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SHORT REPORT

Salinity and prolactin regulate *anoctamin 1* in the model teleost, *Fundulus heteroclitus*

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Abstract

To maintain internal ion balance in marine environments, teleost fishes leverage seawater (SW)-type ionocytes to actively secrete Na^+ and Cl^- into the environment. It is well established that SW-type ionocytes use apically expressed cystic fibrosis transmembrane conductance regulator 1 (Cftr1) as a conduit for Cl^- to exit the gill. Here, we investigated whether the Ca^{2+}-activated Cl^- channel, anoctamin 1 (Ano1), provides an additional path for Cl^--secretion in euryhaline mummichogs (*Fundulus heteroclitus*). Two *ano1* gene isoforms, denoted *ano1.1a* and *-1.1b*, exhibited higher expression in the gill and opercular epithelium of mummichogs long-term acclimated to SW versus fresh water (FW). Branchial *ano1.1b* and *cftr1* expression was increased in mummichogs sampled 24 h after transfer from FW to SW; *ano1.1a* and *-1.1b* were upregulated in the gill and opercular epithelium following transfer from SW to hypersaline SW. Alternatively, the expression of *ano1.1a*, *-1.1b*, and *cftr1* in the gill and opercular epithelium was markedly decreased after transfer from SW to FW. Given its role in attenuating ion secretion, we probed whether prolactin downregulates *ano1* isoforms. In addition to attenuating *cftr1* expression, a prolactin injection reduced branchial *ano1.1a* and *-1.1b* levels. Given how Ano1 mediates Cl^- secretion by mammalian epithelial cells, the salinity- and prolactin-sensitive nature of *ano1* expression reported here indicates that Ano1 may constitute a novel Cl^--secretion pathway in ionocytes. This study encourages a wider evaluation of this putative Cl^--secretion pathway and its regulation by hormones in teleost fishes.

NEW & NOTEWORTHY In this study, we provide evidence in a teleost fish that the Ca^{2+}-activated Cl^- channel, anoctamin 1 may provide an additional path for Cl^- secretion by seawater-type ionocytes. Not only is this the first report of a Cftr-independent Cl^--secreting pathway conferring survival in seawater but also the first description of its regulation by the pituitary hormone prolactin.

gill; ionocyte; mummichog; opercular epithelium; prolactin

INTRODUCTION

Teleost fishes maintain extracellular fluids between 270 and 400 mosmol·kg^{-1}, with Na^+ and Cl^- kept between 130–180 and 120–150 mmol·L^{-1}, respectively (1, 2). Teleosts residing in seawater (SW) must therefore excrete Na^+ and Cl^- into the surrounding environment while simultaneously mitigating the passive loss of water. The gastrointestinal tract engages in water acquisition, whereas specialized cells in the branchial epithelium termed "ionocytes" facilitate the active excretion of Na^+ and Cl^- (3, 4). It was established over 25 years ago that cystic fibrosis transmembrane conductance regulator 1 (Cftr1) within the apical membrane of "SW-type" ionocytes provides a route for Cl^- to pass into the external environment (5, 6). In the basolateral membrane, Na^+-K^+-ATPase (Nka) and Na^+-K^+-$2Cl^-$ cotransporter 1 (Nkcc1) energize and facilitate the Na^+- and K^+-coupled entry of Cl^- into ionocytes (7, 8). Coordination between Cftr1, Nkcc1, and Nka is not restricted to teleost ionocytes; indeed, it constitutes a conserved strategy for Cl^- secretion similarly employed by the elasmobranch rectal gland, avian/reptilian salt glands, and mammalian intestinal crypts, sweat glands, and airways (9, 10).

The active secretion of Cl^- is critical to maintaining fluid/mucus balance in the mammalian airway epithelium. To support Cl^- secretion, pulmonary epithelial cells express CFTR and the Ca^{2+}-activated Cl^- channel, anoctamin 1 (ANO1; TMEM16A) (11). Although Cl^- secretion via CFTR is activated by cAMP in pulmonary ionocytes and ciliated cells (12, 13), Ca^{2+}-activated Cl^- secretion is attributed to ANO1 in ciliated cells (14). Cl^--secreting cells in the airway epithelium are structurally and functionally reminiscent of the SW-type ionocytes found in fishes; however, it stands entirely unresolved whether teleosts use Ano1 as a pathway for Cl^- secretion. This is a significant knowledge gap given that the general paradigm for transcellular Cl^- secretion by teleost ionocytes has remained largely unchanged for decades.

The euryhaline mummichog (*Fundulus heteroclitus*), with its dense ionocyte populations in the gill and opercular epithelium, represents a long-standing model for investigating the physiology of salt secretion (15). Mummichogs are native to salt marshes along the Atlantic coast of North America

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where they experience tidally driven changes in salinity; mummichogs also reside in hypersaline (>100‰) pools isolated from tidal influence (16, 17). Here, we sought to determine whether *ano1* is transcriptionally regulated when mummichogs undergo salinity acclimation. We first identified two *ano1* transcripts with tissue- and salinity-dependent expression patterns that suggest their encoded proteins support SW acclimation. Upon discovering that *ano1* transcripts were rapidly attenuated during freshwater (FW) acclimation, we hypothesized that prolactin (Prl), the classic "FW-adapting hormone" in fishes, inhibits *ano1* expression.

MATERIALS AND METHODS

Animals and Rearing Conditions

Adult mummichogs (*F. heteroclitus*) of both sexes were selected from stocks maintained at the Skidmore College Animal Care Facility. Mummichogs were obtained from Aquatic Research Organisms, Inc. (Hampton, NH). Fish were maintained in FW (5.3 mM Na^+, 5.3 mM Cl^-, 0.1 mM Ca^{2+}) or artificial SW (35‰ Instant Ocean) in recirculating stock tanks with particle and charcoal filtration and continuous aeration at 22–24°C under 12-h light:dark. Fish were fed Omega One mini pellets (Omega Sea) twice daily. All housing and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Skidmore College.

Anoctamin Phylogenetic Analysis

Teleost, mouse, and human *ano1* and *-2* mRNA sequences were retrieved from NCBI, aligned with MAFFT 7 (18), and then cleaned with BMGE on the NGPhylogeny.fr platform (19). The maximum-likelihood phylogeny analysis was analyzed via PhyML 3.0 visualized with iTol v6 (20). Statistical tests for branch support with bootstrap were replicated 100 times (21). Accession numbers for all sequences used in the analysis are provided in Fig. 1*A*.

Tissue and Steady-State Expression of ano1 Gene Transcripts

Tissues were collected from mummichogs maintained in FW for >1 yr ($n = 4$–6). From fish lethally anesthetized with 2-phenoxyethanol (2-PE; 2 mL·L^{-1}, Sigma-Aldrich) the following tissues were collected: whole brain, gill, opercular epithelium, esophagus, stomach, intestine, kidney, muscle, and skin. To compare *ano1.1a* and *-1.1b* levels between SW- and FW-acclimated animals ($n = 9$ or 10), gill filaments were collected from animals acclimated to the two environmental salinities for >2.5 wk. Tissues were stored in TRI Reagent (Molecular Research Corporation) at −80°C until tissue homogenization and RNA isolation.

Effect of Salinity Transfers on ano1 Isoform Gene Expression

The first transfer experiment entailed the direct transfer of mummichogs from FW to SW. At the time of transfer, fish were netted from a FW stock tank and moved into aerated, recirculating 38-L tanks containing either FW (control) or SW ($n = 8$–10). Twenty-four hours after transfer, fish were netted and anesthetized with a lethal dose of 2-PE. Fish were then rapidly decapitated and branchial filaments and opercular epithelium were excised and stored in TRI Reagent at −80°C. White muscle was collected from the caudal musculature and the water content was measured gravimetrically after drying overnight at 90°C. Fish were fasted for the duration of the 24-h posttransfer period. In the second transfer experiment, mummichogs were netted from a SW stock tank and moved into tanks containing either SW (control) or hypersaline SW (2SW; 70‰) ($n = 8$–11). In the third experiment, fish were netted from a SW stock tank and moved into tanks containing either SW (control) or FW ($n = 6$–8).

In Vivo Effects of Prolactin on Branchial ano1.1a and -1.1b Expression

Ovine Prl (oPrl; Sigma-Aldrich) was delivered in saline vehicle (0.9% NaCl; 20 μL·g^{-1} body wt). Mummichogs (2–4 g) were maintained in brackish water (12‰) for 2 wk before the time of the experiment. Fish were lightly anesthetized with 2-PE and administered the saline vehicle or oPrl (1 or 5 μg·g^{-1} body wt) by intraperitoneal injection ($n = 11$–14). oPrl doses were selected based on previous studies in mummichogs and other teleosts (22–25). Fish were then returned to brackish water aquaria (38-L recirculating tanks with filtration and aeration at 24°C) and left undisturbed for 24 h. Fish were fasted for the duration of the postinjection period. At the time of sampling, fish were lethally anesthetized with 2-PE and rapidly decapitated. Gill filaments were collected and stored in TRI Reagent at −80°C. Branchial *cftr1* and *ncc2* data from this experiment were reported in a previous study (26).

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

Total RNA was extracted from homogenized tissues by the TRI Reagent procedure. RNA concentration and purity were assessed by spectrophotometric absorbance (NanoDrop One, ThermoFisher). First-strand cDNA was synthesized by reverse transcribing 100 ng of total RNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies). Relative levels of mRNA were determined by quantitative real-time PCR (qRT-PCR) using the StepOnePlus real-time PCR system (Life Technologies). Primers for *ano1.1a*, *-1.1b*, and *-1.2* were designed using NCBI Primer-BLAST to amplify products of 125, 79, and 135 bp, respectively (Supplemental Table S1). We used previously validated primer sets for all other target and reference genes (27–29). Nonspecific product amplification was assessed by melt-curve analyses. qRT-PCR reactions were set up and cycled as described by Breves et al. (29). Elongation factor 1α (*ef1α*) levels were used to normalize target genes (28). Reference and target genes were calculated by the relative quantification method with PCR efficiency correction (30). Standard curves were prepared from serial dilutions of control gill or opercular epithelium cDNA and included on each plate to calculate the PCR efficiencies for target and reference gene assays.

Statistics

For the tissue expression and Prl-injection experiments, multiple group comparisons were performed by one-way

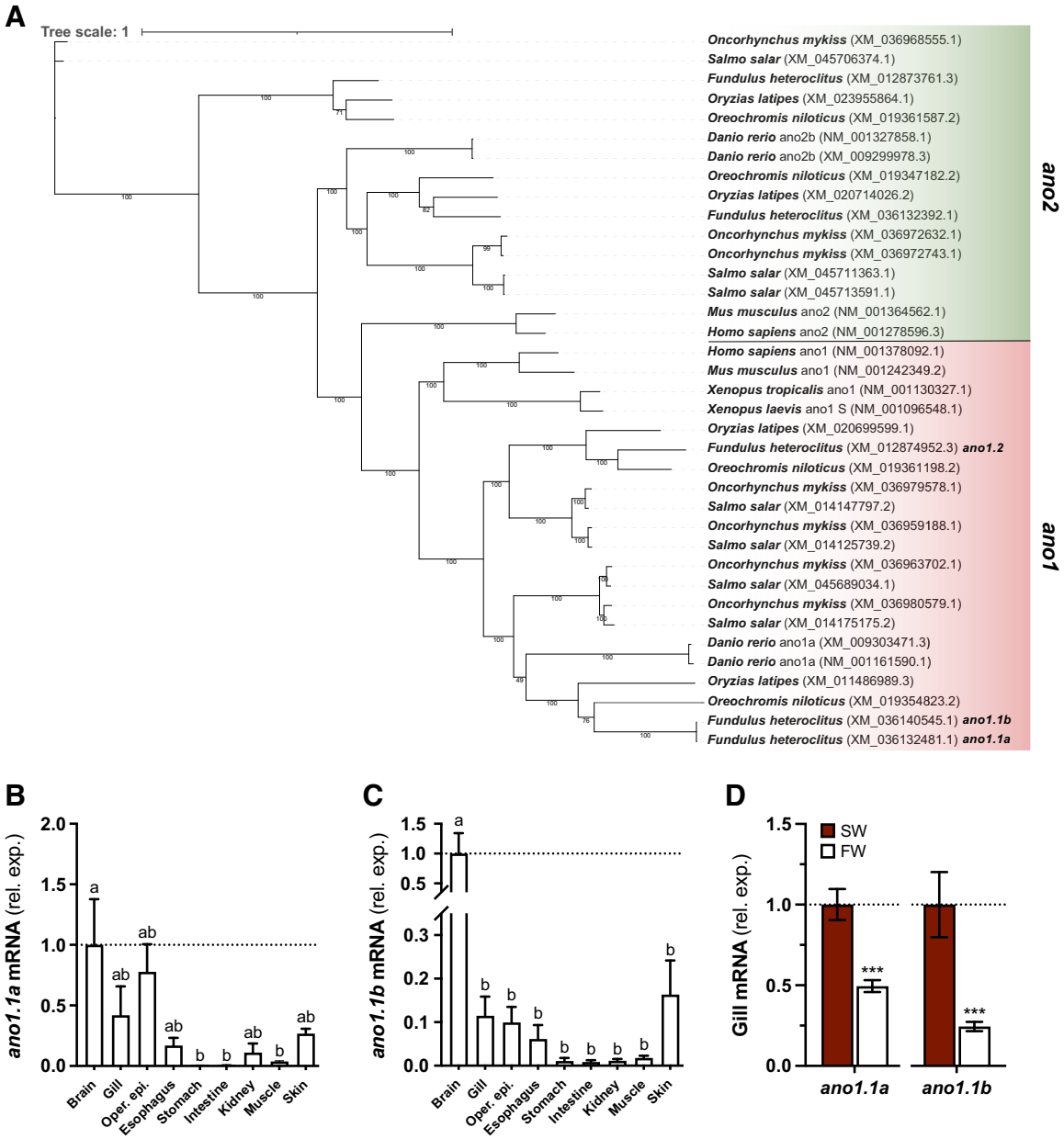

Figure 1. Maximum-likelihood phylogenetic analysis of selected teleost, mouse, and human *ano1* (red shaded area) and *-2* (green shaded area) mRNA sequences. *ano1* genes in *F. heteroclitus* are denoted as *ano1.1a*, *-1.1b*, and *-1.2*. Bootstrap values in percentage are shown on branches (*A*). *ano1.1a* (*B*) and *-1.1b* (*C*) gene expression in various tissues of freshwater (FW)-acclimated mummichogs. Means ± SE ($n = 4$–6). Data were normalized to *ef1α* and are presented relative to brain expression levels. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P < 0.05$). Branchial *ano1.1a* and *-1.1b* gene expression in seawater (SW)- and FW-acclimated mummichogs ($n = 9$ or 10) (*D*). mRNA levels in FW (open bars) are presented as a fold-change from SW (red bars). Asterisks indicate significant differences between salinities (***$P < 0.001$) by Mann–Whitney *U* test.

ANOVA followed by Tukey's HSD test. Significance was set at $P < 0.05$. For single comparisons between treatment groups, either a Student's *t* test or Mann–Whitney *U* test was employed. All statistical analyses were performed using GraphPad Prism 6.

RESULTS

Ano1 and -2 Phylogenetic Analysis

We identified duplicated *ano1* and *-2* genes among all the teleost species included in our phylogenetic analysis. We denoted three mummichog genes within the *ano1* clade as *ano1.1a* (XM_036132481.1), *-1.1b* (XM_036140545.1), and *-1.2* (XM_012874952.3) (Fig. 1*A*).

Tissue and Steady-State Expression of ano1 Gene Transcripts

ano1.1a and *-1.1b* were expressed in the gill and opercular epithelium; the highest expression of *ano1.1b* occurred in the brain (Fig. 1, *B* and *C*). *ano1.2* gene transcripts were not detected in any of the analyzed tissues. Branchial *ano1.1a* and *-1.1b* expression levels were approximately two- and

fourfold higher in long-term SW- versus FW-acclimated mummichogs, respectively (Fig. 1*D*).

Effect of Salinity Transfers on ano1 Isoform Gene Expression

Muscle water content was not impacted following an abrupt transfer from FW to SW (Fig. 2*A*). In the gill, *ano1.1b* and *cftr1* levels were higher following transfer to SW, whereas Na^+-Cl^- cotransporter 2 (*ncc2*) levels were markedly diminished (Fig. 2*B*). Although *ncc2* was similarly diminished in the opercular epithelium following transfer from FW to SW, there were no changes in *ano1.1a*, *-1.1b*, or *cftr1* (Fig. 2*C*). Transfer from SW to 2SW elicited reductions in muscle water content and *ncc2* expression that coincided with elevated *ano1.1a* and *-1.1b* in the gill and opercular epithelium (Fig. 2, *D–F*). Transfer from SW to FW increased muscle water content (Fig. 2*G*) in parallel with *ncc2* expression in the gill and opercular epithelium (Fig. 2, *H* and *I*). In contrast, branchial *ano1.1a*, *-1.1b*, and *cftr1* were diminished following transfer to FW (Fig. 2*H*); *ano1.1b* and *cftr1* were similarly reduced in the opercular epithelium after FW transfer (Fig. 2*I*).

In Vivo Effects of oPrl on Branchial ano1.1a and -1.1b Expression

Branchial *ano1.1a* and *-1.1b* levels were diminished following a single injection of oPrl at either 1 or 5 $\mu g \cdot g^{-1}$ (Fig. 3, *A* and *B*). In parallel with these responses, *cftr1* was similarly diminished by oPrl, but only at the 5 $\mu g \cdot g^{-1}$ dose (Fig. 3*C*). Branchial *ncc2* expression was increased approximately fivefold by oPrl at 5 $\mu g \cdot g^{-1}$ (Fig. 3*D*).

DISCUSSION

The results of this study suggest that Ano1 may constitute an alternate pathway for Cl^- secretion by teleost ionocytes. Our combined phylogenetic and gene expression analyses

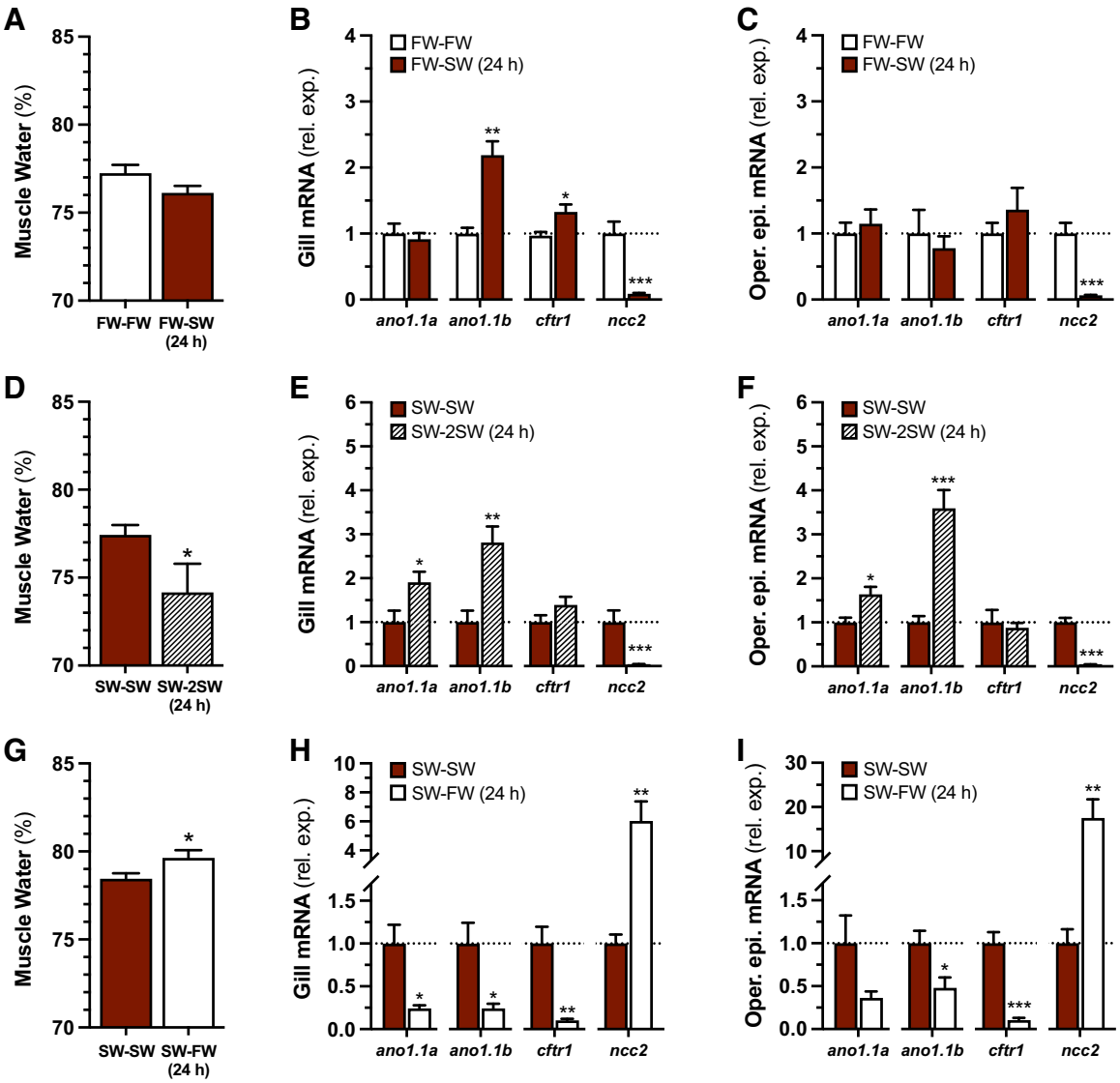

Figure 2. Muscle water content (*A*, *D*, and *G*) and *ano1.1a*, *-1.1b*, *cftr1*, and *ncc2* expression in the gill (*B*, *E*, and *H*) and opercular epithelium (*C*, *F*, and *I*) at 24 h after transfer from freshwater (FW) to seawater (SW) ($n = 8–10$) (*A–C*), SW to hypersaline SW (2SW; 70‰) ($n = 8–11$) (*D–F*), and SW to FW ($n = 6–8$) (*G–I*). Means ± SE. Asterisks indicate significant differences between salinities (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) by Student's *t* test or Mann–Whitney *U* test.

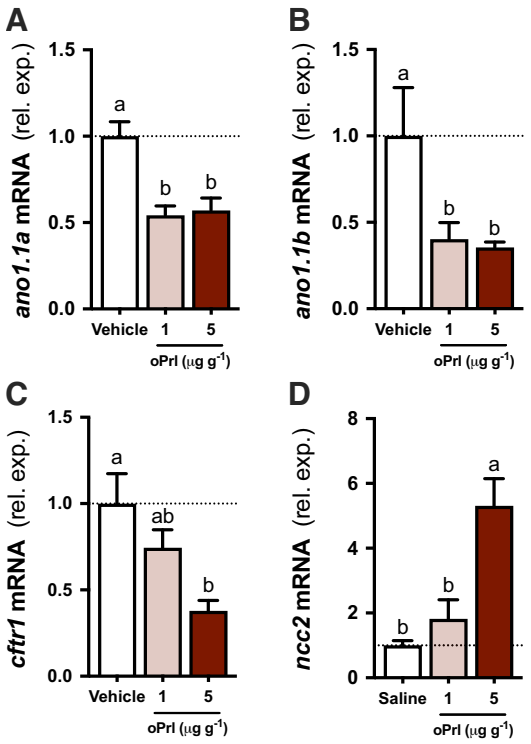

Figure 3. Branchial gene expression of *ano1.1a* (*A*), *-1.1b* (*B*), *cftr1* (*C*), and *ncc2* (*D*) following administration of ovine prolactin (oPrl). Means ± SE (n = 11–14). Fish were administered a single intraperitoneal injection (20 μL·g^{-1} body wt) of saline or oPrl (1 and 5 μg·g^{-1}) (shaded and solid red bars). Fish were sampled 24 h after the injection. Gene expression is presented as a fold-change from saline-injected controls (open bars). Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P < 0.05$).

revealed that mummichogs express two Ano1-encoding genes in tissues harboring robust ionocyte populations (31). Because the functional plasticity exhibited by ionocytes during SW acclimation is incumbent upon the dynamic expression of ion transporters/channels (28, 29, 32), *cftr1* expression was used to contextualize the *ano1.1a* and *-1.1b* responses to increases in salinity. By 24 h after transfer from FW to SW, Cftr1 appears in the apical membranes of ionocytes (7). Accordingly, we detected a modest increase in branchial *cftr1* levels following the transfer from FW to SW, a response that occurred alongside a doubling of *ano1.1b* expression. Given that fish were sampled at a single time point, we cannot rule out that branchial *ano1.1a* may respond to SW. The disparity between *cftr1* and *ano1* isoform responses was more pronounced following transfer from SW to 2SW when both *ano1.1a* and *-1.1b*, but not *cftr1*, levels increased in the gill and opercular epithelium. After transfer to 2SW, posttranslational processes may be sufficient to increase the rate of Cl^- secretion via Cftr1, whereas it is necessary to initiate the de novo synthesis of Ano1 channels (33).

Teleosts in FW are at risk for both excessive hydration and salt loss across body surfaces. To mitigate this situation, "FW-type" ionocytes actively absorb Na^+ and Cl^- from the external environment (34). In mummichogs, FW-type ionocytes express Ncc2 (29); thus, the increase in *ncc2* expression that occurred when fish were transferred from SW to FW reflects the recruitment of FW-type ionocytes associated with low-salinity acclimation. Although the enhanced expression of *ncc2* is necessary to survive in FW, mummichogs must also rapidly attenuate branchial Cl^- secretion. Accordingly, *cftr1, ano1.1a*, and *-1.1b* expression levels were markedly reduced following transfer to FW. These concerted responses suggest that a regulatory system is in place to blunt *cftr1* and *ano1* expression during FW acclimation.

Within the osmoregulatory organs of fishes, Prl is responsible for promoting the cellular and molecular processes that support survival in FW (35). Fittingly, when euryhaline species acclimate to FW conditions, plasma Prl levels rise in parallel with the expression of branchial Prl receptors (36, 37). Heightened Prl signaling at both the ligand and receptor levels promotes the expression of solute transporters expressed in FW-type ionocytes (38). Prl further supports FW acclimation by inhibiting pathways for ion secretion (39). For example, in mummichogs, Prl stimulates *ncc2* while simultaneously inhibiting Nka activity and *nkcc1* and *cftr1* expression (22, 26). Our observation that both *ano1.1a* and *-1.1b* were inhibited by Prl leads us to propose that a Prl-Ano1 regulatory link further contributes to attenuating Cl^-- secretion when mummichogs encounter FW (40).

To our knowledge, this study is the first to describe the regulation of *ano1* isoforms in a teleost during acclimation to FW, SW, and 2SW. The positive relationship between *ano1* and environmental salinity, when considered together with Ano1's role as an apically expressed, Ca^{2+}-dependent Cl^- channel in mammals (11, 41), presents compelling avenues for future study. The development of isoform-specific antibodies is warranted to resolve whether Ano1s are coexpressed with Cftr1 in the apical crypts of SW-type ionocytes (Fig. 4). It must also be determined whether teleost Ano1s function as Ca^{2+}-activated Cl^- channels and if the various

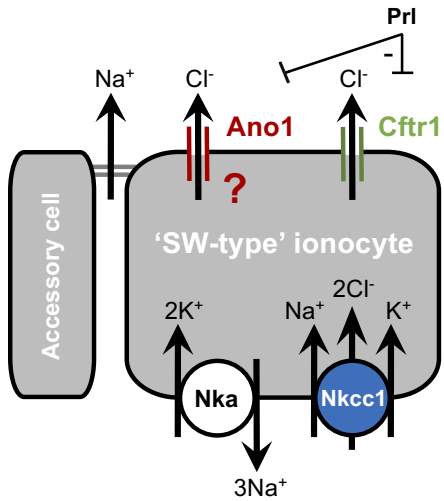

Figure 4. Schematic diagram of "SW (seawater)-type" ionocytes indicating the inhibitory (blocked lines with "-") effects of prolactin (Prl). SW-type ionocytes express Na^+-K^+-ATPase (Nka) and Na^+-K^+-$2Cl^-$ cotransporter 1 (Nkcc1) in the basolateral membrane. Apically located cystic fibrosis transmembrane conductance regulator 1 (Cftr1) enables Cl^- to exit ionocytes, whereas tight junctions between ionocytes and accessory cells provide the pathway for Na^+ to exit the gill. Although the subcellular localization patterns of Nka, Nkcc1, and Cftr1 are firmly established, it remains unresolved whether anoctamin 1 (Ano1) is expressed within the apical membrane of ionocytes.

isoforms exhibit differences in their functional regulation. For studies of this nature, models where Ano1 expression can be manipulated would provide the most conclusive evidence of its role in Cl^- secretion. It is tempting to speculate that elevated Ca^{2+} levels in SW (relative to FW) contribute to the activation of Ano1. As CFTR and ANO1 in mammalian epithelia are functionally interdependent (42), it should also be investigated whether a similar relationship exists in SW-type ionocytes. Lastly, while this study identifies Prl as a regulator of Ano1, there is a plethora of other hormones that regulate ionocytes. In particular, Ano1s should be probed for regulation by cortisol, the primary mineralocorticoid in teleosts. A link of this nature would augment how cortisol stimulates SW-type ionocytes via Nkcc1, Cftr1, and Nka (43, 44). These lines of investigation promise to resolve how Ano1 fits into the scheme for ionocyte-mediated Cl^- secretion, a fundamental paradigm that has remained fixed for decades.

DATA AVAILABILITY

Data will be made available upon reasonable request.

SUPPLEMENTAL MATERIAL

Supplemental Table S1: https://doi.org/10.6084/m9.figshare.26956216.

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DISCLAIMERS

The views expressed herein are those of the authors and do not necessarily reflect the views of the aforementioned granting agencies.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.P.B. and C.A.S. conceived and designed research; J.P.B., M.A.P., and Y.T.T. performed experiments; J.P.B., M.A.P., and Y.T.T. analyzed data; J.P.B., M.A.P., Y.T.T., and C.A.S. interpreted results of experiments; J.P.B. and Y.T.T. prepared figures; J.P.B. drafted manuscript; J.P.B. and C.A.S. edited and revised manuscript; J.P.B., M.A.P., Y.T.T., and C.A.S. approved final version of manuscript.

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