Prolactin 177, prolactin 188 and extracellular osmolality independently regulate the gene expression of ion transport effectors in gill of Mozambique tilapia

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Abstract

This study characterized the local effects of extracellular osmolality and prolactin (PRL) on branchial ionoregulatory function of a euryhaline teleost, Mozambique tilapia (Oreochromis mossambicus). First, gill filaments were dissected from freshwater (FW)-acclimated tilapia and incubated in four different osmolalities, 280, 330, 380 and 450 mOsm/kg. The mRNA expression of Na⁺/K⁺-ATPase α1a (NKA α1a) and Na⁺/Cl⁻ cotransporter (NCC) showed higher expression with decreasing media osmolalities, while Na⁺/K⁺/2Cl⁻ cotransporter 1a (NKCC1a) and PRL receptor 2 (PRLR2) mRNA levels were upregulated by increases in media osmolality. We then incubated gill filaments in media containing ovine PRL (oPRL) and native tilapia PRLs (tPRL177 and tPRL188). oPRL and the two native tPRLs showed concentration-dependent effects on NCC, NKA α1a and PRLR1 expression; Na⁺/H⁺ exchanger 3 (NHE3) expression was increased by 24 h of incubation with tPRLs. Immunohistochemical observation showed that oPRL and both tPRLs maintained a high density of NCC- and NKA-immunoreactive ionocytes in cultured filaments. Furthermore, we found that tPRL177 and tPRL188 differentially induce expression of these ion transporters according to incubation time. Together, these results provide evidence that ionocytes of Mozambique tilapia may function as osmoreceptors as well as directly respond to PRL to modulate branchial ionoregulatory functions.

Keywords: ionocytes; prolactin; extracellular osmolality; tilapia
Introduction

Osmoregulation is essential to maintaining homeostasis, which, in turn, is essential to optimal cell function. In teleost fishes, as in most vertebrates, plasma osmolality and specific ion concentrations are maintained within narrow physiological ranges. Osmoregulation in teleosts is achieved by integrated ion- and water-transporting functions of gill, kidney and intestine. In particular, ionocytes located in the gill are essential for maintaining ion balance in fishes. Ionocytes, also known as chloride cells or mitochondrion-rich cells, are responsible for ion uptake in fresh water (FW) and ion secretion in seawater (SW) (18, 29).

In the currently accepted model, active ion secretion by ionocytes of SW-acclimated fish is mediated by Na⁺/K⁺-ATPase (NKA) and Na⁺/K⁺/2Cl⁻ cotransporter (NKCC) in the basolateral membrane, and by the Cl⁻ channel, cystic fibrosis transmembrane conductance regulator (CFTR), in the apical membrane (18, 29). These ionocytes are referred to as type-IV ionocytes in Mozambique tilapia (Oreochromis mossambicus) (25, 27). By contrast, the mechanisms for ion uptake by ionocytes of FW-acclimated teleosts vary according to species (15, 28, 29). In Mozambique tilapia, three distinct populations of ionocytes, classified as type-I, -II and -III, were identified in FW. Since type-I ionocytes only showed basolateral NKA immunoreactivity and appeared in both FW- and SW-acclimated tilapia, this cell type was first assumed to be an immature ionocyte that would subsequently develop into other types (25). However, a recent study suggested that type-I cells may excrete K⁺, a capacity shared with type-III and -IV cells (21, 22, 65). Type-II ionocytes are characterized by Na⁺/Cl⁻ cotransporter (NCC) localization in the apical membrane, in
addition to basolaterally-located NKA, whereas type-III ionocytes are characterized by the presence of Na\(^+\)/H\(^+\) exchanger 3 (NHE3) instead of NCC in the apical membrane (25, 27, 30).

NKA is a key enzyme for ion-transport by all four ionocyte subtypes in tilapia, as it provides the major driving force for both ion secretion and absorption. Previous studies have shown that the mRNA expression and localization of \(\alpha\) subunits of NKA change according to environmental salinity and/or according to ionocyte subtypes in rainbow trout (\textit{Oncorhynchus mykiss}), Atlantic salmon (\textit{Salmo salar}) and zebrafish (\textit{Danio rerio}) (36, 38, 42, 49). In Mozambique tilapia, transfer from FW to SW reduced branchial NKA \(\alpha\)1a mRNA expression and increased branchial NKA \(\alpha\)1b expression. Conversely, transfer from SW to FW induced branchial NKA \(\alpha\)1a expression and suppressed branchial NKA \(\alpha\)1b expression (68).

Unlike stenohaline fishes, which can only tolerate environmental salinities near to either FW or SW, euryhaline teleosts are able to tolerate a wide range of salinities. In euryhaline fishes, the function and morphology of ionocytes change when animals move between FW and SW environments (32). While the function of these ionocytes is regulated in part by the endocrine system, it has also been suggested that ionocytes are directly affected by extracellular osmolality (33). Studies on the effects of extracellular osmolality on isolated brachial tissue/ionocytes are limited. Because the gill is one of the major osmoregulatory organs in fishes, and experiences direct exposure to changes in environmental salinity, we hypothesize that branchial ionocytes are regulated through complex pathways involving both systemic hormones and direct responses to changes in extracellular osmolality.

Prolactin (PRL) is a FW-adapting hormone in teleosts that acts by reducing
water permeability and promoting ion uptake by osmoregulatory organs, such as gill, gut and kidney (2, 39). PRL is a likely regulator of ionocytes based on the expression of teleost PRL receptors (PRLRs) in gill tissue (16, 35, 51, 52, 79). Consistent with its hyperosmoregulatory actions, PRL release by the pituitary is directly stimulated by a reduction in extracellular osmolality (23, 45, 54, 58, 59, 77, 80). Mozambique tilapia possess two PRL molecules, tPRL\textsubscript{177} and tPRL\textsubscript{188}, which share 69% amino acid identity and are encoded by separate genes (64, 83). Both PRLs were originally believed to play similar roles in hyposmotic acclimation (64). Nevertheless, increasing evidence indicates that the two PRLs are distinctive in their response to changes in extracellular osmolality and exert divergent physiological actions. For example, tPRL\textsubscript{188} was found to be more sensitive to hyposmotic stimulation than tPRL\textsubscript{177} in Mozambique tilapia (3, 59), while in the closely related congener, Nile tilapia (\textit{O. niloticus}), tPRL\textsubscript{188} induced FW-type ionocyte morphology, and was found to be more potent than tPRL\textsubscript{177} in increasing plasma Na\textsuperscript{+} and Cl\textsuperscript{−} levels (1, 47).

Two PRLR isoforms, PRLR\textsubscript{1} and PRLR\textsubscript{2}, have been described in Mozambique tilapia (20). While branchial PRLR\textsubscript{1} expression is induced following transfer from SW to FW, PRLR\textsubscript{2} expression transiently increases after fish are transferred from FW to SW (7, 20). The induction of PRLR\textsubscript{2} expression following an increase in extracellular osmolality was similarly observed in tilapia PRL cells of the pituitary (59). Whether the two tPRLs have differing actions on branchial expression of ion transporters, pumps and PRLRs remains to be elucidated. Numerous studies have employed ovine PRL (oPRL) to evaluate the function of PRL in fishes because of the limited availability of native hormones. In the current study, we simultaneously examined the effects of both native tPRLs and oPRL on the branchial expression of ion pumps, ion transporters and PRLRs.
The aim of this study was to characterize the independent local effects of extracellular osmolality and PRLs on branchial ionoregulatory function. For this purpose, we incubated gill filaments: 1) under various extracellular osmolalities; and 2) with oPRL or tPRLs. We examined the effects of extracellular osmolality and PRLs on mRNA expression of NCC, NKCC1a, NHE3, NKA α1a, NKA α1b, PRLR1 and PRLR2 and the immunolocalization of NKA and NCC.

**Materials and methods**

*Experimental animals.* Male Mozambique tilapia, weighing 76-130 g, were obtained from broodstock tanks at the Hawaii Institute of Marine Biology, University of Hawaii. Fish were maintained in outdoor tanks with a continuous flow of FW at 24-26°C under natural photoperiod. Fish were fed a commercial trout diet (Skretting, Tooele, UT) once a day. All experiments were conducted according to the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

*Purification of tPRL* and *tPRL*. Tilapia PRL177 and PRL188 were purified from media following pituitary tissue incubation (53, 73). 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. Medium was then passed through C-18 cartridges (55-105 µm particle size; Nihon Waters, Tokyo, Japan), equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with 20% acetonitrile (ACN), followed by
80% ACN. The eluates were 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% to 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 western blotted onto a PVDF membrane following (44). 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 tPRL

\[\text{Gill filament preparation and effects of extracellular osmolality and PRL.}\]

After fish were lethally anesthetized with 0.1% 2-phenoxyethanol, gill filaments were removed from the second and third gill arches and incubated following a gill filament incubation method (9, Watanabe S, Itoh K, Kaneko T, unpublished data). The gill filaments were washed in sterilized balanced salt solution (BSS: NaCl, 120 mmol/l; KCl, 4.0 mmol/l; MgSO\(_4\), 0.8 mmol/l; MgCl\(_2\), 1.0 mmol/l; NaHCO\(_3\), 2.0 mmol/l; KH\(_2\)PO\(_4\), 0.4 mmol/l; Na2HPO\(_4\), 1.3 mmol/l; CaCl\(_2\), 2.1 mmol/l; Hepes 10 mmol/l; pH 7.4), and incubated in 0.025% KMnO\(_4\) for 1 min. After washing in BSS, individual gill filaments were cut sagitally along approximately half of their length to allow culture medium to access ionocytes. Leibovitz’s L-15 culture medium (Gibco/Life Technologies, Carlsbad, CA) supplemented with 6.0 mg/l penicillin and 100 mg/l streptomycin (Sigma, St. Louis, MO) was used in all gill filament incubation experiments.

To examine the effect of extracellular osmolality, gill filaments were incubated in four different osmolalities, 280, 330, 380 and 450 mOsm/kg (n = 8). These values reflect plasma osmolality values that Mozambique tilapia can readily tolerate following
abrupt salinity changes (5, 7, 37, 59, 74, 82). The hyposmotic culture medium (280 mOsm/kg) was produced by diluting Leibovitz’s L-15 culture medium with distilled water. Isosmotic (330 mOsm/kg) and hyperosmotic (380, 450 mOsm/kg) media were produced by adding 5 mol/l NaCl solution to the hyposmotic medium. Osmolality of culture media was verified using a vapor pressure osmometer (Wescor 5520, Logan, UT). After adjusting osmolality, all media were sterilized with a 0.2 μm filter. Gill filaments were incubated for 3 and 6 h at 26°C with saturated humidity, and each well (24-well plates; Becton Dickinson, Franklin Lake, NJ) contained three gill filaments and 500 μl culture medium.

To investigate the effect of PRLs, gill filaments were incubated in 330 mOsm/kg culture medium supplemented with oPRL (0.1-5.0 μg/ml, Sigma), tPRL$_{177}$ (0.01-1.0 μg/ml), or tPRL$_{188}$ (0.01-1.0 μg/ml) for 8 and 24 h (8 h incubation with oPRL, n = 8; 8 h incubation with tPRLs, n = 18; 24 h incubation with both oPRL and tPRLs, n = 9). The concentrations of tPRLs were selected based on previous studies that reported plasma tPRL$_{177}$ and tPRL$_{188}$ levels between 0-10 and 0-30 ng/ml, respectively (59, 82).

After incubation, gill filaments were frozen in liquid nitrogen and stored at -80°C prior to RNA extraction and gene expression analyses. For immunohistochemistry, filaments were fixed in 4% paraformaldehyde (PFA) in 0.1 mol/l phosphate buffer (PB, pH 7.4) overnight at 4°C, and stored in 70% ethanol.

**RNA extraction and real-time quantitative PCR.** For each sample, total RNA was extracted from two gill filaments using Tri-Reagent (Molecular Research Center, Cincinnati, OH) and then reverse transcribed using a cDNA reverse transcription kit (Applied Biosystems/Life Technologies, Carlsbad, CA) according to the manufacturers’
protocols. Primer pairs employed in this study have been previously described; EF1α (6), NHE3, NCC and NKCC1a (30), NKA α1a and NKA α1b (68). Briefly, 200 nmol/l of each primer, 1 μl cDNA and 7.5 μl of SYBR Green PCR Master Mix (Applied Biosystems/Life Technologies) were added to a 15 μl final reaction volume. The following cycling conditions were employed for all assays: 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 using the StepOnePlus real-time PCR system (Applied Biosystems/Life Technologies). The cycle threshold (Ct) values and mRNA levels of reference and target genes were determined by the relative quantification method as specified by StepOne Software v 2.0 (Applied Biosystems/Life Technologies). Standard curves were generated from 5-fold serial dilutions of cDNA transcribed from gill mRNA samples. The expression levels of target genes were normalized to EF1α mRNA levels. Data are expressed relative to the mRNA levels of gill filaments sampled prior to incubation (0 h) in media of various osmolalities (Table 1) or reported as fold-change from 3 h 330 mOsm/kg or the 0 μg/ml PRL groups (Fig. 1-5).

Whole-mount immunofluorescence staining. For immunohistochemical detection of NKA-immunoreactive ionocytes, we used a rabbit polyclonal antiserum raised against a synthetic peptide corresponding to part of the highly conserved region of the NKA α-subunit (NAK121) (72). The specificity of this antibody was determined previously (72). A mouse monoclonal antibody directed against 310 amino acids at the C terminus of human colonic NKCC (T4; developed by Christian Lytle and Bliss Forbush III, and obtained from the Developmental Studies Hybridoma Bank, IA, USA) was used to detect NCC. In addition to basolaterally located NKCC1a, the T4 antibody
has been shown to also react with NCC in the apical region of ionocytes of tilapia (25, 27, 30).

Fixed gill filaments were rehydrated in 0.01 mol/l phosphate-buffered saline containing 0.2% Triton X-100 (PBST) for 1 h, and incubated in PBST containing 10% normal goat serum, 0.1% bovine serum albumin, 0.02% keyhole limpet hemocyanin and 0.01% sodium azide (NB-PBS). The filaments were incubated with a mixture of anti-NKA and the T4 antibodies overnight at room temperature. Both anti-NKA and T4 antibodies were diluted 1:500 with NB-PBS. After rinsing with PBST, the samples were incubated overnight at room temperature with a mixture of goat anti-rabbit IgG labeled with Alexa Fluor 488 and goat anti-mouse IgG1 labeled with Alexa Fluor 647 (Molecular Probes/ Life technologies, Carlsbad, CA), both diluted 1:500 with NB-PBS. After rinsing with PBST, the samples were observed with a confocal laser scanning microscope LSM 710 (Zeiss, Jena, Germany) with excitation at 488 and 633 nm, and emission collected with bandpass filters of 493-551 and 638-755 nm.

Statistical analysis. The effect of extracellular osmolality on branchial gene expression was tested by two-way analysis of variance (ANOVA) with osmolality and time as main effects. Significant main effects of osmolality and time were followed up with Tukey’s HSD and two-tailed Student’s t-test, respectively. Data that did not pass normality tests were log transformed. In gill filament incubations with oPRL, group comparisons were performed by one-way ANOVA, followed by Tukey’s HSD. The experiment testing the effects of tPRLs was analyzed by two-way ANOVA with concentration and tPRL isoform as main effects. Significant main effects of concentration and tPRL isoform were followed up with Dunnett's test and one-tailed
Student's t-test, respectively. All data are expressed as mean ± standard error of the mean. All tests were performed using the software Prism, version 6 (GraphPad, San Diego, CA.).

**Results**

**Effects of extracellular osmolality on gene expression.** Gene expression of NCC, NKCC1a, NHE3, NKA α1a, NKA α1b, PRLR1 and PRLR2 was measured in gill filaments incubated under a range of medium osmolalities (280, 330, 380 and 450 mOsm/kg) (Table 1, Fig. 1). The ratios of expression for the seven transcripts at 3 h (330 mOsm/kg) versus 0 h ranged from 17.6% to 61.5%. NCC showed higher expression with decreasing medium osmolality by 3 h, while there was no effect at 6 h. The expression levels of NCC at 3 h were about 3-fold higher at 280, 330 and 380 mOsm/kg than at 450 mOsm/kg. NCC expression significantly decreased with time at 280 mOsm/kg ($P = 0.011$), 330 mOsm/kg ($P = 0.039$) and 380 mOsm/kg ($P = 0.0005$). The Ct values ranged from 27.4-29.6 at 0 h, 30.0-35.9 at 3 h and 31.4-36.7 at 6 h (Fig. 1A). By contrast, NKCC1a expression was initially unresponsive to changes in medium osmolality at 3 h, but was upregulated at 6 h by increasing extracellular osmolality. The mRNA expression of NKCC1a at 450 mOsm/kg medium was 1.6- to 1.8-fold higher than at other osmolalities. NKCC1a expression showed time-dependent decreases at 280, 330 and 380 mOsm/kg ($P = 0.021, 0.049$ and 0.0085, respectively), but an increase at 450 mOsm ($P = 0.047$). The Ct values ranged from 26.7-28.6 at 0 h, 28.0-31.8 at 3 h and 27.5-33.0 at 6 h (Fig. 1B). Two-way ANOVA revealed a significant interaction between medium osmolality and time in NKCC1a expression ($P = 0.001$).
expression of NKA α1a in gill filaments was elevated by exposure to hyposmotic media. Ct values ranged from 26.7-29.1 at 0 h, 28.0-34.4 at 3 h and 29.6-35.9 at 6 h (Fig. 1D). NKA α1a expression significantly decreased with time at 280 mOsm/kg ($P = 0.012$) and 380 mOsm/kg ($P = 0.0031$). NHE3 and NKA α1b mRNA expression was not affected by changes in medium osmolality at both 3 and 6 h (Fig. 1C, E). While PRLR1 mRNA expression was unaffected by medium osmolality at 3 and 6 h, PRLR2 expression rose significantly as medium osmolality increased at both 3 and 6 h (Fig. 1F, G). The expression of PRLR2 at 450 mOsm/kg was 1.8- to 2.7-fold higher than 280, 330 and 380 mOsm/kg, by 3 and 6 h. The Ct values ranged from 26.2-27.7 at 0 h, 27.6-31.8 at 3 h and 27.2-31.9 at 6 h.

Effects of oPRL on branchial gene expression. We tested the in vitro transcriptional responses of gill filaments to various concentrations of oPRL following incubations of 8 and 24 h. The expression of NCC, NKA α1a and PRLR1 increased in response to oPRL in a concentration-dependent manner by 8 h. The expression levels of NCC in 0.1, 1.0 and 5.0 $\mu$g/ml groups were about 2.3-, 12.1- and 19.6-fold higher than those in the 0 $\mu$g/ml group, respectively (Fig. 2A). The Ct value range of NCC expression was 25.8-38.6. NKA α1a expression levels in 1.0 and 5.0 $\mu$g/ml groups were about 2-fold higher than 0 and 0.1 $\mu$g/ml groups (Fig. 2D). The Ct value range of NKA α1a expression was 25.5-37.3. The mRNA expression of PRLR1 in 0.1, 1.0 and 5.0 $\mu$g/ml groups was 1.4-, 2.2- and 3.1-fold higher than the 0 $\mu$g/ml group, respectively (Fig. 2F). The Ct value range of PRLR1 was 25.7-35.1. The expression of NKCC1a, NHE3, NKA α1b and PRLR2 was unaffected by oPRL (Fig. 2B, C, E, G). Unlike the expression patterns observed in 8 h incubations, all genes were significantly stimulated
by 5.0 μg/ml oPRL by 24 h (Fig. 3). The expression of NCC, NKA α1a and PRLR1 were increased by 7.2-, 10.0- and 3.2-fold, respectively, in gill filaments treated with 5.0 μg/ml oPRL when compared with 0 μg/ml controls (NCC, Ct values = 30.2-40.0; NKA α1a, Ct values = 27.5-33.9; PRLR1, Ct values = 27.0-33.0; Fig. 3A, D, F). There was a modest effect of 5.0 μg/ml oPRL on NKCC1a expression with a 1.5-fold increase from the 0 μg/ml group (Ct values = 25.5-28.2; Fig. 3B). The expression of NHE3, NKA α1b and PRLR2 was 2 to 3-fold higher in 5.0 μg/ml groups than in 0 μg/ml groups (NHE3, Ct values = 27.7-32.1; NKA α1b, Ct values = 27.5-33.9; PRLR2, Ct values = 24.8-30.1; Fig. 3C, E, G).

Effects of tilapia PRLs on branchial gene expression. Next, we compared the expression of ion transporters, pumps and PRLRs in gill filaments in response to various concentrations of tPRL_{177} or tPRL_{188} following 8 and 24 h incubations. NCC and NKA α1a expression increased in response to tPRL_{177} in a concentration-dependent manner by 8 h, while tPRL_{188} did not affect the expression of these transporters. Relative to 0 μg/ml controls, NCC expression increased 4.3-fold following incubation with 1.0 μg/ml tPRL_{177} (P < 0.0001). The mRNA expression of NKA α1a was 1.8-fold higher at 1.0 μg/ml tPRL_{177} than that of 0 μg/ml controls (P = 0.001). At 0.5 and 1.0 μg/ml, tPRL_{177} was more potent at inducing NCC and NKA α1a expression than tPRL_{188} (NCC, P = 0.043 at 0.5 μg/ml, P = 0.049 at 1.0 μg/ml; NKA α1a, P = 0.010 at 0.5 μg/ml, P = 0.019 at 1.0 μg/ml; Fig. 4A, D). Both tPRL_{177} and tPRL_{188} produced an increase in PRLR1 mRNA expression in a concentration-dependent manner. Treatment with 1.0 μg/ml of tPRL_{177} and tPRL_{188} induced 1.7- and 1.4-fold higher PRLR1 expression from 0 μg/ml controls (tPRL_{177}, P < 0.0001; tPRL_{188}, P < 0.0001; Fig. 4F).
Expression of NKCC1a, NHE3, NKAα1b and PRLR2 was unaffected by tPRL\textsubscript{177} and tPRL\textsubscript{188} treatment by 8 h (Fig. 4B, C, E, G).

By 24 h of incubation with tPRL\textsubscript{177} and tPRL\textsubscript{188}, significant effects of both PRL isoforms were observed on NCC, NHE3 and PRLR1 gene expression. The expression of NKA α1a was only affected by tPRL\textsubscript{188}. Relative to controls, expression of NCC was 2.7-fold higher with 1.0 µg/ml of tPRL\textsubscript{177} ($P = 0.0034$), and 4.2-fold higher with 1.0 µg/ml of tPRL\textsubscript{188} ($P < 0.0001$) (Fig. 5A). Treatment with 1.0 µg/ml of tPRL\textsubscript{177} and tPRL\textsubscript{188} elicited 1.8- and 2.1-fold increases in NHE3 expression, respectively, from controls ($P = 0.0029$ for tPRL\textsubscript{177}, $P < 0.0001$ for tPRL\textsubscript{188}; Fig. 5C). While the expression of NKA α1a was significantly increased by 1.0 µg/ml of tPRL\textsubscript{188} (7.1-fold increase from controls, $P = 0.0033$), it was not affected by tPRL\textsubscript{177} (Fig. 5D).

Expression of PRLR1 was 1.9-fold higher than controls with 1.0 µg/ml of tPRL\textsubscript{177} or tPRL\textsubscript{188} ($P = 0.0013$ for tPRL\textsubscript{177}, $P = 0.0009$ for tPRL\textsubscript{188}; Fig. 5F). A significant difference between the effects of tPRL\textsubscript{177} and tPRL\textsubscript{188} at the same concentration was only observed in NCC expression. By contrast with the results of our 8 h incubations (Fig. 4A), tPRL\textsubscript{188} was more effective than tPRL\textsubscript{177} at inducing NCC at 1.0 µg/ml by 24 h ($P = 0.048$; Fig. 5A). Neither tPRL\textsubscript{177} nor tPRL\textsubscript{188} effected the mRNA expression of NKCC1a, NKA α1b or PRLR2 (Fig. 5B, E, G).

**NCC and NKA localization patterns.** To examine the effects of incubation time and PRL on the density of ionocytes, NKA and NCC were detected simultaneously by double immunofluorescent staining with anti-NKA and T4 antibodies. A high density of NCC- and NKA-immunoreactive ionocytes was maintained after an 8 h incubation without PRL, although the density was lower than time 0 controls (Fig. 6A, B). By 24 h,
the density of NKA-immunoreactive ionocytes was obviously decreased without PRL added to the media, and NCC immunosignals were hardly observed (Fig. 6C).

Treatment with 5.0 \( \mu \)g/ml oPRL or with 1.0 \( \mu \)g/ml of either tPRL_{177} or tPRL_{188} increased the density of NCC-immunoreactive and NKA-immunoreactive ionocytes (Fig. 6D, E, F) relative to controls by 24 h (Fig. 6C); the number of NCC- and NKA-immunoreactive ionocytes in filaments incubated with PRLs for 24 h, however, was lower than that of time 0 controls (Fig. 6A). There were no overt differences in the densities of NCC-positive and -negative ionocytes among groups treated with oPRL, tPRL_{177} or tPRL_{188} (Fig. 6D, E, F).

**Discussion**

We characterized the effects of osmolality and PRL isoforms on branchial ionoregulatory functions in Mozambique tilapia. To examine the independent effects of extracellular osmolality and PRL on branchial ionoregulatory functions, gill filaments from FW-acclimated tilapia were incubated *in vitro*, and mRNA expression of NCC, NKCC1a, NHE3, NKA \( \alpha 1a \), NKA \( \alpha 1b \), PRLR1 and PRLR2 was measured. To further investigate the effects of PRLs on ionocytes, we examined the branchial localization of NCC and NKA by immunohistochemical observation. We found that, in addition to the previously reported actions of PRL on the expression of genes supporting FW-acclimation responses (6, 10, 68), branchial ionocytes can respond directly to changes in extracellular osmolality by altering the expression of genes involved in ion uptake and secretion. Furthermore, we found that tPRL_{177} and tPRL_{188} differentially activate these genes *in vitro* according to incubation time. Together, these results
provide evidence that ionocytes of Mozambique tilapia may function as osmoreceptors and respond differentially to PRL isoforms.

Branchial ionocytes are responsible for ion absorption in FW and ion secretion in SW to maintain plasma osmolality within a physiological range. In the currently accepted model of ionocyte osmoregulation, ion secretion is mediated by NKA, NKCC and CFTR in SW-acclimated fish, while NKA, NCC and NHE3 are considered to be involved in ion uptake in FW-acclimated tilapia (29). In Mozambique tilapia, expression of NKA $\alpha_{1a}$ is upregulated during FW acclimation, whereas NKA $\alpha_{1b}$ increases during SW acclimation (68). Our results demonstrated that the expression of NKCC1a in vitro was higher in gill filaments exposed to hyperosmotic media, which is consistent with previously observed in vivo patterns in SW transfer experiments (5). A direct response of NKCC1 to osmotic stimulation has been shown in the gill of Japanese eel (*Anguilla japonica*) and killifish (*Fundulus heteroclitus*) (41, 71). For example, in the opercular membrane of killifish, ionocytes respond to hypotonic shock using integrin $\beta_{1}$ as an osmosensor that is connected to the dephosphorylation of focal adhesion kinase (FAK) pY407, which in turn leads to the deactivation of NKCC1 and inhibition of NaCl secretion (41). Furthermore, in Mozambique tilapia, a ‘yolk-ball’ incubation system, in which the yolk sac is separated from the embryonic body and subjected to in vitro incubation, showed that ionocytes formed new SW-specific multicellular complexes following transfer from FW to SW without the presence of embryonic endocrine and nervous systems (26, 63). Taken together, these observations suggest that ion transporter expression and the morphology of ionocytes can be directly regulated by extracellular osmolality, independent of endocrine control, to facilitate acclimation to salinity change.

In the current study, a decrease in extracellular osmolality induced higher
expression of NCC and NKA α1a, two key effectors of ion uptake by FW ionocytes. While increased expression of these genes in response to decreases in environmental salinity has been observed in previous in vivo studies (7, 27, 30, 68), the primary regulator of this response was suggested to be PRL (6, 68). By studying this effect in vitro, our objective was to uncouple hormonal modulation from direct osmotic effects on branchial ionocytes. The apparent decrease in NCC- and NKA-immunoreactive ionocytes in filaments by 24 h incubation indicates that the endocrine system is likely vital to the survival of type-II ionocytes. We found, however, that gill filaments can sustain a high density of ionocytes with functional localization of ion transporters for 8 h in the absence of PRL supplementation in the media. Therefore, our in vitro system reflects the in vivo responses of ionocytes to changes in extracellular salinity at least during short-term (3 and 6 h) conditions. To our knowledge, direct responses of FW branchial ionocytes to a fall in extracellular osmolality in vitro have yet to be reported for a teleost. On the other hand, the osmoreceptivity of PRL cells in the pituitary has been well characterized (24, 61). Decreases in extracellular osmolality elicit changes in PRL release from incubated intact pituitaries and dispersed PRL cells alike (23, 45, 54, 58, 59, 62). PRL release in a hyposmotic environment is induced by an increase in PRL cell volume brought about by the influx of water via a membrane-bound aquaporin (AQP3) (76, 78) and Ca^{2+} influx through the stretch-activated channel, transient receptor potential vanilloid 4 (TRPV4) (55, 56, 57, 60, 77). AQP3 and TRPV4 expression was also observed in tilapia gill (75, 76, 77). Branchial AQP3 and TRPV4 expression increases with decreasing environmental osmolality in several euryhaline species, including Mozambique tilapia (4, 5, 12, 13, 31, 67, 70). It will be interesting to learn whether common osmodetection systems are deployed in both pituitary PRL cells...
and branchial ionocytes.

An interesting question that our current results raise is whether ionocytes respond to environmental osmotic changes via the apical membrane or to plasma osmotic changes via the basolateral membrane. Hyposmotic shock along the basolateral membrane is known to inhibit Cl− secretion by killifish opercular epithelium. This effect was considered to be primarily osmotic because reducing NaCl in the presence of mannitol to maintain constant osmolality had no effect on Cl− secretion rates (40). In Mozambique tilapia, the apical surface of type-II ionocytes completely closes by 6 h after transfer from FW to 70% SW, while plasma osmolality reaches a peak level at 24 h (11). These findings indicate that ionocytes can sense both environmental and plasma osmotic changes to modulate ionocyte structure and/or function.

The two tPRLs were originally shown to be equally effective in their ability to prevent the loss of Na+ from the plasma of hypophysectomized tilapia in FW (64). However, a subsequent study indicated that tPRL188 is more potent than tPRL177 in increasing plasma osmolality of SW-acclimated tilapia (50). Furthermore, the responses of tPRL188 release and mRNA expression to hyposmotic stimulation are more robust than those of tPRL177 (59). Because of the limited availability of native PRLs, oPRL has been frequently used to characterize the function of PRL in fishes. In this study, we examined the effects of oPRL as well as native tPRLs on branchial ionoregulatory function, and found contrasting actions amongst the three forms.

In the present study, oPRL and tPRL177 increased the expression of NCC and NKA α1a by 8 h when compared with controls, but NKCC1a, NHE3 and NKA α1b were not responsive. These results are consistent with previous studies in which the stimulatory effects of oPRL on branchial NCC and NKA α1a gene expression were
demonstrated in vivo (6, 68). Consistent with hyperosmoregulatory actions of PRL, oPRL replacement following hypophysectomy restored NCC and NKA α1a expression in the gill of tilapia in FW, but did not affect the expression of NKCC1a and NKA α1b (6, 68). In zebrafish, branchial NCC expression was elevated by intraperitoneal injection of oPRL and by the addition of oPRL to cultured gill filaments (8). In agreement with these previous studies, oPRL and both tPRLs brought about an increase in the expression levels of NCC and NKA α1a in a concentration-dependent manner by 8 and 24 h. Our results also show that both tPRLs and oPRL promoted NHE3 expression, in addition to NCC and NKA α1a, by 24 h. A specific NHE inhibitor, 5-(N-ethyl-N-isopropyl) amiloride (EIPA), was found to reduce Na⁺ uptake by zebrafish H⁺-ATPase-rich cells (17, 34) and medaka embryonic skin ionocytes (81), indicating that Na⁺ absorption is mediated by NHE3-expressing ionocytes. Together, these findings support the notion that PRL promotes NHE3 expression to facilitate Na⁺ uptake in tilapia.

To our surprise, NKCC1a and NKA α1b were also induced by oPRL by 24 h, especially at higher concentrations. This result indicates that oPRL can increase the expression of NKCC1a and NKA α1b, which are known effectors of ion extrusion. In cell membrane isolated from FW-acclimated Nile tilapia (O. niloticus) liver, oPRL was displaced more effectively by recombinant tilapia growth hormone (GH) than by either form of tPRLs, suggesting that oPRL may interact with both fish GH and PRL receptors (48). PRL and GH belong to a family of pituitary polypeptide hormones that share a common structure. GH is generally regarded as a “SW-adapting hormone” because it stimulates the expression of branchial ion pumps and transporters tied to extrusion pathways. In Atlantic salmon, ovine GH treatment increased NKA and NKCC levels in
the gills (46, 66). One possible explanation for the effects of oPRL on NKCC1a and NKA α1b expression in this study is that oPRL at high concentrations may bind GH receptors and activate branchial ion-secretive transporters. However, in Mozambique tilapia, clear evidence suggesting that GH regulates NKCC and NKA α1b expression is not yet available (6, 68).

Interestingly, the relative effects of tPRL177 and tPRL188 exhibited opposing patterns between 8 and 24 h incubation, although both PRLs induced expression of the same genes. The effect of tPRL177 on NCC and NKA α1a expression was more robust than that of tPRL188 at 8 h, while the effect of tPRL188 on NCC expression was significantly greater than that of tPRL177 at 24 h. This difference in isoform potency indicates that tPRL177 requires a shorter time than tPRL188 to initiate changes in gene expression. When tilapia were transferred from SW to FW, tPRL177 and tPRL188 plasma levels became elevated similarly within 1 day (82). Nevertheless, after 2 days, the levels of tPRL188 were ~3 times higher than those of tPRL177 (59, 82). These results suggest that tPRL188 may play a more important role in long-term FW acclimation while tPRL177 may play a more critical role during early acclimation. Our immunohistochemical observations revealed that both tPRLs possess the capacity to maintain the density of type-II ionocytes in vitro.

In the present study, we also measured gene expression of PRLR1 and PRLR2. Previous studies reported that PRLR1 expression in the gill decreases upon transfer from FW to SW (5) and increases after transfer from SW to FW (7). By contrast, branchial expression of PRLR2 has been shown to be transiently upregulated in tilapia transferred from FW to SW (19, 20). In accordance with these acclimation studies, our results showed that PRLR1 expression was upregulated by oPRL, tPRL177 and tPRL188
in a concentration-dependent manner, while PRLR2 was not affected except following a
24 h incubation with oPRL. An increase in PRLR2 expression after 24 h incubation
with oPRL may be due to a non-specific effect of oPRL, resolving this issue requires
future investigation. The expression of PRLR2 in gill filaments in vitro increased in
response to hyperosmotic stimuli, while PRLR1 expression was unaffected by medium
osmolality. PRLR2 expression was induced by increases in extracellular osmolality by 3
and 6 h, indicating that PRLR2 might play an important role in the initial response to a
rise in external osmolality. Rapid induction of PRLR2 expression following
hyperosmotic stimuli has also been shown to occur in dispersed PRL cells isolated from
both FW- and SW-acclimated tilapia (59). HEK293 cells transfected with tilapia
PRLR2 exhibited greater tolerance to hyperosmotic stress when compared with those
transfected with tilapia PRLR1 or empty vector controls (20). In the gill of Mozambique
tilapia, the expression of a shorter PRLR2 splice variant, which lacks a large part of the
extracellular ligand-binding domain, was reported in addition to a full-length PRLR2
(20). The short form of PRLR2 may sequester functional PRLRs by forming
heterodimers with long form PRLRs, thereby inhibiting PRL-stimulated signal
transduction pathways (20, 33). Together with previous studies, our results suggest that
the direct response of PRLR2 to a hyperosmotic stimulus may render ionocytes less
responsive to circulating PRL, which would in turn facilitate acclimation to higher
salinities.

**Perspectives and Significance**

We showed that extracellular osmolality and two isoforms of tPRL modulated gill
ionoregulatory function directly and independently of one another. While we have shown that ionocytes are directly responsive to changes in extracellular osmolality, PRL is indispensable for survival in FW as demonstrated by hypophysectomy and PRL replacement studies (14). Hyposmotically induced changes in effectors of ion transport, however, may play an important role in the early response of branchial epithelia to a drop in extracellular osmolality, which is later complemented, and possibly sustained, by the actions of circulating PRL. These regulatory capacities are likely to play complementary adaptive roles in accordance with the life history of tilapia. The native distribution of Mozambique tilapia includes estuarine areas, which are subject to frequent salinity variations (69). Hence, mechanisms to respond rapidly to osmotic fluctuations are likely required to cope with changes in environmental salinity. We have recently shown that tilapia reared under a tidally-changing salinity regime exhibited patterns of protein localization and gene expression of ion transporters (NKA, NCC, and NKCC1a) that closely resemble those of SW-acclimated fish (43). While changes in ion transporter expression were observed with fluctuations of plasma osmolality, PRL levels in circulation remained constant through the FW- and SW-phases of the tidal cycle. These findings indicate that the responses of ionocytes to changes in environmental salinity may occur in direct response to changes in extracellular osmolality through the modulation of sub-cellular effectors of ion transport. Altogether, the concerted endocrine and osmotic modulation of branchial ionocytes contributes to the remarkable tolerance of Mozambique tilapia to changes in environmental salinities.

Acknowledgements
This work was funded by grants from the National Science Foundation (IOS-1119693), the Edwin W. Pauley Foundation (2012), and the Binational Agricultural Research Development (BARD) fund (IS-4296-10) to E.G.G. and D.T.L., the National Oceanic and Atmospheric Administration (NA14OAR4170071) which is sponsored by the University of Hawaii Sea Grant College Program, SOEST (Project R/SS-12) to A.P.S., and the University of Hawaii NSF EPSCoR program (EPS-0903833) in support of the Core Genetics Facility at the Hawaii Institute of Marine Biology, University of Hawaii and Research Fellow of Japan Society for the Promotion of Science (No. 2440164) to M.I.. The views expressed herein are those of the authors and do not necessarily reflect the views of the aforementioned granting agencies. University of Hawaii Sea Grant publication number UNIHI-SEAGRANT-JC-14-23.

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812 Complete amino acid sequences of a pair of fish (tilapia) prolactins, tPRL177 and
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815 Figure captions
816
817 Figure 1. Gene expression of Na⁺/Cl⁻ cotransporter (NCC, A), Na⁺/K⁺/2Cl⁻
818 cotransporter 1a (NKCC1a, B), Na⁺/H⁺ exchanger 3 (NHE3, C), Na⁺/K⁺-ATPase α1a
819 (NKAα1α, D), NKA α1b (E), prolactin receptor 1 (PRLR1, F) and PRLR2 (G) in gill
820 filaments cultured in different medium osmolalities for 3 and 6 h. Expression levels are
821 presented as the fold-change (mean ± SEM, n = 8) from 3 h 330 mOsm/kg group.
822 Differences among groups were analyzed by two-way ANOVA. Different letters
823 indicate significant differences among the four osmolalities at P < 0.05 by Tukey’s
824 HSD. †, ††, ††† significant difference between 3 and 6 h at P < 0.05, 0.01 and 0.001,
825 respectively, by student’s t-test.
826
827 Figure 2. Gene expression of Na⁺/Cl⁻ cotransporter (NCC, A), Na⁺/K⁺/2Cl⁻
cotransporter 1a (NKCC1a, B), Na⁺/H⁺ exchanger 3 (NHE3, C), Na⁺/K⁺-ATPase α1a (NKAα1a, D), NKA α1b (E), prolactin receptor 1 (PRLR1, F) and PRLR2 (G) in gill filaments cultured with different ovine prolactin (oPRL) concentrations (0, 0.1, 1.0 and 5.0 μg/ml) for 8 h. Expression levels are presented as the fold-change (mean ± SEM, n = 8) from the 0 μg/ml oPRL group. Different letters indicate significant differences (one-way ANOVA, Tukey’s HSD, P < 0.05).

Figure 3. Gene expression of Na⁺/Cl⁻ cotransporter (NCC, A), Na⁺/K⁺/2Cl⁻ cotransporter 1a (NKCC1a, B), Na⁺/H⁺ exchanger 3 (NHE3, C), Na⁺/K⁺-ATPase α1a (NKAα1a, D), NKA α1b (E), prolactin receptor 1 (PRLR1, F) and PRLR2 (G) in gill filaments cultured with different ovine prolactin (oPRL) concentrations (0, 0.1, 1.0 and 5.0 μg/ml) for 24 h. Expression levels are presented as the fold-change (mean ± SEM, n = 9) from the 0 μg/ml oPRL group. Different letters indicate significant differences (one-way ANOVA, Tukey’s HSD, P < 0.05).

Figure 4. Gene expression of Na⁺/Cl⁻ cotransporter (NCC, A), Na⁺/K⁺/2Cl⁻ cotransporter 1a (NKCC1a, B), Na⁺/H⁺ exchanger 3 (NHE3, C), Na⁺/K⁺-ATPase α1a (NKAα1a, D), NKA α1b (E), prolactin receptor 1 (PRLR1, F) and PRLR2 (G) in gill filaments cultured with different tilapia PRL (tPRL177 and tPRL188) concentrations (0, 0.01, 0.1, 0.5 and 1.0 μg/ml) for 8 h. Expression levels are presented as the fold-change (mean ± SEM, n = 18) from the 0 μg/ml PRL group. Differences among groups were analyzed by two-way ANOVA. *, **, *** significant difference from 0 μg/ml PRL group at P < 0.05, 0.01 and 0.001, respectively, by Dunnett’s test. †, †† significant difference between tPRL177 and tPRL188 at P < 0.05 and 0.01, respectively, by student’s
Figure 5. Gene expression of Na⁺/Cl⁻ cotransporter (NCC, A), Na⁺/K⁺/2Cl⁻ cotransporter 1a (NKCC1a, B), Na⁺/H⁺ exchanger 3 (NHE3, C), Na⁺/K⁺-ATPase α1a (NKAα1a, D), NKA α1b (E), prolactin receptor 1 (PRLR1, F) and PRLR2 (G) in gill filaments cultured with different tilapia PRL (tPRL177 and tPRL188) concentrations (0, 0.01, 0.1, 0.5 and 1.0 μg/ml) for 24 h. Expression levels are presented as the fold-change (mean ± SEM, n = 9) from the 0 μg/ml PRL group. Differences among groups were analyzed by two-way ANOVA. *, **, *** significant difference from 0 μg/ml PRL group at P < 0.05, 0.01 and 0.001, respectively, by Dunnett’s test. † significant difference between tPRL177 and tPRL188 at P < 0.05 by student’s t-test.

Figure 6. Double immunofluorescence staining with anti-Na⁺/K⁺-ATPase (green) and T4 (magenta) antibodies in gill filaments of time 0 control (A), after 8 and 24 h incubation with no prolactin (PRL) (B, C), and 24 h incubation with 5.0 μg/ml ovine PRL (D) and 1.0 μg/ml tPRL177 (E) and tPRL188 (F). Scale bar = 50 μm.
Table 1. Gene expression in gill filaments cultured in different medium osmolalities

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Incubation time</th>
<th>280 mOsm/kg</th>
<th>330 mOsm/kg</th>
<th>380 mOsm/kg</th>
<th>450 mOsm/kg</th>
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<tr>
<td>NCC</td>
<td>3 h</td>
<td>27.7 ± 6.9</td>
<td>23.2 ± 7.3</td>
<td>21.8 ± 3.7</td>
<td>7.5 ± 2.0</td>
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<tr>
<td></td>
<td>6 h</td>
<td>7.3 ± 1.2</td>
<td>6.2 ± 1.3</td>
<td>4.8 ± 0.7</td>
<td>4.5 ± 0.8</td>
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<tr>
<td>NKCC1a</td>
<td>3 h</td>
<td>64.8 ± 6.6</td>
<td>61.5 ± 4.9</td>
<td>70.6 ± 5.1</td>
<td>57.4 ± 5.9</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>43.0 ± 5.2</td>
<td>43.2 ± 7.0</td>
<td>48.3 ± 5.1</td>
<td>79.3 ± 8.2</td>
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<tr>
<td>NHE3</td>
<td>3 h</td>
<td>20.2 ± 3.0</td>
<td>24.5 ± 6.4</td>
<td>22.9 ± 3.1</td>
<td>31.5 ± 10.4</td>
</tr>
<tr>
<td></td>
<td>6 h</td>
<td>9.1 ± 2.3</td>
<td>13.5 ± 1.9</td>
<td>18.0 ± 5.6</td>
<td>14.3 ± 2.3</td>
</tr>
<tr>
<td>NKA α1a</td>
<td>3 h</td>
<td>18.9 ± 5.0</td>
<td>17.6 ± 6.8</td>
<td>14.5 ± 2.7</td>
<td>4.7 ± 1.3</td>
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<td></td>
<td>6 h</td>
<td>4.2 ± 1.3</td>
<td>4.9 ± 1.4</td>
<td>3.8 ± 1.2</td>
<td>3.0 ± 0.5</td>
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<tr>
<td>NKA α1b</td>
<td>3 h</td>
<td>26.6 ± 3.1</td>
<td>23.7 ± 5.9</td>
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<td>26.5 ± 12.2</td>
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<td>6 h</td>
<td>14.2 ± 3.2</td>
<td>16.2 ± 2.4</td>
<td>22.1 ± 5.6</td>
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<tr>
<td>PRLR1</td>
<td>3 h</td>
<td>30.1 ± 3.6</td>
<td>26.5 ± 4.9</td>
<td>25.2 ± 2.9</td>
<td>17.7 ± 2.4</td>
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<tr>
<td></td>
<td>6 h</td>
<td>23.1 ± 4.4</td>
<td>20.2 ± 3.9</td>
<td>16.5 ± 2.7</td>
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<td>59.9 ± 3.7</td>
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Target gene expression levels at 0 h were assigned an arbitrary value of 100 (mean ± SEM, n = 8).
Fig. 1

A. NCC mRNA expression (3 h 330 mOsm/kg = 1)

B. NKCC1a mRNA expression (3 h 330 mOsm/kg = 1)

C. NHE3 mRNA expression (3 h 330 mOsm/kg = 1)

D. NKA α1a mRNA expression (3 h 330 mOsm/kg = 1)

E. NKA α1b mRNA expression (3 h 330 mOsm/kg = 1)

F. PRLR1 mRNA expression (3 h 330 mOsm/kg = 1)

G. PRLR2 mRNA expression (3 h 330 mOsm/kg = 1)
Fig. 2

- **PRLR1 mRNA expression** (0 µg/ml = 1)
- **NKA α1a mRNA expression** (0 µg/ml = 1)
- **NCC mRNA expression** (0 µg/ml = 1)
- **PRLR2 mRNA expression** (0 µg/ml = 1)
- **NKA α1b mRNA expression** (0 µg/ml = 1)
- **NKCC1a mRNA expression** (0 µg/ml = 1)
- **NHE3 mRNA expression** (0 µg/ml = 1)
Fig. 3

A. NCC mRNA expression

B. NKCC1a mRNA expression

C. NHE3 mRNA expression

D. NKA α.1a mRNA expression

E. NKA α.1b mRNA expression

F. PRLR1 mRNA expression

G. PRLR2 mRNA expression

(0 μg/ml = 1)
ABC

**Fig. 4**

**A**

NCC mRNA expression

(0 µg/ml = 1)

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**B**

NKCC1a mRNA expression

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**C**

NHE3 mRNA expression

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**D**

NKα1a mRNA expression

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NKα1b mRNA expression

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**F**

PRLR1 mRNA expression

(0 µg/ml = 1)

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</tbody>
</table>

**G**

PRLR2 mRNA expression

(0 µg/ml = 1)

<table>
<thead>
<tr>
<th>PRL (µg/ml)</th>
<th>tPRL_{177}</th>
<th>tPRL_{188}</th>
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<tbody>
<tr>
<td>0.00</td>
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<tr>
<td>1.00</td>
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</tbody>
</table>
ABC

PRL (ng/ml) PRL (ng/ml) PRL (ng/ml)

NCC mRNA expression (0 ng/ml = 1)

NHE3 mRNA expression (0 ng/ml = 1)

NKCC1a mRNA expression (0 ng/ml = 1)

PRLR1 mRNA expression (0 ng/ml = 1)

PRLR2 mRNA expression (0 ng/ml = 1)

NKA α1a mRNA expression (0 ng/ml = 1)

NKA α1b mRNA expression (0 ng/ml = 1)

Fig. 5
Fig. 6