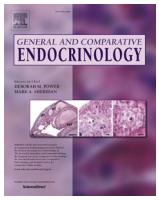
General and Comparative Endocrinology 325 (2022) 114051

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen



Molecular targets of prolactin in mummichogs (*Fundulus heteroclitus*): Ion transporters/channels, aquaporins, and claudins

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ARTICLE INFO

Keywords:
Atlantic killifish
Gill
Intestine
Ionocyte
Prolactin
Tight-junction

ABSTRACT

Prolactin (Prl) was identified over 60 years ago in mummichogs (*Fundulus heteroclitus*) as a "freshwater (FW)-adapting hormone", yet the cellular and molecular targets of Prl in this model teleost have remained unknown. Here, we conducted a phylogenetic analysis of two mummichog Prl receptors (Prlrs), designated Prlra and Prlrb, prior to describing the tissue- and salinity-dependent expression of their associated mRNAs. We then administered ovine Prl (oPrl) to mummichogs held in brackish water and characterized the expression of genes associated with FW- and seawater (SW)-type ionocytes. Within FW-type ionocytes, oPrl stimulated the expression of *Na^+/Cl^- cotransporter 2* (*ncc2*) and *aquaporin 3* (*aqp3*). Alternatively, branchial *Na^+/H^+ exchanger 2* and *−3* (*nhe2* and *−3*) expression did not respond to oPrl. Gene transcripts associated with SW-type ionocytes, including *$Na^+/K^+/2Cl^-$ cotransporter 1* (*nkcc1*), *cystic fibrosis transmembrane regulator 1* (*cftr1*), and *claudin 10f* (*cldn10f*) were reduced by oPrl. Isolated gill filaments incubated with oPrl *in vitro* exhibited elevated *ncc2* and *prlra* expression. Given the role of Aqps in supporting gastrointestinal fluid absorption, we assessed whether several intestinal *aqp* transcripts were responsive to oPrl and found that *aqp1a* and *−8* levels were reduced by oPrl. Our collective data indicate that Prl promotes FW-acclimation in mummichogs by orchestrating the expression of solute transporters/channels, water channels, and tight-junction proteins across multiple osmoregulatory organs.

1. Introduction

Pioneering studies within the field of comparative endocrinology employed mummichogs (*Fundulus heteroclitus*) to identify the fundamental activities of pituitary hormones in fishes (Pickford and Atz, 1957). With respect to linking the pituitary with hydromineral balance, Pickford (1953) and Burden (1956) made two particularly important observations when they found that hypophysectomized mummichogs could not survive in fresh water (FW) and that injection of *Fundulus* pituitary brei rescued them from death. Pickford and Phillips (1959) subsequently discovered that prolactin (Prl) was the pituitary factor that enabled mummichogs to survive in low-salinity conditions. Prl supports the survival of mummichogs in FW by facilitating the maintenance of systemic Na^+ and Cl^- balance (Pickford et al., 1970; Potts and Evans, 1966). While these foundational studies paved the way for Prl to be firmly established as a "FW-adapting hormone" in fishes (Hirano, 1986; Manzon, 2002; Breves et al., 2014), the molecular targets of Prl in mummichogs remain unknown.

Ionocytes harbored within the branchial epithelium play a central role in maintaining Na^+ and Cl^- homeostasis in teleosts. Fish in seawater (SW) must actively secrete Na^+ and Cl^- to mitigate their passive entry across body surfaces, whereas fish in FW must absorb Na^+ and Cl^- to counteract their diffusive loss to the environment (Marshall and Grosell, 2006). In mummichogs, the gill is considered a minor route for the total Na^+ and Cl^- that is acquired from FW environments (Marshall et al., 1997; Patrick et al., 1997; Wood and Laurent, 2003); nonetheless, the brachial epithelium contains ion-absorptive (FW-type) ionocytes equipped to engage in active Na^+ and Cl^- uptake. For example, FW-type ionocytes (potentially as distinct sub-types) express Na^+/H^+ exchanger 2 and 3 (Nhe2 and Nhe3) and Na^+/Cl^- cotransporter 2 (Ncc2) (Edwards et al., 2005; Scott et al., 2005; Katoh et al., 2008; Marshall et al., 2017; Breves et al., 2020). Accordingly, branchial *nhe2* and *ncc2* expression increases when FW-type ionocytes are recruited during low-salinity acclimation (Scott et al., 2005; Breves et al., 2020).

As in other teleosts, the ion-secretory (SW-type) ionocytes of mummichogs express Na^+/K^+-ATPase and $Na^+/K^+/2Cl^-$ cotransporter 1

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https://doi.org/10.1016/j.ygcen.2022.114051
Received 25 February 2022; Received in revised form 25 April 2022; Accepted 2 May 2022
Available online 6 May 2022

Table 1
Specific primer sequences for quantitative real-time PCR.

Gene	Primer Sequence (5′-3′)	Reference/Acc. No.
aqp1a	F: CAATCCGGCCCGGTCCTTCGG R: CAAGAGCATAGACATATAC	Tingaud-Sequeira et al., 2009
aqp3	F: CTCCAAATCTCACCAGCC R: CAGCAGTGGAAGAATCCC	Jung et al., 2012
aqp8	F: CAGCCGTGATGAGCAACTAC R: TATCTCCCAGGATGAGCCTGAC	XM_012866326
cftr1	F: AATCGAGCAGTTCCCAGACAAG R: AGCTGTTTGTGCCCATTGC	Scott et al., 2005
cldn10c	F: CGCACGGAGATCACACATAC R: AGTCTTCCTGGTGGTGTTGG	Marshall et al., 2018
cldn10d	F: CGGTGATCATGTACGTGGAG R: TACTCTGTGGGAAGGGTGGA	Marshall et al., 2018
cldn10e	F: CTCTGCGGAGAAGGAGAAGA R: GAGAAGCTGTGGTGGGCTTA	Marshall et al., 2018
cldn10f	F: ACTTATATCGGCGGAGCAGA R: ATAAGCAGTAGGCGGCAAGA	Marshall et al., 2018
ef1α	F: GGGAAAGGGCTCCTTCAAGT R: ACGCTCGGCCTTCAGCTT	Scott et al., 2005
ncc2	F: AGTCACATCCTGACCGGAAAC R: TCACAGGACTGAGACTGGAT	Breves et al., 2020
nhe2	F: ACAGCATCAGGCGCATTCT R: GCTGGCATCTGCTTGTTGTTAA	Scott et al., 2005
nhe3	F: TGTTGAAGTCGTCAGCGAGAA R: CAGCTCGTGGAAGACGTTGA	Scott et al., 2005
nkcc1	F: CCCGCAGCCACTGGTATT R: GCCATCTGTGGGTCAGCAA	Scott et al., 2004
nkcc2	F: GCCGTGGCCCTTTGC R: CCAGGTCGGTTGTGTTTCCT	Scott et al., 2004
prlra	F: AATGGAGCCCCTCAGTGGA R: GTAGGCAGTACTTCAGACTGTG	XM_021319711
prlrb	F: TCGGAGTTTGCAGCAGTACG R: TCTCCTTCTCTGGGGACCTG	XM_012878477

(Nkcc1) in the basolateral membrane (Marshall et al., 2002; Flemmer et al., 2010). Apically located cystic fibrosis transmembrane conductance regulator 1 (Cftr1) enables Cl^- to exit ionocytes whereas the "tight-junctions" between ionocytes and adjacent accessory cells provide the paracellular pathway for Na^+ to exit the gill (Marshall and Grosell, 2006). Claudin 10 (Cldn10) paralogs provide the cation-permeable pores within the tight-junction complexes adjacent to mummichog SW-type ionocytes; thus, attendant increases in brachial *nkcc1*, *cftr1*, and *cldn10* expression coincide with SW-acclimation (Marshall et al., 2018; Breves et al., 2020; Chen et al., 2021). To our knowledge, there is no information on whether Prl regulates the expression of ion transporters/channels within FW- or SW-type mummichog ionocytes.

In marine/SW-acclimated fishes, the gastrointestinal tract plays a critical role in preventing dehydration because it replaces the water lost by osmosis to the surrounding environment (Takei, 2021). Under marine conditions, euryhaline fishes (including mummichogs) consume ambient SW for desalination in the esophagus (Potts and Evans, 1966; Takei et al., 2017). Upon passing through the stomach and entering the intestine, monovalent ions are further removed from the luminal fluid allowing water to be absorbed through transcellular and paracellular routes (Sundell and Sundh, 2012; Madsen et al., 2015). Nkcc2 mediates the apical entry of Na^+ and Cl^- into enterocytes while aquaporins (Aqps) enhance transcellular osmotic permeability (Madsen et al., 2015; Sundell and Sundh, 2012). Whether Prl supports FW-acclimation by inhibiting molecular mediators of solute-linked fluid absorption, such as Nkcc2 and Aqps, stands unresolved.

Mummichogs populate tide-pools, rivers, and estuaries along the east coast of North America where they readily encounter dynamic salinity conditions. While mummichogs prefer to inhabit hyperosmotic environments, their capacity to withstand FW conditions permits them to select microhabitats suited to food acquisition and reproduction (Able, 2002; Marshall et al., 2016). In the current study, we sought to provide mechanistic insight into how Prl signaling enables mummichogs to tolerate low-salinity conditions. We first conducted a phylogenetic analysis of two predicted mummichog Prl receptors (Prlrs) before assessing the tissue- and salinity-dependent expression patterns of their associated mRNAs. We then determined through a combination of *in vivo* and *in vitro* experimental paradigms whether Prl regulates gene transcripts that encode various ion transporter/channels, Aqps, and Cldn10s.

2. Materials and methods

2.1. Animals and rearing conditions

Adult mummichogs (*Fundulus 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, Blacksburg, VA) in recirculating stock tanks with particle and charcoal filtration and continuous aeration at 22–24 °C under 12L:12D. Fish were fed Omega One mini pellets (Omega Sea, Painesville, OH) twice daily. All housing and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Skidmore College.

2.2. Tissue and steady-state expression of prlra and prlrb

Tissues were collected from mummichogs maintained in FW for > 1 year (n = 4–6). Fish were lethally anesthetized with 2-phenoxyethanol (2-PE; 2 ml l^{-1}, Sigma-Aldrich, St. Louis, MO) and the following tissues were collected: whole brain, gill, opercular epithelium, esophagus, intestine (middle), kidney, liver, and skin. Tissues were stored in TRI Reagent (MRC, Cincinnati, OH) at −80 °C until tissue homogenization and RNA isolation. To compare *prlra* and *prlrb* mRNA levels between SW- and FW-acclimated animals (n = 9–10), gill filaments and intestine were collected from animals acclimated to the two environmental salinities for > 3 weeks.

2.3. In vivo effects of oPrl on branchial and intestinal gene expression

Ovine Prl was purchased from Sigma-Aldrich and delivered in saline vehicle (0.9% NaCl; 20 μl g^{-1} body weight). Mummichogs (2–4 g) were selected from stock tanks and maintained in brackish water (12‰) for 2 weeks prior to the time of the experiment. Fish were lightly anaesthetized with 2-PE and administered the saline vehicle or oPrl (1 or 5 μg g^{-1} body weight) by intraperitoneal (IP) injection (n = 11–14). oPrl doses were selected based on previous studies that employed IP-injection in mummichogs and other teleosts (Pickford et al., 1970; Jackson et al., 2005; Breves et al., 2010; Breves et al., 2013). 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 experiment. At the time of sampling, fish were lethally anaesthetized with 2-PE as described above and rapidly decapitated. Gill filaments and intestine (middle) were collected and stored in TRI Reagent at −80 °C until RNA isolation.

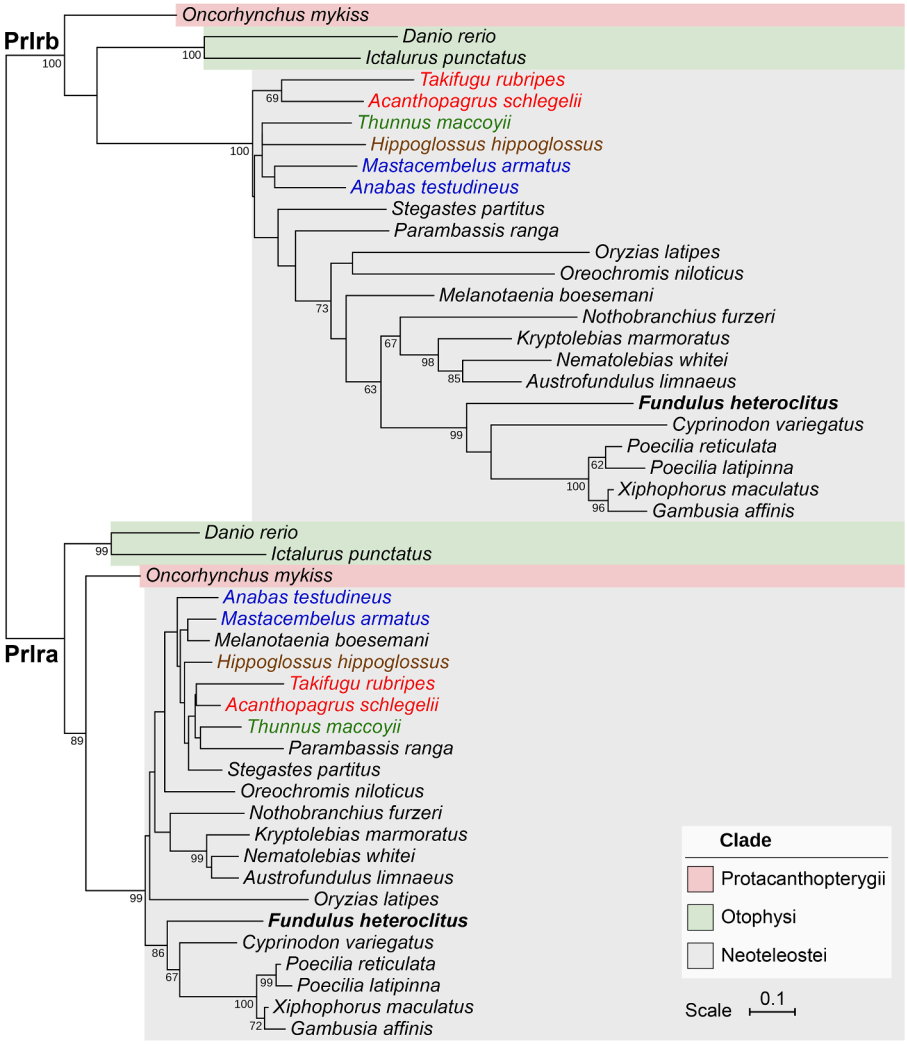

Fig. 1. Maximum likelihood protein phylogeny of teleost Prlra and Prlrb. The best tree was reconstructed using a JTT + G + I model based on an alignment of 312 sites and 48 sequences in Protacanthopterygii, Otophysi, and Neoteleostei clades. Within Neoteleostei, taxon labels for different Percomorphaceae clades are shown in color (red for Eupercaria; green for Pelagiaria; brown for Carangaria, blue for Anabantaria, and black for Ovalentaria). Bootstrap values in percentage (>60%) are shown on branches. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. In vitro effects of oPrl on branchial gene expression

Gill filaments were obtained from mummichogs held in SW and incubated according to McCormick and Bern (1989) with modifications described by Breves et al. (2013). Briefly, gill filaments were severed from the arches at the septum and rinsed with pre-incubation medium (Dulbecco's Modified Eagle Medium; DMEM, ThermoFisher, Waltham, MA) with penicillin and streptomycin (Sigma-Aldrich). Filaments were then placed in 24-well cell culture plates (Corning Inc., Corning, NY) containing pre-incubation medium; gill filaments from a single fish were distributed into two separate wells in a paired design (one well for each treatment). After a 3 h pre-incubation period, medium was replaced with freshly prepared control medium (DMEM + vehicle control: phosphate-buffered saline; PBS) or DMEM supplemented with oPrl (1 μg ml^{-1}) dissolved in PBS. Filaments ($n = 8$) were incubated at 24 °C under atmospheric air for 6 h. The concentration of oPrl was selected following previous studies (Breves et al., 2013; Inokuchi et al., 2015). Incubations were terminated by removing the filaments from the culture plate and placing them in TRI Reagent prior to storage at −80 °C.

2.5. Prlra and prlrb phylogenetic analysis

Prlra (Prlr1) and Prlrb (Prlr2 or Prlr-like) protein sequences in 24 teleost fish were retrieved from GenBank for phylogenetic analysis (Supplemental Table 1). Multiple sequence alignment was performed using MAFFT with the G-INS-I strategy (Katoh et al., 2019). The poorly aligned N- and C-terminus end regions were manually removed. The resulting alignment contained 48 sequences and 312 sites. The maximum likelihood phylogeny was constructed using the PhyML 3.0 program (Guindon et al., 2010) with JTT model, gamma-shaped distribution of the substitution rates (G), and a proportion of invariable sites (I). The best tree search was started with five random trees and the bootstrap analysis was performed 1000 times.

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

Total RNA was extracted from homogenized tissues by the TRI Reagent procedure according to the manufacturer's protocol. RNA

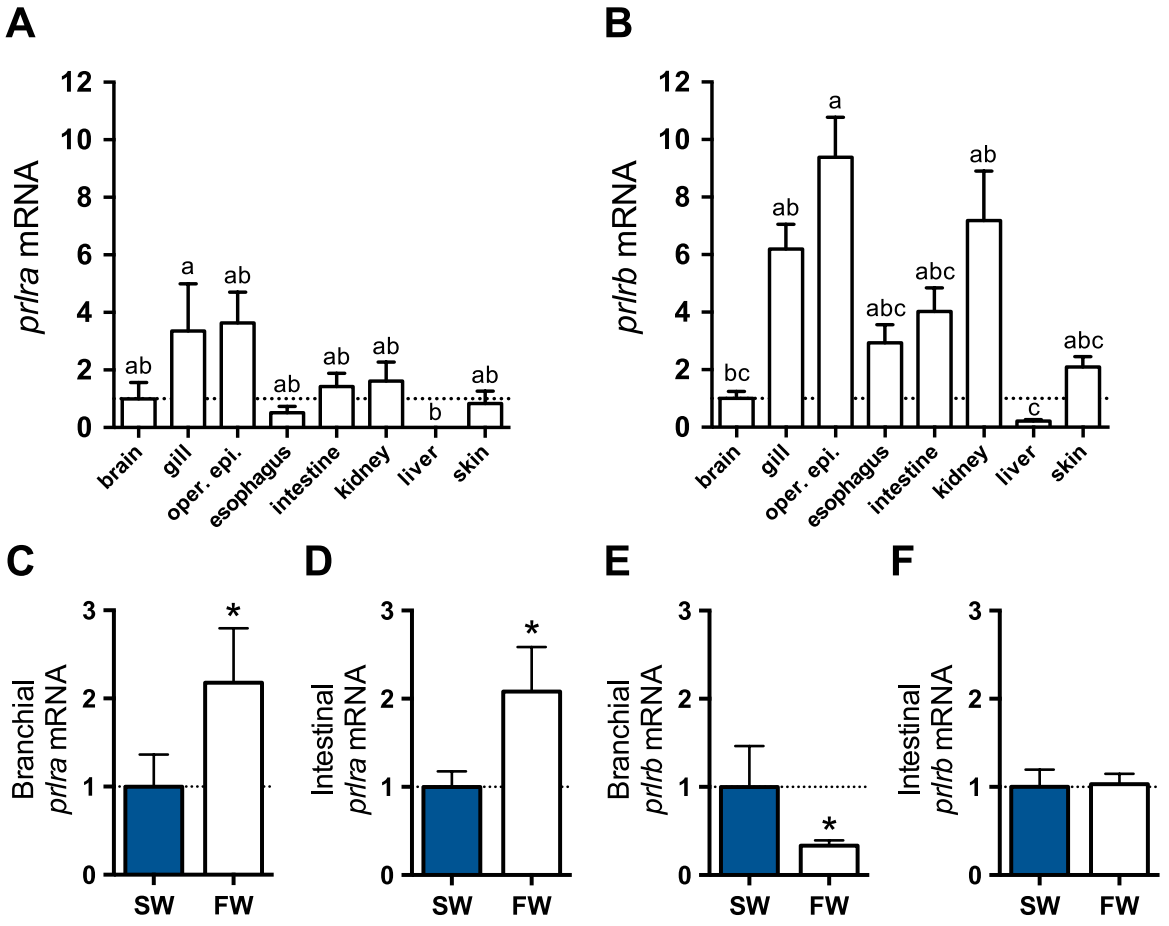

Fig. 2. *prlra* (A) and *prlrb* (B) gene expression in various tissues of freshwater (FW)-acclimated mummichogs. Means ± SEM ($n = 4$–6). Data were normalized to *ef1α* as a reference gene and are presented relative to brain expression levels. Means not sharing the same letter are significantly different (one-way ANOVA, Tukey's HSD test, $P < 0.05$). *prlra* and *prlrb* gene expression in the gill (C, E) and intestine (D, F) of seawater (SW)- and FW-acclimated mummichogs ($n = 9$–10). mRNA levels in FW (open bars) are presented as a fold-change from SW (blue bars). Asterisks indicate significant differences between salinities (*$P < 0.05$) by Mann-Whitney *U* test. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

concentration and purity were assessed by spectrophotometric absorbance (NanoDrop One, ThermoFisher). First strand cDNA was synthesized by reverse transcribing 50–200 ng total RNA with a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, CA). Relative levels of mRNA were determined by qRT-PCR using the StepOnePlus real-time PCR system (Life Technologies). We employed previously validated primer sets for all target and reference genes aside from *aqp8* (XM_012866326), *prlra* (XM_021319711), and *prlrb* (XM_012878477) (Table 1). Primers for *aqp8*, *prlra*, and *prlrb* were designed using NCBI Primer-BLAST to span predicted exon-exon junctions and to amplify products of 105, 150, and 72 base pairs, respectively. Non-specific product amplification was assessed by melt curve analyses. The *aqp8*, *prlra*, and *prlrb* primer sets showed efficiencies of 93%, 98%, and 95%, respectively. qRT-PCR reactions were performed in a 15 µl volume containing 2X Power SYBR Green PCR Master Mix (Life Technologies), 200 nmol/l of each primer, nuclease free water, and 1 µl cDNA template. 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 sec 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 (Scott et al., 2005). 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 control gill or intestine cDNA and included on each plate to calculate the PCR efficiencies for target and reference gene assays. Relative gene expression ratios between groups are reported as a fold-change from controls.

2.7. Statistics

For the tissue profiles of *prlr* expression and the oPrl injection experiment, multiple group comparisons were performed by one-way ANOVA followed by Tukey's HSD test. Significance was set at $P < 0.05$. For a single comparison between SW- and FW-acclimated mummichogs, a Mann-Whitney *U* test was employed and significant

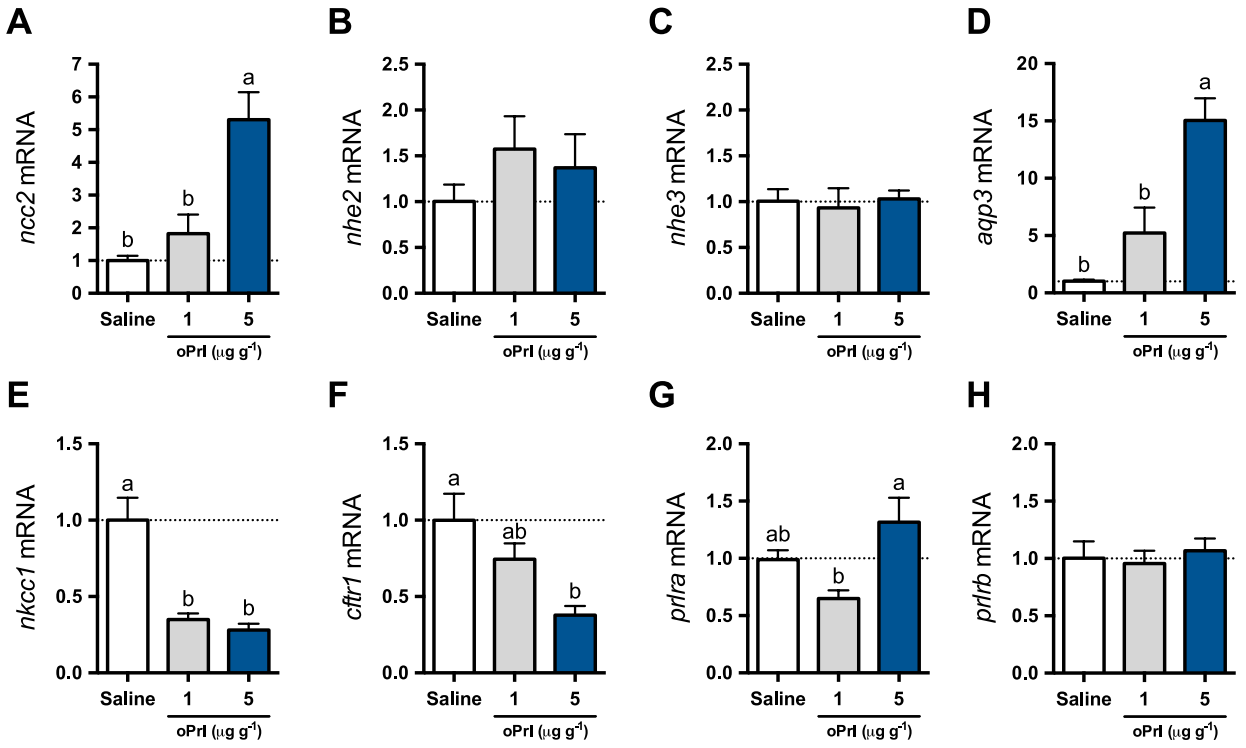

Fig. 3. Branchial gene expression of *ncc2* (A), *nhe2* (B), *nhe3* (C), *aqp3* (D), *nkcc1* (E), *cftr1* (F), *prlra* (G), and *prlrb* (H) following administration of oPrl. Means ± SEM ($n = 11$–14). Fish were maintained in brackish water (12 ppt) and administered a single intraperitoneal injection (20 μl g^{-1} body weight) of saline or oPrl (1 and 5 μg g^{-1}) (shaded and blue 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, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differences are indicated in figures: $*P < 0.05$. For comparing control versus oPrl-treated gill filaments, a Wilcoxon signed rank test was employed and significant differences are indicated in figures: $**P < 0.01$. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

3. Results

3.1. Prlra and prlrb phylogenetic analysis

Two types of Prlrs were identified within all the teleost fishes we examined. In public sequence repositories, the Prlra and Prlrb paralogs are also annotated as Prlr1 and Prlr2, or Prlr and Prlr-like (Supplemental Table 1). Our teleost Prlr phylogeny (Fig. 1) shows that Prlra and Prlrb form two distinct monophyletic groups. Both the Prlra and Prlrb sequences are well separated into three teleost clades (i.e., Protacanthopterygii, Otophysi, and Neoteleostei). Prlra sequences are more conserved than those of Prlrb. The mean ± SEM pairwise distances within Prlra and Prlrb are 0.39 ± 0.02 and 0.90 ± 0.07, respectively. The Prlrb lineage, especially within Neoteleostei, is better resolved than the Prlra lineage in the phylogenetic analysis.

3.2. Tissue and steady-state expression of prlra and prlrb

Following our phylogenetic analyses, we first determined the relative gene expression of mummichog *prlrs* across tissues collected from FW-acclimated animals. Both *prlra* and *prlrb* were highly expressed in the gill and opercular epithelium (Fig. 2A, B). The expression of *prlrb* in the kidney was comparable to levels in the gill and opercular epithelium. In both the gill and intestine, *prlra* expression was ~ 2-fold higher in long-term FW- versus SW-acclimated mummichogs (Fig. 2C, D). On the other hand, *prlrb* expression was lower in the gill, but not in the intestine, of FW- versus SW-acclimated mummichogs (Fig. 2E, F).

3.3. In vivo effects of oPrl on branchial and intestinal gene expression

We next characterized gene expression responses to increased Prl levels in mummichogs held in brackish water. A single IP-injection of oPrl at 5 μg g^{-1} led to a ~ 5-fold increase in branchial *ncc2* expression (Fig. 3A); *nhe2* and *nhe3* were not affected by oPrl (Fig. 3B, C). Branchial *aqp3* expression was increased ~ 15-fold by injection of oPrl at 5 μg g^{-1} (Fig. 3D). *nkcc1* expression was diminished in mummichogs injected with oPrl at 1 or 5 μg g^{-1} (Fig. 3E). *cftr1* levels were similarly diminished

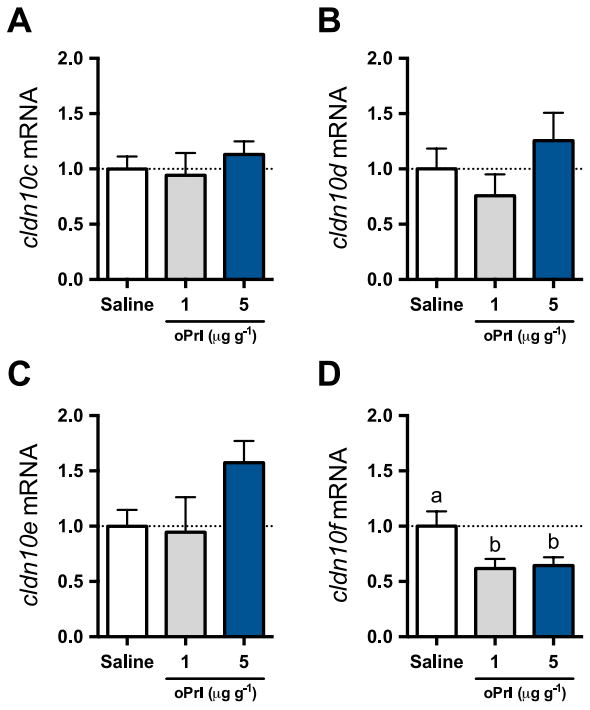

Fig. 4. Branchial gene expression of *cldn10c* (A), *cldn10d* (B), *cldn10e* (C), and *cldn10f* (D) following administration of oPrl. Means ± SEM (n = 11–14). Fish were maintained in brackish water (12 ppt) and administered a single intraperitoneal injection (20 μl g^{-1} body weight) of saline or oPrl (1 and 5 μg g^{-1}) (shaded and blue 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, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

by oPrl, but only at the higher dose (Fig. 3F). oPrl did not affect branchial *prlra* or *prlrb* levels (Fig. 3G, H). Among the four *cldn10* genes targeted in this study, only *cldn10f* was sensitive to oPrl (Fig. 4). *cldn10f* levels were diminished by both doses of oPrl (Fig. 4D).

In the intestine, oPrl did not affect *nkcc2* expression (Fig. 5A). *aqp1a* levels were diminished following injection with oPrl at 5 μg g^{-1} whereas *aqp8* was diminished by 1 and 5 μg g^{-1} of oPrl (Fig. 5B, D). oPrl did not affect intestinal *aqp3* levels (Fig. 5C). Lastly, *prlra*, but not *prlrb*, expression was stimulated by oPrl at 5 μg g^{-1} (Fig. 5E, F).

3.4. *In vitro* effects of oPrl on branchial gene expression

To determine whether oPrl acts directly on gill filaments to regulate *prlra* and *prlrb* expression, as well as gene transcripts that responded to Prl *in vivo*, we incubated mummichog gills in the presence of oPrl. The addition of oPrl to the incubation medium resulted in higher expression of *ncc2* and *prlra*, but not *aqp3* or *prlrb* (Fig. 6). oPrl did not affect *nkcc1*, *cftr1*, or *cldn10f* levels (data not shown).

4. Discussion

In the current study, we addressed how Prl supports the euryhalinity of mummichogs by identifying Prl-regulated gene transcripts in the gill and intestine. Our principal findings include: 1) mummichogs express two Prlr-encoding genes, denoted *prlra* and *prlrb*, in salinity-dependent fashions; 2) Prl acts directly on the gill to promote *ncc2* expression within FW-type ionocytes; and 3) Prl dampens the expression of branchial and intestinal gene transcripts that underlie hyposmoregulatory processes. By resolving how Prl orchestrates the expression of genes linked with iono- and osmoregulation in mummichogs, our study provides new insight into how Prl signaling enables this remarkably osmotolerant model to thrive in FW conditions.

Teleost Prlrs form two distinct clades, Prlra/Prlr1 and Prlrb/Prlr2, and in euryhaline species paralogous Prlrs control the transcription of distinct target genes via JAK/STAT and ERK/MAPK signaling (Huang et al., 2007; Fiol et al., 2009; Chen et al., 2011; Bollinger et al., 2018; Daza and Larhammar, 2018). Our combined phylogenetic and gene expression analyses confirmed that mummichogs express both teleost Prlr paralogs (Figs. 1, 2A, B). Branchial, intestinal, and renal patterns of *prlra* and *prlrb* expression in mummichogs are consistent with reports of Prl binding, Prlr immunoreactivity, and *prlr* mRNA expression in other examined species (Morley et al., 1981; Dauder et al., 1990; Prunet and Auperin, 1994; Weng et al., 1997; Rouzic et al., 2001; Santos et al., 2001; Lee et al., 2006; Huang et al., 2007; Fiol et al., 2009; Breves et al., 2013). Furthermore, the robust expression of *prlrs* in the gill and kidney coincides with how Prl regulates Na^+/K^+-ATPase activity in mummichogs (Pickford et al., 1970). The higher *prlra* levels in the gill and intestine of FW- versus SW-acclimated mummichogs matches how low-salinity conditions induced the expression of *prlra/prlr1* in other euryhaline fishes (Fig. 2C, D) (Sandra et al., 2000, 2001; Pierce et al., 2007; Fiol et al., 2009; Tomy et al., 2009). The elevated *prlra/prlr1* expression in FW occurs in direct response to Prl levels and provides a mechanism to enhance tissue-responsiveness to circulating hormone (Fig. 2C, D, 5E, 6C) (Breves et al., 2013; Inokuchi et al., 2015). On the other hand, higher *prlrb* expression in SW-acclimated mummichogs may promote the osmotic stress tolerance of epithelial cells that are directly exposed to hyperosmotic conditions (Fig. 2E) (Fiol et al., 2009).

Our collective *in vivo* and *in vitro* data show that Prl acts directly on the gill to promote *ncc2*, but not *nhe2* or *nhe3*, expression. Ncc2-expressing ionocytes utilize apically located Ncc2 for the transport of Na^+ and Cl^- from FW into the ionocyte interior (Hiroi et al., 2008). Na^+ and Cl^- then move across the basolateral membrane via Na^+/K^+-ATPase and ClC-family Cl^- channels, respectively (Wang et al., 2015). In turn, Prl supports the survival of mummichogs in FW by promoting a necessary component (Ncc2) of a pathway that ionocytes employ to absorb ions. While not specifically reported yet in mummichogs, it is well established that pituitary *prl* gene expression and plasma Prl levels rise when fish acclimate to FW/ion-poor conditions (Lee et al., 2006; Hoshijima and Hirose, 2007; Fuentes et al., 2010; Seale et al., 2012), and given the connection between Prl and *ncc2* identified here (Fig. 3A, 6A), we propose that Prl signaling is responsible for the increase in *ncc2* expression that occurs when mummichogs encounter FW (Breves et al., 2020). On the other hand, our results indicate that a systemic (or environmental) cue distinct from Prl promotes the expression of *nhe2* and *nhe3* during FW acclimation. The specificity of the Prl-*ncc2* connection in mummichogs is reminiscent of how Prl regulates *ncc2*, but not *nhe3*, in zebrafish (*Danio rerio*) (Breves et al., 2013). Nonetheless, the connection between Prl and *ncc2* is curious given that branchial ion-absorption has a minor impact on systemic Na^+ and Cl^- levels in

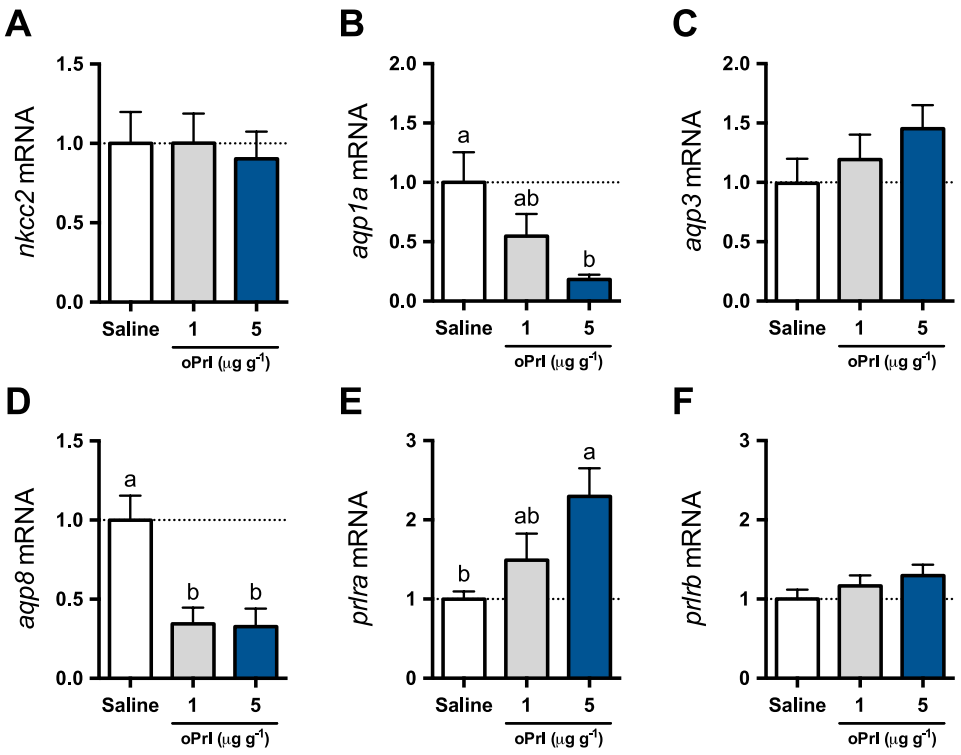

Fig. 5. Intestinal gene expression of *nkcc2* (A), *aqp1a* (B), *aqp3* (C), *aqp8* (D), *prlra* (E), and *prlrb* (F) following administration of oPrl. Means ± SEM (n = 11–14). Fish were maintained in brackish water (12 ppt) and administered a single intraperitoneal injection (20 μl g^{-1} body weight) of saline or oPrl (1 and 5 μg g^{-1}) (shaded and blue 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, $P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mummichogs (Marshall et al., 1997; Patrick and Wood, 1999). Therefore, extra-branchial, and in particular, renal processes also underlie how Prl sustains Na^+ and Cl^- balance (Pickford et al., 1970). Given recent insights into how teleost nephrons support ion conservation in FW (Takvam et al., 2021), a broad suite of renal ion transporters and channels should now be evaluated for their possible regulation by Prl.

Within the osmoregulatory organs of euryhaline teleosts, endocrine factors direct the salinity-dependent expression of particular Aqps (Breves, 2020). For instance, Prl maintains the robust expression of the aquaglyceroporin, Aqp3/*aqp3*, in the gills of FW-acclimated Mozambique tilapia (*Oreochromis mossambicus*) (Breves et al., 2016), Japanese medaka (*Oryzias latipes*) (Ellis et al., 2019), and as shown here, mummichogs (Fig. 3D). While this dependence upon Prl implies that Aqp3 plays an important role in the gills of FW-acclimated fish, how it underlies specific adaptive processes remains unknown. A role for Aqp3 in enhancing transepithelial water movement appears unlikely on the basis that water exchange is disadvantageous for fish in both FW and SW environments (Cerdà and Finn, 2010). Alternatively, Aqp3 may render epithelial cells osmosensitive and/or capable of efficiently regulating their volume in response to fluctuations in extracellular osmolality (Cutler and Cramb, 2002; Watanabe et al., 2005; Tipsmark et al., 2010). More recently, Aqp3 was discovered to mediate the expression of cell-junction proteins (e.g., cadherins) and consequently the adhesive properties of cell-to-cell contacts within epithelia (Edamana et al., 2021). Given that Aqp3 is expressed by multiple branchial cell-types in mummichogs (Jung et al., 2012; Ruhr et al., 2020), we propose that Prl-Aqp3 connectivity contributes to the general "tightening" of branchial surfaces to limit Na^+ efflux in FW (Potts and Evans, 1966). Given that Prl did not stimulate *aqp3* expression in isolated gill filaments (Fig. 6B), albeit assessed with only a single Prl concentration, this regulatory connection in mummichogs seemingly requires the presence of additional systemic factors. We tested the *in vitro* effects of Prl on filaments collected from SW-acclimated animals. Future work is required to confirm that filaments isolated from FW-acclimated mummichogs are also unresponsive to Prl with respect to *aqp3* expression.

Mummichogs rapidly attenuate branchial ion-secretion when they transition from marine to FW environments (Marshall et al., 2000); otherwise, ion-secretion would prove highly deleterious by supplementing the passive losses of Na^+ and Cl^-. It follows that *ncc2* expression is inversely correlated with *nkcc1* and *cftr1* expression (Breves et al., 2020). Our data show that in addition to acting upon Ncc2-expressing ionocytes, Prl promotes FW acclimation by inhibiting the transcription of genes associated with SW-type ionocytes (Fig. 3E, F). As shown previously, Prl inhibits branchial Na^+/K^+-ATPase activity, which like *nkcc1* and *cftr1*, is elevated in SW to support ion-secretion (Pickford et al., 1970). Therefore, the activity of SW-type ionocytes is inhibited by the combination of short-term (local) hyposmotic cues (Marshall et al., 2000) and long-term (systemic) changes in Prl signaling. While Cftr1 permits Cl^- to exit SW-type ionocytes, tight junction complexes between ionocytes and accessory cells provide the path for Na^+ to exit the organism (Marshall et al., 2018; Chen et al., 2021). In mummichogs, the cation-selective tight-junctions adjacent to ionocytes are composed of multiple Cldn10 proteins, and among the *cldn10* transcripts that are up regulated in response to SW/hyper-saline conditions (*cldn10c*, *-d*, *-e*, and *-f*) (Marshall et al., 2018), only *cldn10f* was inhibited by Prl (Fig. 4D). Our observation that Prl regulated a single *cldn10* transcript provides

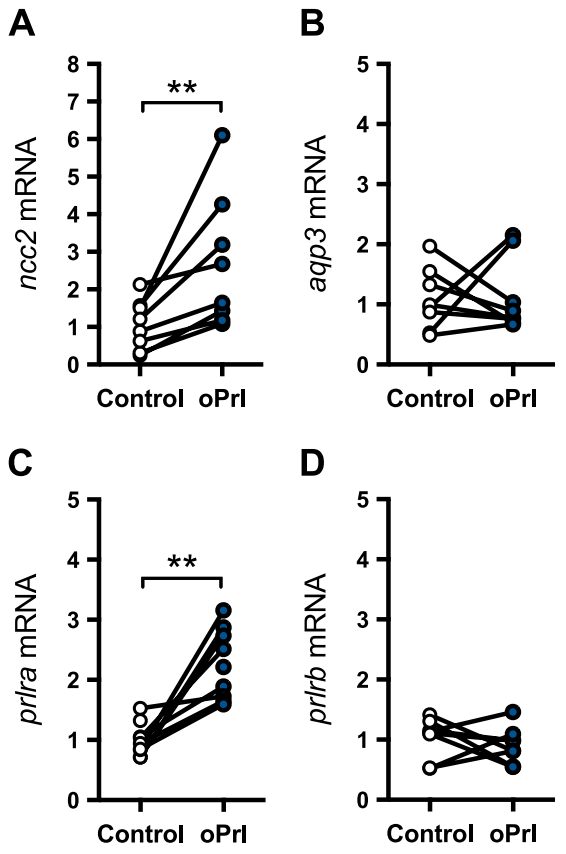

Fig. 6. Effects of oPrl on *ncc2* (A), *aqp3* (B), *prlra* (C), and *prlrb* (D) in incubated gill filaments. Filaments ($n = 8$) were pre-incubated for 3 h, and then incubated in oPrl-supplemented (1 μg ml^{-1}; blue symbols) or vehicle-containing (PBS; open symbols) medium for 6 h. Gene expression is presented relative to the control group mean. Asterisks indicate significant differences between groups (**$P < 0.01$) by Wilcoxon signed rank test.

additional support for the notion that Cldn10s play isoform-specific roles during salinity acclimation (Marshall et al., 2018). Interestingly, Prl promotes *cldn10c* and *cldn10f* expression in medaka (Bossus et al., 2017); thus, Prl regulates branchial Cldns in species-dependent fashions.

To counteract the passive loss of water to the environment, SW-acclimated fish engage in solute-linked water absorption across the intestine (Takei, 2021). Accordingly, osmoregulatory hormones (e.g., arginine vasotocin, cortisol, guanylin, and parathyroid hormone-related protein) modulate intestinal fluid absorption by controlling the activity and/or expression of ion transporters and Aqps, and in particular Nkcc2 and Aqp1 (Breves, 2020; Takei, 2021). Knowing that Prl diminishes intestinal Na^+, Cl^-, and fluid absorption (Utida et al., 1972; Morley et al., 1981), we expected Prl to down regulate *nkcc2* and *aqp* expression in mummichogs. However, we did not find evidence of a regulatory-connection between Prl and *nkcc2*; alternatively, Prl markedly diminished the expression of *aqp1a* and *aqp8* (Fig. 5A, B, D). Prl's regulation of *aqp1a* and *aqp8* is notable given that euryhaline species increase the expression of these particular *aqp* isoforms during SW acclimation as a means to enhance transcellular osmotic permeability (Aoki et al., 2003; Martinez et al., 2005; Giffard-Mena et al., 2007; Raldúa et al., 2008; Kim et al., 2010; Tipsmark et al., 2010; Deane et al., 2011; Madsen et al., 2011; Engelund et al., 2013). In turn, Prlrs in the intestine enable circulating Prl to regulate the permeability characteristics of the intestine in relation to ambient conditions (especially during FW acclimation). Lastly, the fact that intestinal *aqp3* did not respond to Prl suggests that its translated product does not mediate dynamic processes associated with salinity acclimation (Fig. 5C); accordingly, intestinal *aqp3* expression is salinity-independent in mummichogs (Ruhr et al., 2020).

In summary, our collective findings provide new insight into how Prl coordinates the expression of genes that encode mediators of Na^+, Cl^-, and water transport in mummichogs. Prl plays a pivotal role in FW acclimation by activating a putative ion-uptake pathway (via *ncc2*) in tandem with attenuating pathways for ion secretion (via *nkcc1*, *cftr1*, and *cldn10f*) (Fig. 7). Like Nkcc1 and Cftr1 (Marshall et al., 2008, 2009; Flemmer et al., 2010), the sub-cellular distribution of Ncc2 changes in response to dynamic osmotic conditions (Marshall et al., 2017). Mummichogs offer a fitting model to now investigate whether Prl-signaling impacts the post-translational modification of Ncc2 via with-no-lysine kinase 1. In addition to underlying Na^+ and Cl^- balance, Prl is necessary for mummichogs to maintain Ca^{2+} homeostasis (Pang et al., 1978). However, the molecular basis of Prl's hypercalcemic activity in

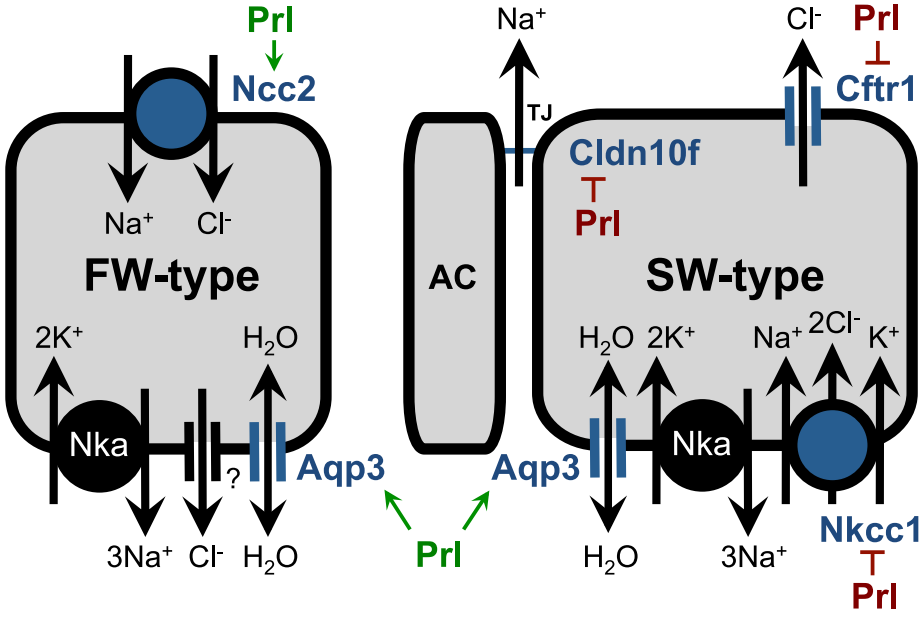

Fig. 7. Schematic diagrams of FW (freshwater)- and SW (seawater)-type ionocytes in mummichogs showing the stimulatory (green) and inhibitory (red) effects of Prl. Apical and basolateral sides are presented at the top and bottom of ionocytes, respectively. Cell models were derived from protein and/or gene transcript expression patterns (Marshall et al., 2002; Katoh et al., 2008; Flemmer et al., 2010; Jung et al., 2012; Marshall et al., 2018; Breves et al., 2020; Ruhr et al., 2020). Given their lack of sensitivity to Prl, Na^+/H^+ exchanger 2 and 3 (Nhe2 and −3) are not included in this schematic. While Aqp3 is illustrated to transport water, it may also potentially transport urea, glycerol, and ammonia. Abbreviations: AC: accessory cell; Aqp3: aquaporin 3; Cldn10f: claudin 10f; Cftr1: cystic fibrosis transmembrane conductance regulator 1; Ncc2: Na^+/Cl^- cotransporter 2; Nka: Na^+/K^+-ATPase; Nkcc1: $Na^+/K^+/2Cl^-$ cotransporter 1; Prl: prolactin; TJ: tight-junction. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

mummichogs is entirely unresolved. Future study is warranted to determine whether Prl regulates the expression of the Ca^{2+} channels/exchangers/pumps (e.g., TRPVs, Na^{+}/Ca^{2+} exchangers, and Ca^{2+}-ATPases) that enable ionocytes to absorb environmental Ca^{2+} (Flik et al., 1996). In this regard, mummichogs will continue to offer a fitting physiological model from which to resolve the mechanistic bases of how Prl supports environmental adaptation in fishes.

Funding

This study was supported by the National Science Foundation [IOS-1755131 to J.P.B.].

CRediT authorship contribution statement

Jason P. Breves: Conceptualization, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Katie M. Puterbaugh:** Formal analysis, Investigation, Visualization. **Serena E. Bradley:** Investigation. **Annie E. Hageman:** Investigation. **Adrian J. Verspyck:** Investigation. **Lydia H. Shaw:** Investigation. **Elizabeth C. Danielson:** Investigation. **Yubo Hou:** Formal analysis, Investigation, Writing – review & editing, Visualization, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We appreciate the excellent fish care provided by Aaron Cordiale and Tracy Broderson.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2022.114051.

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Supplemental Table 1. GenBank accession numbers for teleost prolactin receptor protein sequences.

	Prlra		Prlrb	
Species	**Acc. No.**	**Annotation**	**Acc. No.**	**Annotation**
Acanthopagrus schlegelii	ABR10920	prolactin receptor 1	ABR10921	prolactin receptor 2
Anabas testudineus	XP_026210445	prolactin receptor a	XP_026198563	prolactin receptor b
Austrofundulus limnaeus	XP_013864770	prolactin receptor	XP_013879683	prolactin receptor-like
Cyprinodon variegatus	XP_015231908	prolactin receptor	XP_015230858	prolactin receptor-like
Danio rerio	NP_001122149	prolactin receptor a precursor	NP_001106972	prolactin receptor b
Fundulus heteroclitus	XP_021175386	prolactin receptor a	XP_012733931	prolactin receptor b
Gambusia affinis	XP_043952407	prolactin receptor a	XP_043967357	prolactin receptor b
Hippoglossus hippoglossus	XM_034602350	prolactin receptor a	XP_034450666	prolactin receptor b
Ictalurus punctatus	XP_017348546	prolactin receptor	XP_017306719	prolactin receptor-like
Kryptolebias marmoratus	NP_001316305	prolactin receptor a precursor	NP_001316269	prolactin receptor b precursor
Mastacembelus armatus	XP_026153969	prolactin receptor	XP_026178363	prolactin receptor-like
Melanotaenia boesemani	XP_041826667	prolactin receptor a	XP_041856411	prolactin receptor b
Nematolebias whitei	XP_037548172	prolactin receptor a	XP_037536176	prolactin receptor b
Nothobranchius furzeri	XP_015798368	prolactin receptor	XP_015828737	prolactin receptor-like
Oncorhynchus mykiss	NP_001118071	prolactin receptor a precursor	XP_021461230	prolactin receptor b
Oreochromis niloticus	NP_001266477	prolactin receptor precursor	NP_001266551	prolactin receptor b precursor
Oryzias latipes	XP_011479970	prolactin receptor	XP_004072141	prolactin receptor
Parambassis ranga	XP_028273289	prolactin receptor	XP_028269206	prolactin receptor-like
Poecilia latipinna	XP_014896028	prolactin receptor	XP_014897336	prolactin receptor-like
Poecilia reticulata	XP_008422714	prolactin receptor	XP_008416240	prolactin receptor-like
Stegastes partitus	XP_008295073	prolactin receptor	XP_008292376	prolactin receptor-like
Takifugu rubripes	XP_003965258	prolactin receptor	NP_001072093	prolactin receptor precursor

Thunnus maccoyii	XP_042251239	prolactin receptor a	XP_042277142	prolactin receptor b
Xiphophorus maculatus	XP_005798743	prolactin receptor	XP_023199072	prolactin receptor-like