nka-α1b* (**e**) in brackish water (BW; 23 ‰). Mean ± SEM (*n* = 6–9). Gene expression is presented as a fold-change from the saline-injected sham group. Fish received two intraperitoneal injections of Gh (5 μg g^{-1}) alone or in combination with cortisol (1 μg g^{-1}). The first injection occurred immediately prior to transfer to 23 ‰ BW from isosmotic BW (12 ‰), the second occurred at 24 h. Fish were sampled 3 days after initial transfer to BW. Sham-operated (*shaded bars*) and hypophysectomized fish (Hx; *solid bars*) receiving saline injections served as controls. Means not sharing the same letter are significantly different (ANOVA, Tukey HSD tests, *P* < 0.05)

Effects of hormone replacement in BW

There were no significant differences in plasma osmolality among any of the groups injected with Gh and/or cortisol in BW (Fig. 3a). As observed in the first experiment (Fig. 1b, c, d), there were no effects of hypophysectomy on *ncc*, *nkcc*

or *nka-α1a* expression in BW (Fig. 3b–d); accordingly, there were no effects of Gh or cortisol on the expression of these transcripts. The reduction in *nka-α1b* expression observed in BW following hypophysectomy in the first experiment (Fig. 1e) was apparent in the hormone replacement trial in BW (Fig. 3e), but did not reach statistical significance.
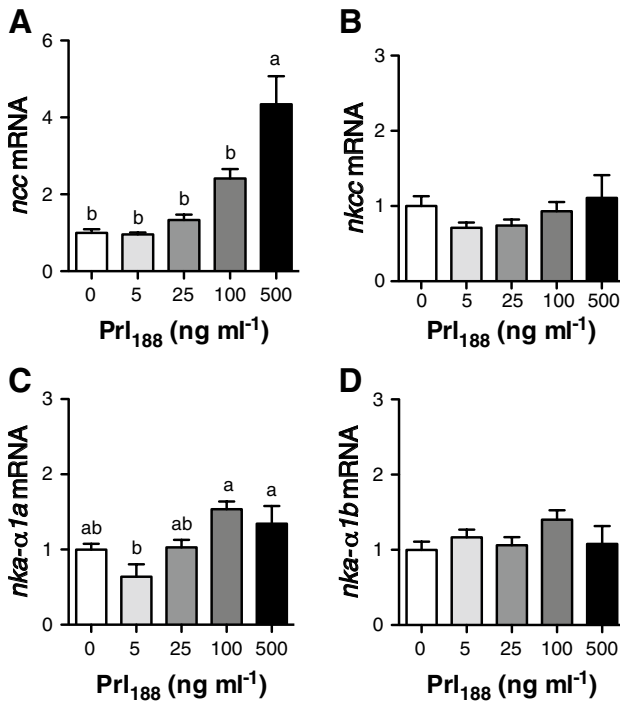
Fig. 4 Effects of Prl_{188} concentration on *ncc* (**a**), *nkcc* (**b**), *nka-α1a* (**c**) and *nka-α1b* (**d**) gene expression in gill filaments cultured for 15 h. Mean ± SEM ($n = 6$). Gene expression is presented as a fold-change from the 0 concentration groups. Means not sharing the same letter are significantly different (ANOVA, Tukey HSD tests, $P < 0.05$)
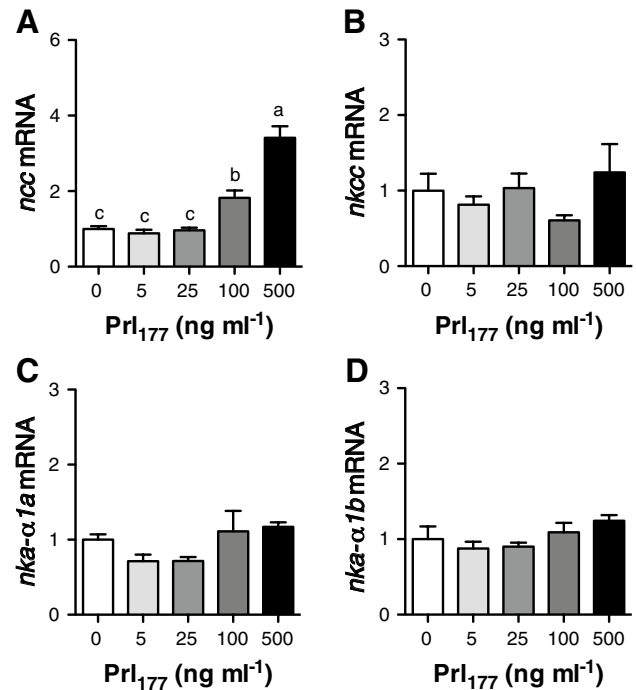
Fig. 5 Effects of Prl_{177} concentration on *ncc* (**a**), *nkcc* (**b**), *nka-α1a* (**c**) and *nka-α1b* (**d**) gene expression in gill filaments cultured for 15 h. Mean ± SEM ($n = 6$). Gene expression is presented as a fold-change from the 0 concentration groups. Means not sharing the same letter are significantly different (ANOVA, Tukey HSD tests, $P < 0.05$)

There were no significant effects of injected hormones on *nka-α1b* expression in hypophysectomized animals.

Effects of hormones on cultured gill filaments

To identify direct actions of hormones to regulate branchial gene expression, we cultured gill filaments in the presence or absence of Prl_{188}, Prl_{177}, Gh or cortisol for 15 h. Prl_{188} and Prl_{177} stimulated the expression of *ncc* from controls at 500 and 100 ng ml^{-1}, respectively (Figs. 4a, 5a). Gh and cortisol did not impact *ncc* expression (Figs. 6a, 7a). There were no effects of Prl_{188}, Prl_{177} or cortisol on *nkcc* expression (Figs. 4b, 5b, 7b). Gh diminished the expression of *nkcc* from controls at 100 and 500 ng ml^{-1} (Fig. 6b). There were no effects of Prl_{188}, Prl_{177}, Gh or cortisol on *nka-α1a* expression (Figs. 4c, 5c, 6c, 7c). There were no clear effects of Prl_{188}, Prl_{177} or Gh on *nka-α1b* expression (Figs. 4d, 5d, 6d, 7d). Cortisol reduced *nka-α1b* expression from controls at all tested doses (Fig. 7d).

Discussion

This study addressed the regulation of *ncc*, *nkcc*, *nka-α1a* and *nka-α1b* transcript expression in Nile tilapia through

in vivo and in vitro approaches including hypophysectomy, hormone replacement and filament culture. Our primary findings include, (1) Prl is both necessary and sufficient to promote *ncc* expression both in vivo and in vitro, and (2) the pituitary mediates the salinity-dependent expression patterns for *nka-α1a* and *nka-α1b* during acclimation to FW and BW, respectively. We compare and contrast these patterns with results previously reported in Mozambique tilapia to describe both the conserved and divergent actions of hypophyseal hormones in tilapias.

Freshwater-type ionocytes are the main conduit for active ion-uptake across the gill to counteract diffusive losses to a hyposmotic external environment. Varying and contending models for the cellular mechanisms that underlie ion-uptake in FW have been presented for a host of teleost species (Evans 2011). In tilapia, however, convincing biochemical, morphological and pharmacological evidence supports the operation of *ncc*-expressing ionocytes as key effectors of Cl$^-$ uptake (Hiroi et al. 2008; Inokuchi et al. 2008; Horng et al. 2009). Accordingly, by 3 days after transfer, we observed markedly higher branchial *ncc* expression in intact FW-fish versus intact BW-fish (Fig. 1b), a pattern consistent with longer-term acclimation (Breves et al. 2010b). Hypophysectomy clearly disrupted this expression pattern by preventing enhanced *ncc*
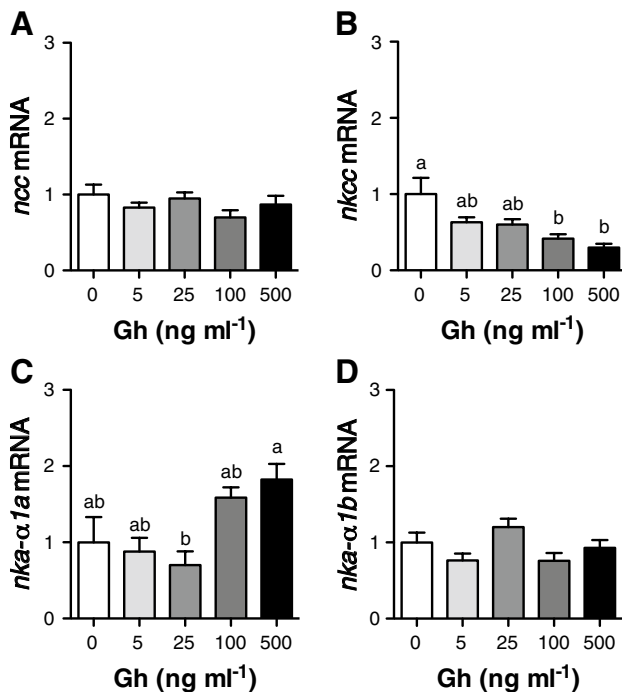
Fig. 6 Effects of Gh concentration on *ncc* (**a**), *nkcc* (**b**), *nka-α1a* (**c**) and *nka-α1b* (**d**) gene expression in gill filaments cultured for 15 h. Mean ± SEM ($n = 6$). Gene expression is presented as a fold-change from the 0 concentration groups. Means not sharing the same letter are significantly different (ANOVA, Tukey HSD tests, $P < 0.05$)
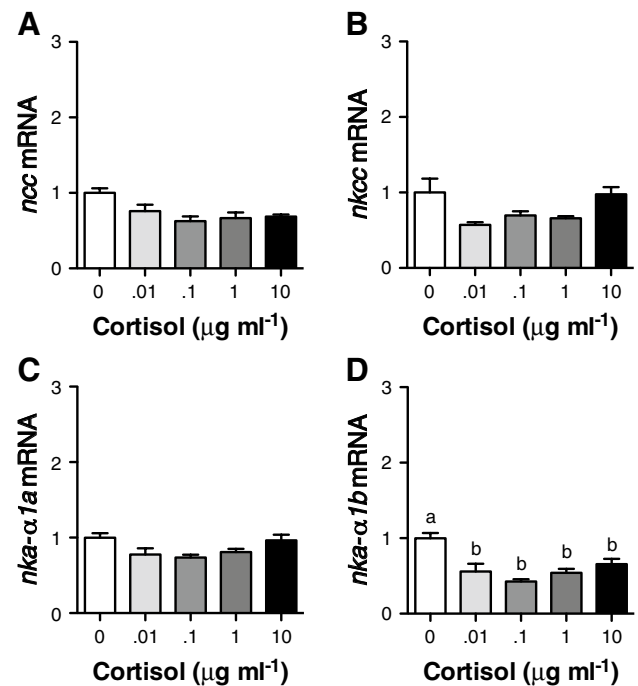
Fig. 7 Effects of cortisol concentration on *ncc* (**a**), *nkcc* (**b**), *nka-α1a* (**c**) and *nka-α1b* (**d**) gene expression in gill filaments cultured for 15 h. Mean ± SEM ($n = 6$). Gene expression is presented as a fold-change from the 0 concentration groups. Means not sharing the same letter are significantly different (ANOVA, Tukey HSD tests, $P < 0.05$)

expression in FW. Prl_{188}, administered alone or in combination with cortisol, restored *ncc* expression (Fig. 2b). A dependence upon Prl for *ncc* expression in Nile tilapia is identical to the pattern in Mozambique tilapia, suggesting that a conserved Prl-Ncc connection, at least in part explains the dependence upon a pituitary for FW survival (Dharmamba and Maetz 1972). Prl-stimulated *ncc* expression may also contribute to the deleterious effects of Prl during acclimation to hyperosmotic environments (Herndon et al. 1991; Pisam et al. 1993). Recently, branchial and epidermal *ncc* expression was linked with Prl signaling in the stenohaline zebrafish (*Danio rerio*) (Breves et al. 2013, 2014), suggesting that the operation of a Prl-Ncc connection is not restricted to tilapiine species. Hiroi and McCormick (2012) recently described the prevalence of Ncc-expressing ionocytes across teleost groups and suggest Ncc-dependent ion-uptake pathways operate in at least some *Ostariophysi* and *Acanthopterygii* species. From a comparative perspective, it will be interesting to learn the extent to which Prl is linked with Ncc in various euryhaline and stenohaline fishes of these taxa.

Previously, we reported that Mozambique tilapia undergo a switch in the catalytic isoform of a branchial Na⁺, K⁺-ATPase during transitions between FW- and SW-environments (Tipsmark et al. 2011). Here, we observed a similar pattern in Nile tilapia with *nka-α1a* and *nka-α1b*, showing enhanced expression in FW and BW, respectively (Fig. 1d). For both Nile and Mozambique tilapia, enhanced expression of *nka-α1a* expression in FW requires an intact pituitary; Prl replacement can either partially or fully restore expression of this isoform (Fig. 2d; Tipsmark et al. 2011). Alternatively, *nka-α1b* expression is regulated in a pituitary-independent fashion in Mozambique tilapia (Tipsmark et al. 2011). This is not the case in Nile tilapia, where in the first experiment, hypophysectomy significantly disrupted *nka-α1b* expression in BW (Fig. 1e). A similar, albeit non-significant, pattern was observed in the replacement therapy trial (Fig. 3e). In Mozambique tilapia, transfer to a hyperosmotic environment is followed by increases in plasma Gh levels and branchial *gh receptor* expression (Yada et al. 1994; Seale et al. 2002; Breves et al. 2010a, b). In addition to stimulating branchial Na⁺, K⁺-ATPase activity, native Gh improves the SW-adaptability of hypophysectomized Mozambique tilapia (Sakamoto et al. 1997). Collectively, these responses suggest that Gh promotes ion extrusion in Mozambique tilapia gill (Sakamoto et al. 1997), a pattern well documented in salmonids (Sakamoto et al. 1993). In the current study, Gh failed to impact *nka-α1b* expression in both hypophysectomized Nile tilapia (Fig. 3e) and cultured filaments (Fig. 6d). Accordingly,

Auperin et al. (1995) found no evidence of enhanced Na^+, K^+-ATPase activity following Gh administration in Nile tilapia. Thus, it appears that Gh may only impact Na^+, K^+-ATPase activity in Mozambique tilapia, the species able to acclimate to full-strength SW. Since cortisol failed to impact *nka-α1b* expression in vivo (Figs. 2e, 3e), and surprisingly diminished expression in vitro (Fig. 7d), we were unable to identify the factor(s) under pituitary control that directs branchial *nka-α1b* expression in BW.

Gill filament culture is a useful method to identify transcriptional responses to endocrine cues that do not require input from the whole animal, with gill-autonomous functions maintained for 1–4 days depending upon the technique employed (McCormick and Bern 1989; Kiilerich et al. 2007; Breves et al. 2013). The tilapia gill expresses receptors for Prl (Sandra et al. 1995; Fiol et al. 2009), Gh (Pierce et al. 2007) and cortisol (Dean et al. 2003), making it plausible that cognate ligands for these receptors directly control ionoregulatory functions. Mozambique and Nile tilapia Prl cells secrete two Prls, Prl_{188} and Prl_{177}, which are encoded by different genes (Specker et al. 1985; Yamaguchi et al. 1988; Rentier-Delrue et al. 1989b). While in Mozambique tilapia Prl_{188} and Prl_{177} are differentially responsive to changes in extracellular osmolality (Borski et al. 1992; Seale et al. 2012b), both possess similar ion-retaining activities (Specker et al. 1985). Here, we found that *ncc* expression was stimulated by both Prl_{188} and Prl_{177} (Figs. 4a, 5a). In turn, we propose that increased Prl levels in FW (Auperin et al. 1994; Yada et al. 1994; Seale et al. 2002) play a key role in maintaining adaptive *ncc* expression. In IP-injected Nile tilapia, Prl_{188} was more potent than Prl_{177} with respect to ion-retaining activity (Auperin et al. 1994). Nonetheless, our current data suggest that Prl_{188} and Prl_{177} at least share the capacity to stimulate *ncc* expression in vitro, an action likely mediated via Prl receptors in the gill (Sandra et al. 1995; Weng et al. 1997; Breves et al. 2013). On the other hand, we failed to identify any in vitro effects of Gh (Fig. 6a) or cortisol (Fig. 7a) on *ncc* expression in cultured gill filaments, patterns consistent with the results of replacement therapy (Figs. 2b, 3b). In light of previous work suggesting that hormone-induced transcriptional responses may be impacted by the salinity in which donor animals are maintained (Kiilerich et al. 2007), we cannot exclude the possibility that filaments collected from BW (12 ‰)-acclimated tilapia may exhibit contrasting responses to Prl, Gh and/or cortisol relative to filaments collected from FW- or BW (23 ‰)-acclimated fish.

In conclusion, this study provides evidence that Nile and Mozambique tilapia possess both conserved and divergent means to regulate branchial ionoregulatory function. For example, when both species undergo acclimation to FW, the enhanced expression of *ncc* to promote ion-uptake is entirely Prl-dependent. On the other hand, during acclimation to hyperosmotic environments, the expression of *nka-α1b* appears to be pituitary-dependent in Nile tilapia, while this is not the case in *O. mossambicus* (Tipsmark et al. 2011). These patterns indicate that while both species employ conserved mechanisms of adapting to low environmental salinities they differ in how the endocrine system mediates acclimation to hyperosmotic environments. Interestingly, the ability to acclimate to salinities exceeding 25 ‰ is where the osmoregulatory capacities of these two congeners generally diverge (Watanabe et al. 1985). If the notion that ancestral tilapias were of a marine origin and that FW-adaptability is a derived characteristic is indeed true, then with colonization of FW habitats *O. niloticus* may have lost the capacity to constitutively (i.e., devoid of pituitary-mediation) activate branchial transcriptional responses supporting ion extrusion. In the future, we believe these two *Oreochromis* species will prove to be valuable model systems to further identify points of divergence in how hypophyseal hormones direct salt and water balance.

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