Special Issue: The Multifunctional Fish Gill

Endocrine control of gill ionocyte function in euryhaline fishes

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Abstract:

The endocrine system is an essential regulator of the osmoregulatory organs that enable euryhaline fishes to maintain hydromineral balance in a broad range of environmental salinities. Because branchial ionocytes are the primary site for the active exchange of Na^+, Cl^-, and Ca^{2+} with the external environment, their functional regulation is inextricably linked with adaptive responses to changes in salinity. Here, we review the molecular-level processes that connect osmoregulatory hormones with branchial ion transport. We focus on how factors such as prolactin, growth hormone, cortisol, and insulin-like growth-factors operate through their cognate receptors to direct the expression of specific ion transporters/channels, Na^+/K^+-ATPases, tight-junction proteins, and aquaporins in ion-absorptive (freshwater-type) and ion-secretory (seawater-type) ionocytes. While these connections have historically been deduced in teleost models, more recently, increased attention has been given to understanding the nature of these connections in basal lineages. We conclude our review by proposing areas for future investigation that aim to fill gaps in the collective understanding of how hormonal signaling underlies ionocyte-based processes.

Keywords: cortisol; growth hormone; ion transporter; prolactin; receptor; salinity

1. Introduction

Fishes, the most numerous and diverse vertebrates, consist of three major classes: Agnatha (jawless fishes), Chondrichthyes (cartilaginous fishes), and Osteichthyes (bony fishes) (Moyle and Cech 2004). Teleosts (class Osteichthyes; subclass Actinopterygii; infraclass Neopterygii; division Teleostei) and lampreys (members of Class Agnatha) typically maintain extracellular fluids between 270 and 400 mOsm/kg, with Na^+ and Cl^- constituting the major dissolved ions (Hwang and Lin 2014; Ferreira-Martins et al. 2016). Therefore, when residing in dilute freshwater (FW) environments, they are at risk for both excessive hydration and salt loss across body surfaces. To counterbalance this situation, the gill actively absorbs ions (Na^+, Cl^-, and Ca^{2+}) from the external environment, while the kidney and urinary bladder produce large volumes of dilute urine (Marshall and Grosell 2006; Kaneko et al. 2008). Lampreys and teleosts residing in seawater (SW), on the other hand, must excrete ions gained by passive diffusion from the surrounding environment and replace water that is lost via osmosis. While multiple segments of the gastrointestinal tract work in concert to promote solute-linked water absorption (Barany et al. 2020; Takei 2021), the gills and kidney secrete monovalent (Na^+, Cl^-) and divalent (Mg^{2+}, Ca^{2+}, and SO_4^{2-}) ions into the external environment, respectively (Kaneko et al. 2008). Cartilaginous fishes are typically marine in their distribution and operate as osmoconformers by retaining urea and trimethylamine oxide while maintaining internal Na^+ and Cl^- concentrations below those of SW (Hwang and Lin 2014). Hagfishes (members of Class Agnatha) are marine osmoconformers with limited capacities to regulate internal ion concentrations.

While most fishes inhabit a single aquatic environment characterized as either FW (≤0.5‰) or SW (30-40‰), a relatively small percentage of species (~5%) are considered “euryhaline” and can withstand both conditions (Schultz and McCormick 2013). Euryhaline species possess the capacity to rapidly modulate ion- and water-transporting activities within the gill, gastrointestinal tract, kidney, and urinary bladder following changes in salinity (Takei et al. 2014). In turn, they offer valuable opportunities to resolve how cellular and molecular processes within osmoregulatory organs enable fish to transition between environmental salinities. As the branchial exchange of ions with the external environment is critical for maintaining osmoregulatory balance, decades of focused investigations have pursued how “ionocytes”, cells specialized for Na^+, Cl^-, and Ca^{2+} transport, operate in the gills relative to environmental salinity (Evans et al. 2005; Dymowska et al. 2012).

2. Molecular aspects of ionocyte function in the gills

2.1 Freshwater-type ionocytes in teleosts

Historically, various models have been put forth to explain how the branchial ionocytes of FW-acclimated fishes actively absorb ions against strong electrochemical gradients (Hwang and Lin 2014). The contrasting models of FW-type ionocytes reflect, in part, the evolution of different strategies for Na^+ and Cl^- uptake across the teleost lineage (Dymowska et al. 2012; Takei et al. 2014; Yan and Hwang, 2019). For euryhaline teleosts, the most comprehensive models of FW-type ionocytes are derived from rainbow trout (*Oncorhyncus mykiss*), Mozambique tilapia (*Oreochromis mossambicus*), and Japanese medaka (*Oryzias latipes*) (Dymowska et al. 2012; Hsu et al. 2014; Inokuchi et al. 2022). For basal fishes, recent progress has been made in the development of FW-type ionocyte models for sea lamprey (*Petromyzon marinus*) (Ferreira-Martins et al. 2021). Without question, insights into how ionocytes operate in stenohaline zebrafish (*Danio rerio*) have supported progress in the euryhaline species listed above (Guh et al. 2015).

In FW-type ionocyte models for salmonids, largely conceived from findings in rainbow trout, two distinct subtypes absorb environmental Na^+, Cl^-, and Ca^{2+}. In one subtype, termed peanut lectin agglutinin positive (PNA^+) cells, Na^+/H^+ exchangers 2 and 3 (Nhe2 and -3; Slc9a2 and -3), epithelial Ca^{2+} channel (ECaC), and an Slc26-family anion exchanger are expressed in the apical membrane. Na^+/K^+-ATPase (Nka) mediates the basolateral movement of Na^+, while an uncharacterized pathway allows for the exit of Cl^- (Ivanis et al. 2008; Dymowska et al. 2012). The other ionocyte subtype, termed PNA^- cells, expresses an apical Na^+ channel, purported to be acid-sensing ion channel 4

(Asic4), along with apical H^+-ATPase. Na^+/HCO_3^- cotransporter 1 (Nbce1; Slc4a4) and Nka are also expressed in PNA$^-$ cells to mediate the basolateral exit of Na^+ (Parks et al. 2007; Dymowska et al. 2014).

Like in trout, there are multiple FW-type ionocytes operating within the branchial epithelium of euryhaline Mozambique tilapia. “Type II” ionocytes express a Na^+/Cl^- cotransporter in the apical membrane to transport Na^+ and Cl^- into the cell interior (Hiroi et al. 2008). This Ncc is denoted Ncc2 (Slc12a10) and is not a member of the “conventional” Ncc1 (Slc12a3) clade (Motoshima et al. 2023). Nka and Clc family Cl^- channel 2c (Clc2c) support the basolateral transport of Na^+ and Cl^- from the ionocyte interior into the blood plasma, respectively (Pérez-Ruis et al. 2015; Wang et al. 2015; Breves et al. 2017b). While Ncc2-expressing ionocytes operate in euryhaline and stenohaline species spanning teleost clades (Wang et al. 2009; Hsu et al. 2014; Inokuchi et al. 2017; Lema et al. 2018), they are conspicuously absent in salmonids (Hiroi and McCormick 2012). In tilapia, a second type of Na^+-absorptive ionocyte which expresses Nka, coined “Type III” ionocytes, is characterized by the apical localization of Nhe3 (Hiroi et al. 2008). The density of Type III ionocytes (along with *nhe3* expression) increases in the gills of tilapia exposed to low-Na^+ conditions (Inokuchi et al. 2008, 2009).

2.2 Freshwater-type ionocytes in basal fishes

In lampreys, two FW-adaptive ionocytes have been proposed to support ion uptake (Bartels and Potter 2004; Reis-Santos et al. 2008; Ferreira-Martins et al. 2021). These two ionocytes differ most notably in their expression of Nka and H^+-ATPase. A “larval FW ionocyte” highly expresses H^+-ATPase but shows low expression of Nka, whereas a “FW ionocyte” (observed in larvae as well as post-metamorphic and adult stages) strongly expresses both H^+-ATPase and Nka. H^+-ATPase E subunit (*atp6v1e*) expression markedly decreases in the gills when lamprey acclimate to elevated salinities (Reis-Santos et al. 2008; Ferreira-Martins et al. 2016). The ionoregulatory role of H^+-ATPase in FW gills typically involves its co-expression with a pathway for the electrochemically neutral uptake of environmental Na^+. The absorption of environmental Na^+ by lampreys appears to involve the epithelium Na^+ channel (ENaC) (Ferreira-Martins et al. 2016), while Ncc supports both Na^+ and Cl^- uptake (Barany et al. 2021b). Accordingly, both ENaC and Ncc are highly expressed in the gills of FW-acclimated lamprey and exhibit decreased expression during SW acclimation, although which particular cell-types express these transporters has not been fully elucidated. The co-involvement of an apical carbonic anhydrase-powered Cl^-/HCO_3^- exchanger and a basolateral Cl^--channel in Cl^- uptake has also been proposed, but the molecular identities of these transporters are unresolved (Bartels and Potter 2004; Ferreira-Martins et al. 2021).

2.3 Seawater-type ionocytes in teleosts

Within the branchial epithelium of marine/SW-acclimated teleosts, SW-type ionocytes actively secrete excess Na^+ and Cl^- into the environment. SW-type ionocytes express Nka and $Na^+/K^+/2Cl^-$ cotransporter 1 (Nkcc1; Slc12a2) in the basolateral membrane to energize and facilitate the Na^+- and K^+-coupled passage of Cl^- from blood plasma into the cell interior (Marshall and Grosell 2006; Kaneko et al. 2008). The catalytic α-subunit of the Nka enzyme contains binding sites for ATP, Na^+, and K^+ (Geering 2008). Two distinct isoforms of the α-subunit (α1a and α1b) were identified in salmonids, first by Richards et al. (2003). In salmonids and cichlids, these isoforms have functional capacities exclusive to either FW (α1a) or SW (α1b), with branchial expression "switching" from one to the other during salinity transitions (Bystriansky et al. 2006; Nilsen et al. 2007; McCormick et al. 2009; Tipsmark et al. 2011; Dalziel et al. 2014). Apically located cystic fibrosis transmembrane conductance regulator 1 (Cftr1) enables Cl^- to exit SW-type ionocytes and to enter the external environment (Marshall and Grosell 2006). With Nkcc1 and Cftr1 forming the pathway for transcellular Cl^- excretion, tight-junction complexes composed of claudins (Cldns) between ionocytes and adjacent accessory cells provide the paracellular route for Na^+ to exit the gill (Marshall and Grosell 2006; Tipsmark et al. 2008b; Bui and Kelly 2014). Attendant increases in branchial Nka, Nkcc1, and Cftr1 expression coincide with SW-acclimation. For this reason, all three ion transporters are widely used as key markers of branchial ion-secretory capacity.

2.4 Seawater-type ionocytes in basal fishes

The pathways for branchial Cl^- secretion are far less resolved in basal fishes than in teleosts. Cftr orthologs are present in the genomes of sturgeon, bichir, and coelacanth (Shaughnessy and Breves 2021), yet none of these orthologs have been functionally characterized. A single Cftr ortholog was identified in sea lamprey; however, *cftr* expression is low in all larval, juvenile, and adult tissues aside from intestine (Ren et al. 2015). Moreover, compared with human Cftr, lamprey Cftr exhibits limited Cl^-

conductance and reduced activation by cAMP (Cui et al. 2019). Given the limited Cl^- conductance of lamprey Cftr and the lack of a *cftr* transcriptional response to SW exposure (Shaughnessy et al. unpublished), it is questionable whether Cftr mediates the secretion of Cl^- by lamprey ionocytes known to express Nka and Nkcc1 (Shaughnessy and McCormick 2020). A recent analysis of the updated inshore hagfish (*Eptatretus burgeri*) genome assembly (Yu et al. 2023; Marlétaz et al. 2023) indicates that a *cftr* ortholog may be absent in hagfishes altogether (Yamaguchi et al. 2023).

3. Hormones and ionocytes

The endocrine system has long been appreciated as a central player in the homeostatic regulation of salt and water balance in fishes. Perturbations in internal osmotic and ionic conditions caused by changes in environmental salinity elicit the secretion of hormones that modulate ion- and water-transport by key osmoregulatory organs. Because these regulatory connections are indispensable to maintaining hydromineral balance, there is no shortage of literature that discusses how hormones impact the osmoregulatory physiology of fishes at the organismal, organ, and cellular levels (Hirano 1986; McCormick 2001; Manzon 2002; Evans et al. 2005; Sakamoto and McCormick 2006; Takei and McCormick 2013; Takei et al. 2014). Therefore, in this review, we do not address all established hormonal actions within the gills of fishes; rather, we focus on how hormones control the molecular components of ionocytes. We focus on the regulatory connections identified in euryhaline species but, in several instances, reference stenohaline zebrafish for added context. An expansive collection of endocrine factors undeniably contributes to regulating branchial ionocytes (Evans et al. 2005; Takei et al. 2014); however, the identification of molecular endocrine targets is largely based on studies that focused upon the “classical” FW- and SW-adapting hormones in fishes, namely prolactin (Prl), growth hormone (Gh), and cortisol. While this review is heavily weighted toward describing the actions of these three hormones, we also highlight promising areas for future investigations into how additional endocrine factors regulate ionocytes.

4. Freshwater-adaptive endocrine control

4.1 Prolactin

Euryhaline models, and most famously, mummichog (*Fundulus heteroclitus*), supported the discovery that pituitary hormones are key regulators of osmoregulatory organs (Pickford and Atz 1957). Pickford (1953) and Burden (1956) reported that hypophysectomized mummichogs could not survive in FW, and that pituitary brei injections rescued them from death. Prl was subsequently identified as the pituitary factor that enables individuals to reside in dilute environments (Pickford and Phillips 1959). Over the succeeding decades, it was firmly established that through its highly conserved actions on teleost osmoregulatory organs, Prl stimulates a spectrum of activities befitting FW-acclimation (Loretz and Bern 1982; Hirano 1986; Manzon 2002; Sakamoto and McCormick 2006; Breves et al. 2014a, 2020). Accordingly, pituitary *prl* expression and plasma Prl levels rise when fish acclimate to low-salinity conditions (Lee et al. 2006; Hoshijima and Hirose 2007; Fuentes et al. 2010; Seale et al. 2012). The notion that ionocytes are targets of Prl signaling was supported decades ago by the observation that Prl influences ionocyte populations in Mozambique and Nile (*O. niloticus*) tilapia (Herndon et al. 1991; Pisam et al. 1993; Flik et al. 1994). With respect to directing ionoregulatory function, Zhou et al. (2003) showed that exogenous Prl stimulated ion uptake in rainbow trout branchial epithelium. Patterns of Prl binding and *prl receptor* (*prlr*) gene expression reported in both euryhaline and stenohaline FW species further associated Prl signaling with ionocytes (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 Prlr was localized to branchial ionocytes of Mozambique tilapia and sea bream (*Sparus aurata*) (Weng et al. 1997; Santos et al. 2001).

Only recently have investigations into the actions of Prl become unencumbered by a paucity of molecular tools to study FW-type ionocytes. For example, the characterization of tilapia Type II ionocytes by Hiroi et al. (2008) provided an opportunity to link Prl with a specific molecular pathway for ion uptake, particularly Na^+/Cl^- cotransporter 2 (Ncc2; Slc12a10). Prl enables hypophysectomized tilapia to recruit Ncc2-expressing ionocytes during FW acclimation, an activity that does not require systemic intermediaries (Breves et al. 2010c; Inokuchi et al. 2015; Watanabe et al. 2016) (Fig. 1). Prl similarly regulates branchial *ncc2* expression in euryhaline mummichog (Breves et al. 2022) and Japanese medaka (Bossus et al. 2017), as well as in stenohaline zebrafish (Breves et al. 2013). Activated Prl receptors (Prlrs) can modulate the transcription of target genes through JAK/STAT and ERK/MAPK signaling (Huang et al. 2007; Fiol et al. 2009; Chen et al. 2011). In medaka, Prl stimulates *ncc2* via STAT5 activation rather than through ERK- or AKT-dependent pathways (Bollinger et al. 2018).

Since Clc2c is expressed within Ncc2-expressing ionocytes to facilitate basolateral Cl^- movement (Pérez- Ruis et al. 2015; Wang et al. 2015), it is fitting that Prl coordinately promotes *clc2c* and *ncc2* expression (Breves et al. 2017b; Breves 2019) (Fig. 1). In contrast, *clc3* expression in tilapia ionocytes is not under Prl control (Tang and Lee 2011; Breves et al. 2017b).

The potential for Ncc-dependent pathways to operate in the osmoregulatory organs of cartilaginous and jawless fishes has recently received increased attention. In Japanese-banded houndshark (*Triakis scyllium*), a “conventional” *ncc1* (*slc12a3*) is expressed within a subpopulation of gill ionocytes, termed type-B cells, where its expression increases upon transfer from full-strength SW to 30% SW (Takabe et al. 2016). Given that elasmobranch genomes are devoid of Ncc2-encoding genes (Motoshima et al. 2023), Ncc1 may assume a role in branchial Na^+ and Cl^- absorption in elasmobranchs. Similarly, the branchial expression of *ncca* (*ncc1*) in sea lamprey is attenuated during SW acclimation (Ferreira-Martins et al. 2016; Barany et al. 2021b). Given the expression of the Prlr in lamprey gills, the next step is to assess whether the recently found Prl participates in modulating *ncca* when lamprey transition between FW and marine environments (Gong et al. 2020).

In two lampreys (*P. marinus* and *Lethenteron reissneri*), the expression of gene transcripts encoding ENaC subunits increases under low-Na^+ conditions (Ferreira-Martins et al. 2016; Tseng et al. 2022). Thus, ENaC may provide a means for lampreys to absorb Na^+ from FW; this strategy for Na^+ absorption is absent in cartilaginous and ray-finned fishes (Ferreira-Martins et al. 2021). Curiously, branchial gene expression of an ENaC subunit, *scnn1a*, decreases when inshore hagfish experience high-salinity conditions (Yamaguchi et al. 2023). Despite hagfishes exhibiting ionoconformity, this response suggests that Na^+ movement in the gill may be more complex than previously thought. To our knowledge, endocrine control of ENaC subunit expression has not been addressed in any cyclostome and, in an analogous fashion as *ncca*, should be probed for links to the Prlrs identified in hagfish and lamprey (Gong et al. 2020).

While branchial ionocytes leveraging Ncc operate in species across the three major fish lineages, they are not found within salmonids (Hiroi and McCormick 2012). In turn, an apically located Cl^-/HCO_3^- exchanger (Slc26a6) may provide a pathway for Cl^- absorption by PNA^+ ionocytes in rainbow trout and other salmonids (Boyle et al. 2015; Leguen et al. 2015). Branchial *slc26a6a2* is elevated in FW- versus SW-acclimated Atlantic salmon (Takvam et al. 2021) and is a transcriptional target of Prl signaling

(Breves et al. unpublished). Therefore, Slc26a6a2 may constitute a pathway for Prl-stimulated Cl^- uptake in species lacking Ncc-expressing ionocytes (Zhou et al. 2003). Because Leguen et al. (2015) reported *clc2* expression in trout ionocytes (putative PNA^+ cells), Prl-based control of salmonid *clc2* isoforms also warrants investigation. Studies of this nature will enable comparisons of Prl-Clc2 connectivity between species that do, and do not, leverage Ncc2-expressing ionocytes.

Within the PNA^- ionocytes of trout, Nbce1 supports the absorption of environmental Na^+ by cotransporting Na^+ and HCO_3^- across the basolateral membrane (Parks et al. 2007; Leguen et al. 2015). The apical entry of Na^+ into PNA^- cells was proposed to occur via Asic4 through its electrochemical linkage to H^+-ATPase (Dymowska et al. 2014). Under this scenario, intracellular HCO_3^- is supplied by carbonic anhydrase (Parks et al. 2007). In tilapia, Nbce1 operates in the basolateral membrane of Ncc2-expressing ionocytes (Furukawa et al. 2011). To our knowledge, Nbce1, Asic4, H^+-ATPase, and carbonic anhydrase have not been associated with Prl signaling in trout or tilapia.

In addition to Type II ionocytes, a second type of Na^+-absorptive ionocyte in tilapia (Type III ionocytes) is characterized by the apical expression of Nhe3 (Hiroi et al. 2008). Prl promotes *nhe3* gene expression in tilapia gill filaments (Inokuchi et al. 2015; Watanabe et al. 2016) whereas it has no such effect in mummichog or zebrafish (Breves et al. 2013, 2022) (Fig. 1). Because salmonids express Nhe2 and -3 within PNA^+ ionocytes, they will prove key in resolving the extent to which Prl regulates Nhes across teleosts (Ivanis et al. 2008; Hiroi and McCormick 2012). Unfortunately, the lack of information on Nhes in lamprey ionocytes precludes consideration of a Prl-Nhe connection (Ferreira-Martins et al. 2021). Recent pharmacological experiments performed in zebrafish implicated K^+-dependent Na^+/Ca^{2+} exchangers (Nckxs) in mediating Na^+ absorption (Clifford et al. 2022). Should roles emerge for Nckxs in supporting Na^+ uptake by euryhaline species, Nckx isoforms would be additional candidates for regulation by Prl.

Nka plays a critical role in energizing ion transport by FW- and SW-type ionocytes, with the reciprocal expression of *nka-α1a* and *-α1b* first described in salmonids transitioning between FW and SW environments (Richards et al. 2003; Mackie et al. 2005; Bystriansky et al. 2006; Madsen et al. 2009; McCormick et al. 2009; Dalziel et al. 2014). Tilapia also undergo *nka-α1a* and *-α1b* “switching” upon salinity changes, and Prl stimulates the “FW-inducible” *nka-α1a* isoform (Tipsmark et al. 2011;

Breves et al. 2014b; Inokuchi et al. 2015; Watanabe et al. 2016) (Fig. 1). Thus far, the capacity for Prl to promote *nka-α1a* expression seems specific to tilapia, as Prl fails to stimulate *nka-α1a* in Atlantic salmon (Tipsmark and Madsen 2009; Breves et al. unpublished). In zebrafish, *nka-α1a1a.2* is expressed in Ncc2-expressing ionocytes responsible for Cl^- uptake (Liao et al. 2009); however, Prl has no effect on branchial *nka-α1a1a.2* expression (Breves 2019). The auxiliary γ-subunit of Nka (also called Fxyd) participates in the regulation of enzymatic activity by associating with the Na^+/K^+ pump complex (Geering 2008; Pavlovic et al. 2013). Among the Fxyd isoforms identified in teleosts, Fxyd11 is predominately expressed in the gills where it interacts with Nka (Tipsmark 2008; Wang et al. 2008; Saito et al. 2010). In tilapia, Prl and cortisol synergistically promote *fxyd11* expression in FW (Tipsmark et al. 2011).

For teleosts residing in FW, greater than 90% of whole-body Ca^{2+} uptake is mediated by branchial/epidermal ionocytes (Flik et al. 1996; Lin and Hwang 2016). Transcellular Ca^{2+} uptake entails the apical entry of Ca^{2+} through ECaC (Trpv5/6) followed by basolateral exit via Ca^{2+}-ATPase 2 (Pmca2) and Na^+/Ca^{2+} exchanger 1 (Ncx1) (Flik et al. 1996; Liao et al. 2007). Prl is hypercalcemic in multiple teleosts (Pang et al. 1978; Fargher and McKeown 1989; Flik et al. 1989, 1994; Kaneko and Hirano 1993; Chakraborti and Mukherjee 1995; Wongdee and Charoenphandhu 2013), at least in part by stimulating branchial Pmca activity (Flik et al. 1996). Future investigations employing both euryhaline and stenohaline FW models are needed to determine whether Prl promotes ECaC and Ncx1 expression in parallel with promoting Pmca activity to sustain Ca^{2+} uptake.

Aquaporins (Aqps) constitute a superfamily of integral membrane proteins that facilitate passive movements of water and small non-ionic compounds across cell membranes (Cerdà and Finn 2010). Multiple branchial cell types, including ionocytes, express a subset of Aqps (Lignot et al. 2002; Hirata et al. 2003; Watanabe et al. 2005; Tse et al. 2006; Brunelli et al. 2010; Tingaud-Sequeira et al. 2010; Tipsmark et al. 2010; Jung et al. 2012; Breves et al. 2016; Ruhr et al. 2020). Prl stimulates the expression of the aquaglyceroporin, Aqp3, in Mozambique tilapia (Breves et al. 2016) (Fig. 1), Japanese medaka (Ellis et al. 2019), and mummichog (Breves et al. 2022). On the other hand, Prl does not promote branchial *aqp1* expression (Ellis et al. 2019). Although the Aqp-specific effects of Prl suggest that Aqp3 plays an important role in FW-acclimated fish, there is still no clear picture of how it underlies adaptive processes. A role for Aqp3 in enhancing transepithelial water movement appears unlikely because branchial water exchange is disadvantageous to systemic hydromineral balance. Alternatively, Aqp3 may render ionocytes osmosensitive to extracellular conditions and/or capable of efficiently regulating their volume (Cutler and Cramb 2002; Watanabe et al. 2005; Tipsmark et al. 2010).

Prl has long been recognized for its effects on membrane permeability which result in a general "tightening" to minimize diffusive ion loss (Potts and Evans 1966; Hirano 1986). Paracellular solute movements across epithelia are governed in large part by the barrier properties of tight-junction complexes composed of Cldn and occludin proteins (Chasiotis et al. 2012). In tilapia and medaka, FW acclimation entails the increased expression of branchial *cldn28a* and *-28b*, respectively (Tipsmark et al. 2008a; Bossus et al. 2015). In Atlantic salmon and medaka, Prl stimulates *cldn28a* and *-28b* gene expression (Tipsmark et al. 2009; Bossus et al. 2017). Prl-Cldn28 connectivity thus provides a means to regulate tight-junction properties for minimizing ion loss in FW. *Occludin* expression is also correlated with environmental salinity (Chasiotis et al. 2009; Kumai et al. 2011; Whitehead et al. 2011), making it a good candidate for regulation by Prl; however, to our knowledge, this link has yet to be examined.

Teleosts express two separate Prlrs, denoted Prlr1 (Prlra) and -2 (Prlrb), that differ in their responses to salinity changes (Huang et al. 2007; Pierce et al. 2007; Fiol et al. 2009; Tomy et al. 2009; Rhee et al. 2010; Breves et al. 2011; Chen et al. 2011; Flores and Shrimpton 2012; Breves et al. 2013). Branchial *prlr1* has emerged as a transcriptional target of Prl in tilapia, mummichog, and zebrafish (Inokuchi et al. 2015; Breves et al. 2013, 2022). In turn, Prl seemingly upregulates the expression of Prlr1 to enhance the sensitivity of ionocytes to circulating hormone during FW acclimation (Weng et al. 1997). Alternatively, *prlr2/b* is typically insensitive to Prl (Breves et al. 2013, 2022; Inokuchi et al. 2015), which is not surprising given that its expression is upregulated by the hyperosmotic extracellular conditions associated with SW acclimation (Fiol et al. 2009; Inokuchi et al. 2015; Seale et al. 2019).

In tandem with initiating active ion uptake, euryhaline species must attenuate branchial ion secretion when transitioning from SW to FW. While promoting the recruitment of FW-type ionocytes and the expression of their associated ion transporters, Prl simultaneously dampens cellular and molecular phenotypes appropriate for SW conditions. For instance, Herndon et al. (1991) observed that Prl reduced the size and number of SW-type ionocytes in tilapia. At the molecular level, Prl inhibits the transcription of *nkcc1* and *cftr1* within the SW-type ionocytes of medaka and mummichog (Tipsmark and Madsen 2009; Bossus et al. 2017; Breves et al. 2022) (Fig. 2). Prl also inhibits branchial Nka activity and *nka-α1b* expression (Pickford et al. 1970; Sakamoto et al. 1997; Shrimpton and McCormick 1998; Kelly et al. 1999; Mancera et al. 2002; Tipsmark and Madsen 2009), which, like *nkcc1* and *cftr1*, are elevated in SW to support ion secretion. Recall that while Cftr1 is the conduit for Cl^- to exit SW-type ionocytes, tight junction complexes between ionocytes and accessory cells provide the paracellular path for Na^+ to exit the organism. The cation-selective tight-junctions adjacent to ionocytes are composed of multiple Cldn10 isoforms (Tipsmark et al. 2008b; Bui and Kelly 2014). Among the four mummichog *cldn10* genes (*cldn10c*, *-10d*, *-10e*, and *-10f*) upregulated in response to SW (Marshall et al. 2018), *cldn10f* is the only transcript downregulated by Prl (Breves et al. 2022) (Fig. 2). Collectively, these *nkcc1*, *cftr1*, and *cldn10f* responses illustrate the various means by which Prl inhibits branchial salt secretion.

4.2 Growth hormone and somatolactin

As discussed in Section 5.1, Gh is conventionally regarded as a “SW-adapting hormone” because it promotes the survival of euryhaline fishes (and especially salmonids) in hyperosmotic environments (Björnsson 1997; McCormick et al. 2002; Takei et al. 2014). To our knowledge, there is no direct evidence that Gh plays a role in regulating FW-type ionocytes. Nonetheless, Gh receptors (Ghrs) are expressed in the gills of euryhaline species regardless of whether they are acclimated to FW or SW (Pierce et al. 2007; Poppinga et al. 2007; Breves et al. 2011; Link et al. 2010); therefore, Ghrs are at least present to mediate any direct regulatory connections between circulating Gh and FW-type ionocytes. It is certainly plausible that Gh may indirectly regulate FW-type ionocytes through the synthesis of insulin-like growth-factors (Igfs) (Reinecke et al. 1997; Berishvili et al. 2006; Reindl and Sheridan 2012). In fact, black-chinned tilapia (*Sarotherodon melanotheron*) exhibit enhanced *ghr* and *igf1* expression in the gill during FW acclimation (Link et al. 2010). Similarly, zebrafish exhibit elevated pituitary *gh* and branchial *ghr* (*ghra* and *-b*), *igf1a*, and *-2a* expression when challenged with ion-poor conditions (Hoshijima and Hirose 2007; Breves et al. unpublished). However, whether the Gh/Igf system supports the molecular responses of tilapia and zebrafish ionocytes to FW/ion-poor conditions has yet to be determined.

Somatolactin (Sl), a member of the Gh/Prl-family of pituitary hormones, is a putative regulator of various physiological processes in fishes, particularly Ca^{2+}

homeostasis (Kaneko and Hirano 1993). Rainbow trout transferred to Ca^{2+}-rich FW exhibit reduced *sl* gene expression in the pituitary, a response that is consistent with Sl having hypercalcemic activity (Kakizawa et al. 1993). Given the substantial progress made toward understanding how ionocytes absorb environmental Ca^{2+} (Lin and Hwang 2016), a reassessment of whether Sl is indeed hypercalcemic is warranted by probing targets such as ECaC, Pmca2, and Ncx1.

4.3 Cortisol

Cortisol is typically deemed a "SW-adapting hormone" because it directly stimulates the activities and/or expression of transporters tied to branchial ion-secretion (Section 5.2). The recognition that cortisol also promotes ion uptake in some teleosts arrived after its SW-adaptive role was firmly established (McCormick 2001; Takei and McCormick 2013). Morphological responses to cortisol in the gills of rainbow trout and American eel (*Anguilla rostrata*) suggested that FW-type ionocytes are targets of cortisol signaling (Perry et al. 1992), a notion that would be later supported with the development of molecular tools to more precisely study FW-type ionocytes. In tilapia, medaka, and zebrafish, Nhe3 and Ncc2 are expressed in distinct ionocyte subtypes (Hiroi and McCormick 2012; Hsu et al. 2014; Guh et al. 2015). In zebrafish, cortisol stimulates Na^{+} uptake in a fashion dependent upon the presence of Nhe3b-expressing ionocytes and promotes the differentiation of Ncc2-expressing ionocytes from a progenitor population (Kumai et al. 2012; Cruz et al. 2013a). While cortisol similarly promotes *ncc2* expression in medaka (Bossus et al. 2017; Ellis et al. 2019), this is not the case in tilapia (Breves et al. 2014b; Watanabe et al. 2016).

The FW-adaptive role of cortisol in zebrafish appears to be mediated solely by the glucocorticoid receptor (Gr) rather than the mineralocorticoid receptor (Mr) (Cruz et al. 2013b). The zebrafish Gr is expressed by Nka-rich branchial and epidermal ionocytes, with knockdown of *gr*, but not *mr*, disrupting the development of FW-type ionocytes through the action of forkheadbox I3 transcription factors (Foxi3a and -b) (Cruz et al. 2013b). Exogenous cortisol increases *nhe3b*, *H^{+}-ATPase α-subunit* (*atp6v1a*), and *ecac* expression in zebrafish embryos. In medaka embryos, knockdown of *gr2,* but not *gr1* or *mr*, decreases the total number of epidermal ionocytes (Trayer et al. 2013). Conversely, in FW-acclimated tilapia, it was suggested that the Mr, rather than the Gr, controls cortisol-mediated development of Nka-rich branchial ionocytes (Wu et al.

2023). Accordingly, *mr* expression occurs in ionocyte precursors/epidermal stem cells (Wu et al., 2023).

In Atlantic salmon, cortisol upregulates gene transcription and protein abundance of the “FW-inducible” Nka-α1a isoform (Kiilerich et al. 2007b; McCormick et al. 2008, 2012; Tipsmark and Madsen 2009). Cortisol also upregulates the “SW-inducible” Nka-α1b isoform (Kiilerich et al. 2007b; Tipsmark and Madsen 2009; Breves et al. 2024), and thus, the capacity of cortisol to increase the expression of both Nka-α1a and -α1b is indicative of its dual role in promoting FW- and SW-adaptive processes. While cortisol was shown to stimulate branchial carbonic anhydrase activity in trout (Gilmour et al. 2011), to our knowledge, no ion transporters expressed in salmonid FW-type ionocytes outside of Nka (e.g., Nhe2, -3, Asic4, ECaC, and Nbce1) have been linked with cortisol. This is a significant knowledge gap, especially given that cortisol is known to stimulate Ca^{2+} uptake by ECaC-expressing ionocytes in zebrafish (Lin and Hwang 2016). Reminiscent of the scenario for Prl (Section 4.1), future work is warranted to resolve whether cortisol affects Ca^{2+} uptake pathways in euryhaline species.

In addition to promoting key mediators of ion uptake (e.g., Ncc2, Nhe3, and Nka-α1a), cortisol promotes FW acclimation by decreasing the paracellular permeability of the branchial epithelium (Kelly and Wood 2002; Zhou et al. 2003; Kolosov and Kelly 2017). This important contribution to FW acclimation is achieved through the regulation of specific tight-junction proteins. For instance, cortisol increases the expression of *cldn8d*, *-10c*, *-10d*, *-10e*, *-10f*, *-11a*, *-27a*, *-30c*, and *-33b* in various euryhaline species (Tipsmark et al. 2009; Bui et al. 2010; Bossus et al. 2017; Kolosov and Kelly 2017). Finally, it certainly must be recognized that cortisol can promote FW acclimation by acting in concert with Prl (Eckert et al. 2001; McCormick 2001). For instance, from a molecular perspective, Prl and cortisol act synergistically to promote branchial *nka-α1a* and *cldn28b* expression in tilapia and medaka, respectively (Watanabe et al. 2016; Bossus et al. 2017).

4.4 Thyroid hormones

Although limited, there is evidence that thyroid hormones are involved in the control of FW-adaptive branchial processes. Unfortunately, information is particularly scant regarding plasma thyroxine (T_4) and 3-3′-5-triiodothyronine (T_3) levels in euryhaline species undergoing FW acclimation. In sea bream, plasma T_4 levels increase following transfer from SW to FW (Klaren et al. 2007). Alternatively, Mozambique tilapia acclimating to FW exhibit rapid declines in both plasma T_4 and T_3 (Seale et al. 2021). While the dynamics of T_4 and T_3 in tilapia suggest a hyposmotically-induced suppression of thyroid hormone production at the systemic level, at the level of the gill, these changes coincide with an increase in the outer-ring deiodination activity of deiodinase 2 (Dio2). As shown in mummichog, Dio2 expression/activity is activated by hyposmotic stress (López-Bojórquez et al. 2007). Thus, increased branchial Dio2 activity supports the local production of T_3 at a time when the recruitment of ionocytes is activated following entry into FW (Hiroi et al. 2008; Breves et al. 2021). Accordingly, tilapia treated with T_4 exhibit an increase in the density and size of presumed FW-type ionocytes (Peter et al. 2000). It remains to be seen whether these cellular responses to T_4 manifest changes in branchial *ncc2*, *nhe3*, and *clc2c* expression.

5. Seawater-adaptive endocrine control

5.1 Growth hormone and insulin-like growth-factors

Although much of the early attention given to the Gh/Igf system in fishes was driven by its potential application to understanding growth in aquaculture settings, the osmoregulatory actions of both Gh and Igf1 have emerged as important aspects of the hormonal control of osmoregulation. In salmonids, Gh is integral to the timing of parr-smolt transformation and the associated development of SW tolerance (Hoar 1988; Björnsson 1997; McCormick 2013), and accordingly, plasma Gh levels increase during smolting (Boeuf et al. 1989; Prunet et al. 1989; Young et al. 1989; McCormick et al. 2007, 2013; Nilsen et al. 2008). The SW-adaptive role for Gh is not restricted to salmonids, as in both salmonid and non-salmonid teleost species, exposure to SW corresponds with elevated plasma Gh levels and increased *gh* gene expression, Gh protein content, and somatotroph numbers in the pituitary (Deane and Woo 2009). As shown in Mozambique tilapia, somatotrophs release Gh in direct response to hyperosmotic extracellular conditions (Seale et al. 2002). Importantly, treatment with Gh upregulates branchial Nka activity and improves the SW tolerance of several euryhaline teleosts (Madsen 1990a, b; McCormick 1996; Xu et al. 1997; Mancera and McCormick 1998; Pelis and McCormick 2001). Intraperitoneal injection with Gh also increases Nkcc1 protein abundance within SW-type ionocytes (Pelis and McCormick 2001) and stimulates *nka-α1b* and *nkcc1* expression (Tipsmark and Madsen 2009), although these effects were most pronounced when Gh was co-administered with cortisol.

Ghrs are present in teleost gills (Gray et al. 1990; Yao et al. 1991; Sakamoto and Hirano 1991); however, they have yet to be localized to any discrete branchial cell-types. It was initially reported that rainbow trout acclimating to SW do not exhibit changes in branchial Gh binding (Sakamoto and Hirano 1991). More recent molecular analyses describe variable branchial *ghr* expression patterns with respect to SW acclimation. In Atlantic salmon, *ghr* expression has been seen to increase (Kiilerich et al. 2007a; Nilsen et al. 2008) or not change at all (Breves et al. 2017a) during smolting. Likewise, there is little consistency in branchial *ghr* patterns following SW exposure, with increases, decreases, and no changes in expression all having been observed across several species (Kiilerich et al. 2007a; Nilsen et al. 2008; Breves et al. 2010a, b; Flores and Shrimpton 2012; Einarsdóttir et al. 2014; Breves et al. 2017a; Link et al. 2022). Additionally, Gh-treated gill explants from coho salmon (*Oncorhynchus kisutch*) and Nile tilapia did not exhibit changes in Nka activity, or *nka-α1b* and *nkcc1* gene expression (McCormick et al. 1991; Breves et al. 2014b). Rather than directly regulating the expression of specific ion-transporters, Gh may exert cytogenic effects that promote the recruitment of branchial ionocytes (Madsen 1990a, b; Flik et al. 1993; Prunet et al. 1994). For instance, Gh- elicited increases in Nka activity and Nkcc1 in Atlantic salmon were coincident with an increased abundance of ionocytes (Pelis and McCormick 2001).

Gh is the primary regulator of the production and release of Igf1 and -2 from the liver (Pierce et al. 2011; Reindl and Sheridan 2012). Branchial *igf1 receptor* (*igf1r*) expression increases during smolting and upon exposure to SW (Nilsen et al. 2008; Shimomura et al. 2012), and increased circulating Igf1 levels correlate with elevated branchial Nka activity (Agustsson et al. 2001; McCormick et al. 2007; Shimomura et al. 2012). However, not all studies have observed rises in plasma Igf1 during smolting (Nilsen et al. 2008; Breves et al. 2017a). Intraperitoneal injection of Atlantic salmon with Igf1 increases SW tolerance but only marginally impacts gill Nka activity (McCormick 1996) whereas Nkcc1 in isolated Japanese eel (*Anguilla japonica*) gill cells is stimulated by Igf1 (Tse et al. 2007). In addition to exerting osmoregulatory actions as endocrine signals (i.e., secreted from the liver and acting upon ionocytes) (Madsen and Bern 1993), Igf1 and -2 may also operate as autocrine/paracrine signals (i.e., produced by and acting upon ionocytes) (Berishvili et al. 2006; Tipsmark and Madsen 2009). In Atlantic salmon, Nilsen et al. (2008) reported increases in gill *igf1* and *igf1r* during smolting and SW acclimation, even when no increase in circulating Igf1 was detected.

Similarly, Breves et al. (2017a) observed increases in branchial *igf2* and *igf1ra* expression in smolts following SW exposure.

The promotion of SW-adaptive ionoregulatory capacities by Gh may be best explained by its interaction with cortisol to promote both the proliferation of ionocytes and their responsiveness to cortisol (McCormick 2013). Studies using salmonids demonstrated that cortisol interacts with the Gh/Igf system to affect SW-type ionocytes. The co-administration of cortisol with either Gh or Igf1 increases gill Nka activity to levels beyond those induced by treatment with either hormone individually (Madsen 1990a, b; Madsen and Korsgaard 1991; McCormick 1996). Scenarios proposed to underlie the apparent synergistic actions of cortisol and Gh include, 1) Gh promotes Gr abundance in ionocytes, thereby increasing the capacity for cortisol to affect ion transporter expression, and 2) Gh promotes ionocyte proliferation while cortisol promotes the differentiation of ionocytes (McCormick 2013). Thus, future work should leverage recent insights into the regulators of ionocyte differentiation, such as forkhead box transcription factors (Hsiao et al. 2007), to elucidate how Gh and cortisol shape SW-type ionocyte populations.

Recent studies also describe the potential for Gh and Igf1 to regulate SW-adaptive branchial processes in lampreys. Kawauchi et al. (2002) were the first to identify a lamprey Gh capable of stimulating hepatic *igf1* expression. Later, Gh-like cells in the lamprey pituitary were shown to increase in abundance during metamorphosis (Nozaki et al. 2008). The discovery of Ghr, Prlr, and Prl itself in sea lamprey spurred recent investigations into their regulatory roles (Gong et al. 2022). Although pituitary *gh* and *prl* expression are upregulated during sea lamprey metamorphosis (Gong et al. 2022), it was later shown that *gh* also increases in the pituitary of non-metamorphosing larvae over the same period (Ferreira-Martins et al. 2023). Thus, such increases in *gh* expression may be seasonal, and it remains unclear whether the same is true for pituitary *prl* expression. In any case, branchial *ghr* and *prlr* gene expression also increases during metamorphosis (Gong et al. 2020; Ferreira-Martins et al. 2023). Because similar increases do not occur in non-metamorphosing larval lamprey (Ferreira-Martins et al. 2023), heightened *ghr* and *prlr* expression likely underlies developmental (as opposed to seasonal) processes. Substantial increases in hepatic and branchial *igf1* expression also occur throughout metamorphosis, and therefore, endocrine as well as autocrine/paracrine actions of Igf1 may operate in lamprey (Ferreira-Martins et al. 2023). Surprisingly, SW exposure does not affect pituitary *gh* expression, hepatic *igf* expression, or branchial *ghr* and *igf1* expression (Gong et al. 2020, 2022; Ferreira-Martins et al. 2023) and treatment with recombinant Gh does not affect branchial ion transporters (Gong et al. 2022). Future studies in lamprey are warranted to assess whether Gh and Igf1 promote the recruitment of SW-type ionocytes through cytogenic actions.

5.2 Corticosteroids

In lobe-finned fishes (Sarcopterygii) and tetrapods, cortisol (or, in some cases, corticosterone) and aldosterone are the products of the corticosteroid biosynthesis pathway and the predominant circulating hormones. Cortisol and aldosterone separately regulate carbohydrate metabolism and osmoregulation by interacting with the Gr and Mr, respectively. In all other fishes, corticosteroids and their receptors mediate both carbohydrate metabolism and osmoregulation. However, important differences exist between fish groups, particularly with respect to the milieu of corticosteroids in circulation and the identity and expression of receptors that mediate the actions of these steroids. Here, we focus on corticosteroids that are known to directly regulate branchial processes in fishes.

Non-sarcopterygian fishes lack aldosterone synthase (Cyp11b2) and consequently the ability to synthesize aldosterone (Baker 2003; Takahashi and Sakamoto 2013). In actinopterygian fishes, cortisol is the predominant corticosteroid present in circulation, with 11-deoxycorticosterone and corticosterone present at far lower concentrations (Prunet et al. 2006). Among the circulating corticosteroids in actinopterygians, cortisol has both glucocorticoid and mineralocorticoid activity. To a far lesser extent, 11-deoxycorticosterone also exhibits mineralocorticoid-like actions (Takahashi and Sakamoto 2013). Chondrichthyan fishes produce a novel steroid biosynthetic product, 1α-hydroxycorticosterone, which exhibits some mineralocorticoid-like action (Anderson 2012). However, chondrichthyans do not utilize branchial processes for bulk ion secretion but rather use the salt-secretory rectal gland (Wright and Wood 2015); therefore, the potential ionoregulatory actions of 1α-hydroxycorticosterone will not be discussed here. Lampreys apparently lack 11β-hydroxylase (Cyp11b1) and cannot produce cortisol or corticosterone (Bridgham et al. 2006; Close et al. 2010; Rai et al. 2015). Thus, 11-deoxycortisol and 11-deoxycorticosterone are the most abundant circulating corticosteroids in lampreys and exhibit capacities to regulate branchial ionoregulatory activities (Close et al. 2010; Shaughnessy et al. 2020).

Chondrichthyan and actinopterygian fishes express both classes of corticosteroid receptors (Gr and Mr). In actinopterygians, it has long been held that the ionoregulatory actions of corticosteroids result from cortisol acting through the Gr. While this remains true, recent discoveries have added some nuance to this perspective. For instance, particular teleosts express two distinct Gr orthologs (Bury et al. 2003) as well as an Mr (Colombe et al. 2000). Knowledge of these three corticosteroid receptor subtypes has motivated investigations into how the actions of cortisol and 11-deoxycorticosterone are differentially mediated by these receptors (see discussion below). Interestingly, lamprey do not express Gr or Mr but rather an ancestral “corticoid receptor” (Cr) that facilitates the osmoregulatory actions of 11-deoxycortisol (Bridgham et al. 2006; Close et al. 2010; Shaughnessy et al. 2020).

Using adult sea lamprey, Close et al. (2010) demonstrated that 11-deoxycortisol elicits an increase in branchial Nka activity. Later, Shaughnessy et al. (2020) described how 11-deoxycortisol supports the acquisition of SW tolerance during metamorphosis. Plasma 11-deoxycortisol levels and gill Cr abundance both increase during metamorphosis and are positively correlated with gill Nka activity. Accordingly, the treatment of mid-metamorphic lamprey with 11-deoxycortisol improves SW tolerance and increases gill Nka and Nkcc1 protein expression (Shaughnessy et al. 2020; Barany et al. 2021a). Likewise, 11-deoxycortisol increases the expression of *nka* and *nkcc1* transcripts in lamprey gill explants (Shaughnessy et al. 2020). Interestingly, 11-deoxycorticosterone can elicit modest increases in branchial *nka* and *nkcc1* expression but is far less potent than 11-deoxycortisol (Shaughnessy et al. 2020). Future studies are warranted to further elucidate the ionoregulatory roles of 11-deoxycortisol and 11-deoxycorticosterone, and particularly whether they interact with Gh and Prl.

Cortisol has long been known to support the acclimation of teleosts to SW. Multiple lines of evidence have described this role, including early studies demonstrating that circulating cortisol increases during salmonid parr-smolt transformation and upon exposure to SW (Fontaine and Hatey 1954; Specker and Schreck 1982; Langhorne and Simpson 1986; Shrimpton et al. 1994), and that SW tolerance is increased following cortisol treatment (Bisbal and Specker 1991). Elevations in plasma cortisol following exposure to SW also occur in numerous non-salmonid species (McCormick 2001). Early work described the direct action of cortisol to increase gill Nka activity, which correlated with the development of SW tolerance during smolting (Langhorne and Simpson 1986; McCormick and Saunders 1987). Additional studies showed that gill Nka activity can be impacted *in vivo* by the administration of cortisol (Pickford et al. 1970; Bisbal and Specker 1991; McCormick et al. 1991) and *in vitro* by exposing gill explants to cortisol (McCormick and Bern 1989).

More recently, cortisol was shown to regulate proteins and gene transcripts expressed by SW-type ionocytes, such as Nka, Nkcc1, and Cftr (Fig. 3). Atlantic salmon interperitoneally injected with cortisol increase the expression of *nka-α1b* (McCormick et al. 2008; Tipsmark and Madsen 2009; Breves et al. 2020, 2024) and the protein abundance of Nka and Nkcc1 (Pelis and McCormick 2001). In gill explants from FW- and SW-acclimated Atlantic salmon, cortisol increases *nka-α1b* and *nkcc1* expression (Tipsmark et al. 2002; Kiilerich et al. 2007b, 2011a, b, c). *In vivo* treatment with cortisol increases *cftr1* expression in Atlantic salmon parr and smolts (Singer et al. 2003; Breves et al. 2020, 2024), and *in vitro* exposure of gill explants to cortisol increases *cftr1* and *nkcc1* (Kiilerich et al. 2007b). Likewise, cortisol promotes *cftr1* and *nkcc1* expression in the gills of FW-acclimated trout and medaka (Tipsmark et al. 2002; Kiilerich et al. 2011a; Bossus et al. 2017). In tilapia and striped bass (*Morone saxatilis*), cortisol similarly promotes branchial *nkcc1* expression (Kiilerich et al. 2011c). Cortisol also promotes components of SW-type ionocytes in non-teleost models, such as Nka and Nkcc1 in Atlantic (*Acipenser oxyrhynchus*) and Persian sturgeon (*A. persicus*) (Khodabandeh et al. 2009; McCormick et al. 2020).

Fewer studies have examined the molecular actions of 11-deoxycorticosterone, as it circulates at far lower concentrations than cortisol. Intraperitoneal injection of 11-deoxycorticosterone has no effect on SW tolerance or branchial *nka-α1a* and *-α1b* expression in Atlantic salmon (McCormick et al. 2008). The *in vitro* effects of 11-deoxycorticosterone vary depending on whether treated filaments are collected from salmon acclimated to either FW or SW. 11-deoxycorticosterone is more effective in stimulating *nka-α1a* versus *-α1b* expression (Kiilerich et al. 2007b, 2011a, b), although this effect is generally far less consistent than that of cortisol.

The role of the Gr in mediating the ionoregulatory actions of cortisol in teleosts has also received considerable attention. Early studies demonstrated that a corticosteroid receptor expressed in the gills with high binding affinity for cortisol increases during parr-smolt transformation and SW acclimation (Weisbart et al. 1987; Maule and Schreck 1990; Shrimpton and Randall 1994; Shrimpton et al. 1994;

Marsigliante et al. 2000). Moreover, Gr expression is strongly correlated with the capacity for cortisol to stimulate branchial Nka activity (Shrimpton and McCormick 1999). Following the discovery of two distinct Grs (Bury et al. 2003) and an Mr (Colombe et al. 2000; Sturm et al. 2005) in teleost fishes, studies using selective receptor antagonists investigated their individual roles in mediating the actions of cortisol and 11-deoxycorticosterone. It was proposed that the Gr and Mr underlie the duality of cortisol operating as a FW- and SW-adapting hormone (Prunet et al. 2006). In support of this, the upregulation of *gr* expression occurs in the gills of several species during smolting or following SW exposure (Mazurais et al. 1998; Mizuno et al. 2001; Kiilerich et al. 2007a; Nilsen et al. 2008; Yada et al. 2014; Bernard et al. 2020), and a potential role for the Mr in FW ionoregulation has been suggested (Sloman et al. 2001; Scott et al. 2005; Kiilerich et al. 2011a). The ionoregulatory role of the Mr in FW may entail activation by both cortisol and 11-deoxycorticosterone, as the Mr is potently activated by both hormones (Sturm et al. 2005; Katsu et al. 2018). Investigations into the regulation of *gr* and *mr* during smolting or SW acclimation have generally presented mixed results. In some studies, only *gr* is upregulated during smolting (Kiilerich et al. 2007a, 2011b; Nilsen et al. 2008), and in others, the transcriptional upregulation of both receptors occurred (Yada et al. 2014; Bernard et al. 2020). Similarly, there seems to be little consistency in how *gr* and *mr* are transcriptionally regulated during SW acclimation in salmonids (Kiilerich et al. 2007b, 2011a; Nilsen et al. 2008; Flores and Shrimpton 2012) as well as non-salmonids (Aruna et al. 2012a, b).

Several *in vivo* and *in vitro* studies have employed receptor blockade approaches, including the cotreatment of corticosteroids with mammalian Gr and Mr antagonists (e.g., RU486 and spironolactone, respectively). Cotreatment with RU486 blocks the upregulation of branchial *nka-α1a* and *-α1b* by cortisol, whereas cotreatment with spironolactone has no effect on SW tolerance or *nka-α1a* and *-α1b* expression (McCormick et al. 2008). Kiilerich et al. (2007b) demonstrated using Atlantic salmon gill explants that both RU486 and spironolactone can block the ability of cortisol to upregulate *nka-α1a*, *-α1b*, and *cftr1*. However, these results were not consistent across species or salinities (Kiilerich et al. 2007b, 2011b, c). In teleosts, RU486 antagonizes both Gr1 and -2, with more potent effects on Gr1 (Bury et al. 2003). On the other hand, spironolactone is now known to act as an agonist of the fish Mr, activating it with similar potency to cortisol, 11-deoxycorticosterone, and aldosterone (Sugimoto et al. 2016; Fuller et al. 2019). Thus, studies which use RU486 and spironolactone to differentially block the Mr and Gr should be interpreted with caution. Considering the challenges associated with pharmacologically targeting the fish Gr and Mr, advanced molecular approaches using transcriptional knockdown or transgenic knockout have emerged to investigate the Gr and Mr (Faught and Vijayan 2018; Yan and Hwang 2019). To date, these approaches have mostly been leveraged to investigate the metabolic, developmental, and ionoregulatory actions of corticosteroids in zebrafish (Faught and Vijayan 2018; Yan and Hwang 2019), which cannot tolerate SW. However, Japanese medaka offer a promising euryhaline model for knockdown or knockout approaches (Yan and Hwang 2019) and is therefore poised to delineate the Gr- and Mr-mediated actions of corticosteroids on SW-type ionocytes.

In tetrapods, the interaction of aldosterone with the Mr is facilitated by coexpression of the Mr with the cortisol-inactivating enzyme, 11β-hydroxylase 2 (Cyp11b2). Interestingly, a strong transcriptional upregulation of *cyp11b2* occurs in the gills of smolting Atlantic salmon (Kiilerich et al. 2007a; Nilsen et al. 2008). It was also shown in trout branchial epithelial cells that cortisol increases *cyp11b2* expression (Kolosov and Kelly 2019). These findings suggest the operation of a tissue-level mechanism to regulate cortisol signaling. A better understanding of which branchial cell-types specifically express *cyp11b2* is needed to assess its role in tuning the actions of cortisol on ionocytes.

The role of corticosteroids in regulating permeability of the branchial epithelium has also received considerable attention. This work has largely focused on the FW-adaptive, rather than the SW-adaptive, roles of corticosteroids, as the increased expression of tight-junction proteins generally promotes epithelial tightening. However, “leaky” tight-junction complexes composed of Cldn10s contribute to SW-adaptation by facilitating the paracellular excretion of Na^+ (Tipsmark et al. 2008b; Bui and Kelly 2014). Acclimation to SW increases the expression of *cldn10* isoforms in puffer fish (*Tetraodon nigroviridis*) (Bui et al. 2010) and exposure of gill explants to cortisol stimulates multiple *cldn10s* in medaka (Bossus et al. 2017). Cortisol and 11-deoxycorticosterone generally upregulate the expression of Cldns through processes mediated by both the Gr and Mr (Tipsmark et al. 2009; Bui et al. 2010; Chasiotis and Kelly 2011, 2012; Kelly and Chasiotis 2011; Bossus et al. 2017; Kolosov et al. 2017b; Kolosov and Kelly 2019). In sea lamprey, multiple claudins have been identified that are expressed in the gill, and among those investigated, *cldn3* and *-10* orthologs increase their expression after exposure to ion-poor water and exhibit decreases during SW acclimation (Kolosov et al.

2017a, 2020). Future studies in lamprey should seek to address whether 11-deoxycortisol and 11-deoxycorticosterone control branchial barrier functions via Cldns.

Cortisol was the first hormone linked with the expression of branchial Aqps. FW-acclimated eels infused with cortisol show a marked decrease in the expression of *aqp3* in the gill (Cutler et al. 2007) (Fig. 3). Choi et al. (2013) subsequently reported that cortisol diminishes branchial *aqp3* and *-8* expression in sockeye salmon (*Oncorhynchus nerka*). These patterns suggest that SW-induced increases in plasma cortisol are responsible for rapidly attenuating *aqp3* expression upon entry into hyperosmotic environments (Cutler and Cramb 2002; Cutler et al. 2007). Furthermore, cortisol blocks the stimulatory action of Prl on *aqp3* (Breves et al. 2016). The regulation of branchial Aqp3 is a clear example of antagonistic, rather than synergistic, actions of cortisol and Prl in support of salinity acclimation.

5.3 Thyroid hormones

In addition to supporting FW acclimation (Section 4.4), there is evidence that thyroid hormones promote SW-adaptive processes by acting directly on ionocytes and through interactions with the Gh/Igf system (McCormick 2001). For example, coho salmon and mummichog increase plasma T_4 levels in response to SW (Knoeppel et al. 1982; Specker and Kobuke 1987), and Atlantic salmon and summer flounder (*Paralichthys dentatus*) treated with T_4 or T_3 exhibit increased SW tolerance (Refstie 1982; Saunders et al. 1985; Schreiber and Specker 1999). Accordingly, when summer flounder and mummichog are treated with thiourea (an inhibitor of T_4 synthesis), they exhibit diminished hyposmoregulatory capacities (Knoeppel et al. 1982; Schreiber and Specker 1999). Thiourea diminishes the SW tolerance of flounder by disrupting the development of SW-type ionocytes during metamorphosis (Schreiber and Specker 2000). To our knowledge, there has been no direct assessment of whether the rapid recruitment of SW-type ionocytes that occurs in euryhaline species when they encounter SW is linked with thyroid hormone signaling.

6. Future perspectives

The availability of genomic resources and molecular tools over the last two decades has given rise to an increasingly mechanistic understanding of how hormones regulate ionocytes. This trend will undoubtedly continue with manipulative molecular tools such as gene editing ushering in new opportunities to link hormones and their cognate receptors with specific ion transporters. Zebrafish have already proven to be a valuable model for this purpose, supporting progress toward understanding the ontogeny and function of ion-absorptive ionocytes (Chen et al. 2019). Nonetheless, the poor salinity tolerance of zebrafish precipitates the need for a similarly amenable euryhaline model, a need that Japanese medaka seem poised to fill (Yan and Hwang 2019). In a similar vein, refined methods for primary cell culture of the branchial epithelium would accelerate the use of advanced molecular manipulations; however, progress in this endeavor has been limited.

The various modes by which endocrine factors can affect branchial processes deserve continued attention. For example, it is necessary to better resolve the cytogenic (controlling ionocyte abundance), molecular (controlling the expression of ion transporters), and physiological (controlling the function of ion transporters) actions of hormones (Breves et al 2014a; Shir-Mohammadi and Perry 2020). Important in this endeavor will be the characterization of, 1) the factors influencing the differentiation of SW-type ionocytes from precursor cells (analogous to how Foxi3a and -b regulate FW-type ionocyte differentiation in zebrafish), 2) the regulatory elements in the promoters and distal regulatory regions of genes encoding ion transporters, and 3) the functional elements of the ion transporters themselves (such as the motifs facilitating ATP binding and phosphorylation).

Despite the recent progress, there are still many gaps to fill in the collective understanding of how ionocytes operate – this is especially true for non-teleost fishes. For example, it stands unresolved whether Slc26-family anion exchangers, Clc family Cl^- channels, and Cftr sustain Cl^- transport in the ionocytes of lampreys and sturgeons (Ferreira-Martins et al. 2021; Shaughnessy and Breves 2021). We foresee that some of these transporters/channels will emerge as hormone targets. The recent expansion of genomic resources in non-teleosts will certainly support work of this nature (Amemiya et al. 2013; Smith et al. 2013; Braasch et al. 2016; Vialle et al. 2018; Smith et al. 2018; Cheng et al. 2019; Du et al. 2020; Yamaguchi et al. 2020; Marlétaz et al. 2023).

Finally, future work should seek to better understand how systemic hormones interact with the osmotic stress signaling cascades that permit ionocytes to directly perceive salinity changes (Fiol and Kültz 2007). For instance, cortisol promotes the expression of osmotic stress transcription factor 1 (Ostf1) during the acute phase of SW acclimation (McGuire et al. 2010). While Prl inhibits the activity of SW-type ionocytes (Fig. 2), it remains to be seen whether Prl dampens the expression of intracellular and paracrine factors that respond to hyperosmotic conditions (e.g., Ostf1, serum- and glucocorticoid-inducible kinase 1, 14-3-3 proteins, MAPKs, endothelin 1, interleukins, and tumor necrosis factor α) (Fiol and Kültz 2007; Notch et al. 2012; Kültz 2015; Lai et al. 2015). Given the multifactorial nature of intracellular osmotic stress signaling (Fiol and Kültz 2007), and the myriad hormones that impact branchial processes (Evans et al. 2005; Takei et al. 2014), it will be interesting to learn the extent to which ionocytes are a hub for interactions between intracellular, paracrine, and systemic signals.

Competing interests

The authors have no competing interests to declare that are relevant to the content of this article.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analyzed.

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Figure legends

Figure 1. Schematic diagrams of “Type II” and “Type III” ionocytes in Mozambique tilapia showing the stimulatory (arrows with a “+”) effects of prolactin (Prl) (see text for citations). Nka-α1a and Clc2c are included in these models based upon the expression of their associated gene transcripts; however, they have yet to be definitively assigned to tilapia ionocytes. Apical and basolateral sides are presented at the top and bottom of cells, respectively. Abbreviations: Aqp3: aquaporin 3; Clc2c: Clc family Cl^- channel 2c; Ncc2: Na^+/Cl^- cotransporter 2; Nka: Na^+/K^+-ATPase; Prl: prolactin.

Figure 2. Schematic diagrams of FW (freshwater)- and SW (seawater)-type ionocytes in mummichogs showing the stimulatory (arrows with a “+”) and inhibitory (blocked lines with a “-”) effects of prolactin (Prl) (see text for citations). Where Cl^- transport is indicated with a question mark, a pathway is presumed to exist but remains uncharacterized. Apical and basolateral sides are presented at the top and bottom of cells, respectively. Abbreviations: Aqp3: aquaporin 3; Cftr1: cystic fibrosis transmembrane conductance regulator 1; Cldn10f: claudin 10f; Ncc2: Na^+/Cl^- cotransporter 2; Nka: Na^+/K^+-ATPase; Nkcc1: Na^+/K^+/$2Cl^-$ cotransporter 1; Prl: prolactin; TJ: tight-junction. Figure adapted from Breves et al. (2022).

Figure 3. Schematic diagram of SW (seawater)-type ionocytes showing the stimulatory (arrows with a "+") and inhibitory (blocked lines with a "-") effects of cortisol (Cort) (see text for citations). Apical and basolateral sides are presented at the top and bottom of cells, respectively. Abbreviations: Aqp3: aquaporin 3; Cftr1: cystic fibrosis transmembrane conductance regulator 1; Cldn10s: claudin 10 isoforms; Cort: cortisol; Nka: Na^+/K^+-ATPase; Nkcc1: $Na^+/K^+/2Cl^-$ cotransporter 1; TJ: tight-junction.