The effects of osmotic challenges: involvement of ion transport proteins NKA, NKCC, and CFTR in the Gulf killifish, Fundulus grandis

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THE EFFECTS OF OSMOTIC CHALLENGES: INVOLVEMENT OF ION TRANSPORT PROTEINS NKA, NKCC, AND CFTR IN THE GULF KILLIFISH, *FUNDULUS GRANDIS*

A Dissertation

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in

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by

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5.1. Putative ion transport of the intestine of seawater and freshwater acclimated _F. grandis_........................................................................................................................................103
LIST OF ABBREVIATIONS

CFTR: cystic fibrosis transmembrane conductance regulator
ECF: extracellular fluid
ELISA: enzyme-linked immunosorbent assay
FW: fresh water
H&E staining: hematoxylin & eosin staining
IHC: immunohistochemistry
\( I_s \): short circuit current
MRC: mitochondria rich cell
NCC: \( \text{Na}^+/\text{Cl}^- \) cotransporter
NKA: \( \text{Na}^+/\text{K}^+ \) ATPase
NKCC: \( \text{Na}^+/\text{K}^+/2\text{Cl}^- \) cotransporter
PCR: polymerase chain reaction
q-PCR (real time PCR): quantitative polymerase chain reaction
RT - PCR: reverse transcriptase - polymerase chain reaction
\( R_t \): transmembrane resistance
SW: sea water
This dissertation investigated the cellular and molecular basis of osmoregulation in developing and adult Gulf killifish, *Fundulus grandis*, acclimated to salinities ranging from fresh water to sea water. In chapter 2, *F. grandis* embryos were reared in 0.1, 5, and 32 ppt water from 2 days (d) post fertilization until late embryogenesis. There were no discernable differences among salinities in the morphology of osmoregulatory organs, including the pharyngeal arches, digestive tract, or kidney. The localization and abundance of Na\(^+\)/K\(^+\) ATPase (NKA), Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter (NKCC), and the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) on the external surfaces of these tissues support a role in osmoregulatory as early as prior to hatch. In addition, *F. grandis* larvae were exposed from hatch to 0.1, 5, and 32 ppt water until 6 weeks post hatch (wph). Differential localization of NKCC and CFTR during osmotic challenges indicated putative altered functions of the intestine and gills, but not of the kidney in larval *F. grandis*. In Chapter 3, adult *F. grandis* were acutely transferred from 5 ppt water to 0.1, 1, and 5 ppt water for 7 d, or were acclimated to 0.1 ppt water then acutely transferred to 0.1 and 32 ppt water for 7 d. The mRNA and protein levels of NKA, NKCC, and CFTR in the intestine were differentially affected by the hypo- or hyperosmotic challenges. Localization of transport proteins suggested differences in intestine function associated with salinity acclimation. The anterior and posterior intestine may function in HCO\(_3\)\(^-\) or Cl\(^-\) secretion during exposure to 0.1 ppt water; the anterior intestine from 32 ppt acclimated fish may facilitate both Cl\(^-\) and water absorption; and the posterior intestine during exposure to 32 ppt water may facilitate HCO\(_3\)\(^-\) or Cl\(^-\) secretion. In chapter 4, the roles of these proteins in the anterior intestine of *F. grandis* were assessed using an electrophysiological approach coupled with pharmacological inhibition of transporters. A high apical CFTR activity in the anterior
intestine during acclimation in fresh water was observed. In conclusion, salinity acclimation affected the possible functions of osmoregulatory tissues of larval *F. grandis*, as well as the putative functions in ion/water regulation in the intestine of adult *F. grandis*. 
1.1. Literature review

1.1.1. Osmoregulation and active ion transport in teleost fish

Teleost fish are subjected to environments that pose risks to the maintenance of their internal water and salt balance. Since the beginning of 20th century, fish have been known to have active ion transport systems to maintain their extracellular fluids in steady state disequilibrium with the external environment (Keys, 1933; Krogh, 1939, 1946; Smith, 1931, 1932; Smith et al., 1930; Smith and Smith, 1931). This active ion transport is mediated predominantly by mitochondrion rich cells (MRCs) in transporting epithelia (Evans, 2008; Evans, 2011; Evans et al., 2005; Karnaky, 1980; Karnaky et al., 1976; Marshall et al., 1999; Zadunaisky, 1996). These cells, by virtue of their abundance of mitochondria, have high levels of ATP to fuel pumps that help establish electrochemical gradients that permit passive solute movement across membranes. Of particular importance is the Na⁺/K⁺ ATPase (NKA) pump. NKA creates a gradient for Na⁺ that helps transport solutes using passive Na⁺-dependent facilitated transporters. Localization of these transport proteins to the apical or basolateral surfaces of an epithelium can mediate either ion secretion or ion absorption across epithelia.

In fresh water (FW), the extracellular fluid (ECF) of a fish is hyperosmotic compared to its ambient environment; therefore, a fish must actively absorb ions from water via the gills and produce large amounts of hypoosmotic urine in the kidney to compensate for passive ion loss and osmotic water gain (Evans et al., 2005). In sea water (SW), the ECF of a fish is hypoosmotic compared to its aquatic environment. As a result, seawater fish absorb salt from ingested SW to promote water absorption in the intestine; these salts are subsequently excreted.
actively at the gills, while the kidney produces small volumes of an isoosmotic urine (Wood and Marshall, 1994).

1.1.2. Regulation of transporting proteins on the epithelium of osmoregulatory tissues during osmotic challenge

Research into the mechanisms of solute transport has a long and controversial history, often complicated by the diversity of mechanisms among fish species. The field of study has been revolutionized in recent years by emergence of increasingly sophisticated cellular and molecular approaches (Berdan and Fuller, 2012; Burnett et al., 2007; Evans, 2008; Hiroi and McCormick, 2012; Scott and Brix, 2013). Ion transporting proteins localized primarily on the plasma membrane of MRCs of transporting epithelia are critical to the maintenance of ion and water balance during osmotic challenges. Among the large repertoire of ion transport proteins in fish epithelia, NKA, Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) are particularly important in ion transport and in conferring osmoregulatory capacity in euryhaline fishes (Evans et al., 2005; Hiroi and McCormick, 2012; Hwang and Lee, 2007). Different isoforms of these three proteins mediate much of the transepithelial movements of Na⁺ and Cl⁻ required for hypoosmoregulation in seawater fish and hyperosmoregulation in freshwater fish. As such, there has been extensive interest in studying the regulation, function, abundance, and cellular distribution of these transport proteins during osmotic challenges. There is also considerable interest in understanding the mechanisms by which these transport proteins are regulated in euryhaline fishes during acute salinity transfers.

1.1.2.1. NKA

NKA consists of two α subunits and two β subunits that are non-covalently associated to form two αβ heterodimers (Skou and Esmann, 1992). The α subunits are the main catalytic units of the pump and contain all of the main binding sites for ATP, Na⁺, and K⁺. β subunits are
glycosylated polypeptides that help in the folding and translocation of the protein into the basolateral plasma membrane (Skou and Esmann, 1992). NKAα subunit has at least 4 isoforms (Blanco and Mercer, 1998). The NKAα1 isoform has been sequenced in several teleost species including the European eel, Anguilla anguilla (Cutler et al., 1995) and Atlantic salmon, Salmo salar (GenBank accession no. BT058747.1), and complete NKAα1 and α2 isoform sequences are available for common killifish, Fundulus heteroclitus (GenBank accession no. AY057072.1, AY057073.1). Three isoforms of NKAα1, including NKAα1a (GenBank accession no. CK878443), NKAα1b (GenBank accession no. CK879688), and NKAα1c (GenBank accession no. CK885259), are expressed in the gills of S. salar (Nilsen et al., 2007) and inanga, Galaxias maculatus, (Urbina et al., 2013). The mRNA levels of these isoforms are differentially regulated by hyperosmotic challenge. Branchial NKAα1a mRNA is decreased in G. maculatus (Urbina et al., 2013) and in anadromous/landlocked S. salar (Nilsen et al., 2007) during seawater exposure, whereas NKAα1b mRNA is increased by seawater acclimation in landlocked S. salar (Nilsen et al., 2007), and NKAα1c mRNA is decreased by seawater acclimation (Urbina et al., 2013). The branchial NKA activity level is elevated by freshwater exposure in F. heteroclitus (Scott and Schulte, 2005) and in S. salar (Bystriansky and Schulte, 2011). NKA activity is also elevated by seawater acclimation in the brown trout, salmo trutta and in the juvenile marble goby, Oxyelotris marmorata (Chew et al., 2009; Tipsmark et al., 2002).

Protein expression of NKA has been mostly studied using antibodies against epitopes of the NKAα subunit. Branchial NKAα protein abundance is elevated in the gills of juvenile O. marmorata and S. trutta during seawater acclimation (Chew et al., 2009; Tipsmark et al., 2002). With the use of antibodies against specific isoforms, NKAα1a protein abundance is decreased,
while NKAα1b abundance is increased in the gills of *S. salar* during seawater acclimation (McCormick *et al.*, 2009). The isoforms of NKAα1 also have been expressed in the intestine of teleosts, including NKAα1a mRNA in the intestine of *F. heteroclitus* (Scott *et al.*, 2006). The expression of NKAα protein is elevated following SW transfer in the intestine of European sea bass, *Dicentrarchus labrax*, four-eyed sleeper, *Bostrychus sinensis*, and *A. anguilla* (Cutler *et al.*, 2000; Jensen *et al.*, 1998; Peh *et al.*, 2009).

The NKAα subunit is localized on the basolateral membrane of all teleost epithelia and will energize either active ion absorption or active secretion depending on the passive-mediated transport proteins expressed in the epithelium (Choi *et al.*, 2010; Evans *et al.*, 2005; Fisher *et al.*, 2013; Grosell, 2006; Hiroi and McCormick, 2012; Lin *et al.*, 2004; Sucre *et al.*, 2013; Sucre *et al.*, 2011; Zadunaisky, 1996). Furthermore, isoform-specific antibodies against NKAα1a and NKAα1b have shown discrete localization of these isoforms in freshwater and seawater MRCs in the gills of *S. salar* (McCormick *et al.*, 2009).

1.1.2.2. NKCC

NKCC is widely distributed among different species of vertebrates (Haas, 1994), playing an important role in cell volume regulation (Hoffmann *et al.*, 2007) and transepithelial ion/water movement in epithelial cells (Cutler and Cramb, 2002). The NKCC subfamily is comprised of two isoforms, including the secretory NKCC1 isoform and the absorptive NKCC2 isoform (Evans *et al.*, 2005; Marshall *et al.*, 2002a), providing unique transport characteristics to an epithelium. In *A. anguilla*, two isoforms of NKCC1 and two isoforms of NKCC2 have been found. NKCC1a mRNA (GenBank accession no. AJ486858.1) is expressed in various tissues, while NKCC1b mRNA (GenBank accession no. AJ486859.1) is only found in the brain (Cutler and Cramb, 2002). NKCC2α mRNA (GenBank accession no. AJ564602.1) is
specifically found in the kidney and NKCC2β mRNA (GenBank accession no. AJ564603.1) is expressed in the urinary bladder and the intestine (Cutler and Cramb, 2008). Partial NKCC1 and NKCC2 mRNA sequences are also seen in F. heteroclitus gills (GenBank accession no. AY533706.1 and AY533707.1).

The expression and cellular distribution of NKCC isoforms are tissue specific and salinity-dependent in teleost epithelia. NKCC1 mRNA level in the gills of adult D. labrax decreases during freshwater acclimation (Lorin-Nebel et al., 2006). NKCC1 mRNA level is increased during hypoosmotic challenge in the anterior intestine of gilthead sea bream, Sparus aurata, but causes a decrease in the rectum; while the NKCC2 mRNA level is increased in the anterior intestine during hyperosmotic challenge (Gregorio et al., 2013). The level of NKCC2a mRNA is increased with seawater acclimation in the intestine of A. anguilla (Cutler and Cramb, 2008). Unfortunately, the protein expression of NKCCs has been mostly studied using the relatively non-specific T4 antibodies against the carboxy-terminal amino acids of the human colonic NKCC (Lytle et al., 1995), which possibly binds NKCC1, NKCC2 and Na⁺/Cl⁻ cotransporter (NCC). As a result, it is hard to discriminate among isoforms. Total protein expression of NKCCs/NCC in the gills of D. labrax decreases during freshwater exposure (Lorin-Nebel et al., 2006) and increases in the gills of juvenile O. marmorata during seawater acclimation (Chew et al., 2009).

As described previously, NKCC utilizes the favorable electrochemical gradient of Na⁺, established by NKA, to facilitate the entry of Na⁺, K⁺ and 2Cl⁻ into a cell. In coordination, NKA and NKCC form a mechanism for transepithelial Na⁺ and Cl⁻ transport. NKCC2 on the apical membrane of an epithelium provides one of several mechanisms for transepithelial Na⁺ movement. Conversely, when NKCC1 is localized to the basolateral membrane, Na⁺ is
predominately cycled across that membrane, helping to drive Cl\(^-\) entry into a cell against the electrochemical gradient of Cl\(^-\). Under this scenario, Cl\(^-\) transporting proteins such as CFTR localized on the opposing membrane will help to mediate transepithelial Cl\(^-\) movement.

Assessment of the cellular localization of NKCCs using the T4 antibody (Lytle et al., 1995) reveals basolateral NKCCs/NCC in the MRCs of the branchial epithelium during seawater acclimation in various species including *O. marmorata* (Chew et al., 2009), *F. heteroclitus* (Katoh et al., 2008), and *D. labrax* (Lorin-Nebel et al., 2006). In the freshwater gill, localization of NKCCs/NCC varies between species. For instance, apical localization of the cotransport is reported in *F. heteroclitus* (Katoh et al., 2008) and *D. labrax* (Lorin-Nebel et al., 2006), whereas a basolateral localization of the contranport is described in rainbow trout, *Oncorhynchus mykiss* (Katoh et al., 2008) and *Oxyelotris. marmorata* (Chew et al., 2009). In the intestine, NKCCs/NCC are localized on the apical membrane in the intestine of *O. marmorata* (Chew et al., 2009) and in the anterior intestine of *S. aurata* (Gregorio et al., 2012) during seawater acclimation. Apical NKCC localization has been reported in the gut of both freshwater and seawater acclimated juvenile *D. labrax* using the C-14 NKCC1 antibody (Sucre et al., 2013). In conclusion, the apically localized NKCC (or potentially NKCC2 or NCC) are related to Cl\(^-\) and Na\(^+\) absorption, while basolaterally localized NKCC (presumably NKCC1) are involved in ion secretion in the epithelium.

1.1.2.3. CFTR

After being initially cloned in *F. heteroclitus* (GenBank accession no. AF000271.1, Singer et al., 1998), CFTR has been found in various teleost species such as *S. salar* (GenBank accession no. AF155237.1, AF161070.1), Japanese eel, *Anguilla japonica* (GenBank accession no. AB705416), and *S. aurata* (GenBank accession no. GU183822).
Hypo- or hyperosmotic challenges have been found to affect CFTR mRNA and protein expression levels. Branchial CFTR mRNA and protein expression decrease during freshwater acclimation in *F. heteroclitus* (Scott *et al.*, 2005) and juvenile *D. labrax* (Bodinier *et al.*, 2009b). During hypoosmotic challenge, the level of CFTR mRNA remains unchanged in the intestine of *F. heteroclitus* (Scott *et al.*, 2006), whereas the level increases in the anterior intestine but decreases in the rectum of *S. aurata* following transfer from SW to brackish water (Gregorio *et al.*, 2013). In contrast, CFTR mRNA and protein levels increase in posterior intestine of juvenile *D. labrax* (Bodinier *et al.*, 2009b). Thus, the regulation in the expression of CFTR mRNA and protein seems to be tissue specific during osmotic challenge.

In coordination with NKCC, CFTR is one of the major routes of transcellular Cl̄ secretion (Marshall *et al.*, 2002b; Marshall and Singer, 2002; Singer *et al.*, 1998) in the gills and operculum of marine fish (Bodinier *et al.*, 2009b; Marshall and Singer, 2002; Ouattara *et al.*, 2009; Scott and Schulte, 2005; Singer *et al.*, 1998). These proteins may also be involved in the cellular excretion of Cl̄ across the basal membrane during ion absorption in the intestine (Bodinier *et al.*, 2009b; Hwang and Lee, 2007; Peh *et al.*, 2009). Cl̄ movement is passive through CFTR following its prevailing electrochemical gradient (Hwang and Lee, 2007; Marshall, 2002). CFTR has been recognized as a regulator of the Cl̄/HCO₃⁻ exchanger (Singh *et al.*, 2010; Singh *et al.*, 2008), the major path for HCO₃⁻ secretion in marine teleosts (Grosell, 2006; Taylor and Grosell, 2006). When HCO₃⁻ is secreted into the lumen, the Cl̄ secreted by CFTR can be cycled back into the intestinal epithelial cells (enterocytes) by the Cl̄/HCO₃⁻ exchanger, whereas luminal HCO₃⁻ can react with Ca²⁺ and Mg²⁺ to form CaCO₃ and MgCO₃ precipitates (Faggio *et al.*, 2011; Kurita *et al.*, 2008; Mekuchi *et al.*, 2013; Whittamore, 2012; Whittamore *et al.*, 2010). Divalent ion precipitation decreases the osmolality of the intestinal
fluid in the lumen, facilitating water absorption in the posterior intestine (Carvalho et al., 2012; Whittamare, 2012; reviewed by Takei and Loretz, 2011).

Besides facilitating HCO$_3^-$ secretion, Cl$^-$ secretion through CFTR may function in acid/base regulation together with the apical V-type ATPase and Cl$^-$/OH$^-$ exchanger. Collaco et al. (2013) found that the activity of V-type ATPase involved in the secretion of H$^+$ to the lumen is decreased during inactivation of CFTR. The activity of Cl$^-$/OH$^-$ exchanger that excretes OH$^-$ to the lumen also decreases CFTR inhibition (Mount and Romero, 2004).

1.1.2.4. Post-translational regulation of NKCC and CFTR

Post translational regulation of NKCC and CFTR proteins during osmotic stress can potentially decrease the cost of transcriptional regulation during osmotic challenges (Bodinier et al., 2009b). In mammals, trafficking and activation of CFTR in the enterocytes is regulated by cAMP and cGMP-dependent phosphorylation, including protein kinase A (PKA), protein kinase C (PKC) and AMP-activated kinase (AMPK) (Golin-Bisello et al., 2005; Hallows et al., 2003).

In the branchial MRCs of *F. heteroclitus*, apical CFTR can be activated by cAMP independent focal adhesion kinase (FAK) (Marshall et al., 2009). In *F. heteroclitus*, cAMP-protein kinase A (cAMP-PKA) activated the phosphorylation of basolateral NKCC1 on the branchial epithelium during acute hyperosmotic acclimation (Flemmer et al., 2010). In opercular MRCs of *F. heteroclitus* during hypoosmotic challenge, dephosphorylation of FAK leads to basolateral NKCC1 deactivation (Marshall et al., 2008). The basolaterally localized NKCC may also be involved in volume-sensitive ion regulation (Hoffmann et al., 2007). Although not specifically addressed in this thesis, posttranslational regulation of CFTR and NKCC is an important mechanism in controlling osmoregulation and warrants further consideration.
1.1.3. Osmoregulatory organs

As discussed in section 1.1.1, euryhaline fish exposed to FW passively lose ions and gain water, facing the risk of cell swelling and plasma osmolality dilution. Freshwater acclimated fish need to actively take up ions from the gills to compensate the passive loss of ions. Dilute urine is excreted from the kidneys to remove excess water. In hyperosmotic environments, fish tend to lose water and gain ions, facing the risk of cellular shrinkage and plasma osmolality increase (Evans, 2005; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Marshall, 1994). In contrast to freshwater fish, marine fish actively drink SW, from which they actively accumulate ions from the intestinal lumen to facilitate water absorption. Monovalent ions absorbed from the intestine are subsequently secreted at the gills, whereas divalent ions are secreted at the kidney, forming an isoosmotic urine in the process (Evans, 2005). Furthermore, these divalent ions have relatively poor absorptivity in the gastrointestinal tract of marine teleosts due to the low membrane permeability of the epithelium. Divalent ions such as Ca\(^{2+}\) and Mg\(^{2+}\) are trapped in carbonate precipitates in the gut of marine fish (Evans, 2008; Evans, 2011; Evans et al., 2005; Grosell, 2006).

1.1.3.1. Gills

The gills of teleosts are located in the branchial chamber and covered by opercula. The gills contain four pairs of holobranchs, with filaments attached to the arches (Evans et al., 2005). For most fish, the gill is a multifunctional organ that mediates various physiological functions including gas exchange, ion transport, nitrogenous waste excretion and acid-base regulation (Evans, 2008; Evans et al., 2005). In seawater acclimated fish, active salt secretion is mediated by MRCs that are rich in basolaterally-localized NKA, NKCC1, and apical CFTR (Evans et al., 2005). When fish are transferred to FW, the gill reduces active ion secretion,
minimizes paracellular ion loss through regulation of tight junction proteins, and stimulates active ion absorption in the MRCs (Evans et al., 2005). Although the seawater gill model of ion transport has been long established (Figure 1.1), various working freshwater gill models have been proposed (Evans, 2011; Hiroi and McCormick, 2012; Hirose et al., 2003; Hwang and Lee, 2007). These transport models suggest that the cellular distribution of proteins such as NKCC is inconsistent among species (Katoh et al., 2008), and that other ion transport proteins such as the Cl⁻/HCO₃⁻ exchanger and Na⁺/H⁺ exchanger may be involved in both ion-transport and acid-base regulation. The mRNA and protein levels of NKA, NKCC, and CFTR, and the localization of these proteins in the gills of teleosts can be divergently regulated by osmotic challenges.

Figure 1.1. Working model for the extrusion of NaCl by the marine teleost gill epithelium, modified from Evans et al. (2005). In the gills of seawater acclimated fish, active NaCl secretion is mediated by MRCs that are rich in basolaterally localized NKA, NKCC1, and apical CFTR. MRC = mitochondrion rich cell. NKA = Na⁺/K⁺ ATPase, NKCC = Na⁺/K⁺/2Cl⁻ cotransporter, CFTR = cystic fibrosis transmembrane conductance regulator. SW = sea water.

1.1.3.2. Intestine

Smith et al. (1930) first proposed that marine teleosts ingest SW to counteract the dehydrating effects of SW via osmosis at the gills. Water and NaCl absorption across the marine teleost intestine are tightly co-regulated (Scott et al., 2006), and occur against an osmotic
gradient (Curran and Solomon, 1957). The osmotic pressure of the fluid decreases along the gastrointestinal tract in several marine teleost including Buffalo sculpin, *Enophrys bison* (Sleet and Weber, 1982) and European flounder, *Platichthys flesus* (Bury et al., 2001), suggesting that active NaCl ingestion and water absorption occurs along the intestine (reviewed by Grosell and Taylor, 2007). This ion and water transport in seawater acclimated fish is higher than in freshwater acclimated fish (Smith et al., 1975). Freshwater fish have substantially reduced rates of water ingestion (Scott et al., 2006; Wood and Marshall, 1994), with this cessation of drinking occurring at a salinity approaching FW.

There is evidence that the reduction in drinking during hypoosmotic exposure correlates with the salinity at which the transition between a seawater to freshwater fish gill physiology occurs (Copeland, 1947, 1950; Philpott and Copeland, 1963), suggesting possible interaction between physiological changes in intestine and gill physiologies during osmotic challenges. In *Fundulus species*, no mechanism of active Cl$^-$ uptake has been found in the freshwater fish gill, indicating that there may be a Cl$^-$ absorption mechanism in other osmoregulatory organs such as the intestine. In *F. heteroclitus*, during hypoosmotic challenge, the intestinal Na$^+$ and Cl$^-$ concentrations are relatively constant, although Na$^+$ and Cl$^-$ flux across the intestinal epithelium increases 12 hours after transfer compared to the pre-transfer level (Scott et al., 2006). This result suggests that a change in ion transport processes in the intestine occurs during hypoosmotic challenge, however, the function of the freshwater intestine in ion and acid-base regulation is still not elucidated.

1.1.3.3. Kidney

The nephrons of most teleosts are composed of a glomerulus, proximal tubule (in the pronephron or head kidney), distal tubule, and collecting tubule (Hickman and Trump, 1969).
Since the kidney of teleosts lacks a Loop of Henle and has no zonation such as the cortex and medulla (Hickman and Trump, 1969), marine teleosts are not able to excrete hyperosmotic urine (Evans, 2005). Excretion of excess salts is primarily mediated by the gills (Evans, 2005). In seawater acclimated fish, glomeruli are typically small and much less abundant (Wong and Woo, 2006), or are completely absent (e.g. Plainfin midshipman, *Porichthys notatus*), making nephrons more heavily reliant on tubular secretion than on filtration for the production of an isosmotic urine (Ozaka *et al.*, 2009). Seawater acclimated fish may excrete $\text{SO}_4^{2-}$ and $\text{Ca}^{2+}$ from the kidney via $\text{Na}^+$/Ca$^{2+}$ exchangers on the apical membrane (Islam *et al.*, 2011). In contrast, freshwater acclimated fish have abundant glomeruli, which together with an increase in the diameter and thickness of the collecting tubules, suggest an increase in glomerular filtration leading to an increased volume of dilute urine production (Wong and Woo, 2006).

NKA has been localized in the proximal, distal, and collecting tubules, but not in the glomeruli of milkfish, *Chanos chanos* (Tang *et al.*, 2010a) and spotted green pufferfish, *Tetraodon nigroviridis* (Lin *et al.*, 2004), *F. heteroclitus*, and *O. mykiss* (Katoh *et al.*, 2008) acclimated to different environmental salinities. NKA, which is almost exclusively localized on the basal membrane of tubular cells, maintains a favorable electrochemical gradient for $\text{Na}^+$. This gradient helps facilitate the secretion and/or absorption of other solutes. In *T. nigroviridis*, the relative abundance of kidney NKA $\alpha$-subunit and NKA activity were elevated in FW, but not in brackish water or SW. (Lin *et al.*, 2004).

NKA$\alpha$ mRNA level increases in the kidney of seawater acclimated black porgy, *Acanthopagrus schlegeli* (Tomy *et al.*, 2009). In *C. chanos*, the highest abundance of relative mRNA level of NKA$\alpha$ subunit is found in freshwater acclimated milkfish rather than in seawater acclimated fish. Furthermore, the relative protein abundance of renal NKA$\alpha$ subunits,
as well as NKA activity, are also higher in freshwater fish than in seawater fish (Tang et al., 2010a).

The NKCC2α mRNA levels in kidney are similar between freshwater and seawater acclimated A. anguilla, whereas NKCC2β is only found in the urinary bladder, although its regulation by osmotic challenges is not known (Cutler and Cramb, 2008). NKCCs/NCC proteins are localized to the apical membrane of the distal and collecting tubules in F. heteroclitus and O. mykiss, whereas NKCCs/NCC proteins are only found basolaterally in the proximal tubules of F. heteroclitus (Katoh et al., 2008). Apical localization is also found in the collecting tubule and urinary bladder of freshwater acclimated D. labrax (Lorin-Nebel et al., 2006). By using antibodies specific to NKCC2 or NCC of pufferfish megugu, Takifugu obscurus, NaCl reabsorption is proposed to occur in the distal nephron of the fish kidney mediated by both apical NCC and apical NKCC2 in freshwater acclimated fish but only by apical NKCC2 in seawater acclimated fish (Kato et al., 2011).

In the larval D. labrax, CFTR is localized to the apical membrane of renal tubules, and only to the basolateral membrane in the collecting ducts of seawater acclimated larvae. During freshwater acclimation, the relative CFTR protein level is increased in the kidney of larval D. labrax (Bodinier et al., 2009a; Bodinier et al., 2009b).

1.1.4. Fundulus grandis in osmoregulation study

Fundulus sp. are considered model teleosts in the study of the physiological basis of environmental stress tolerance (Burnett et al., 2007). Griffith (1974) found that among the 27 Fundulus sp in North America, most Fundulus sp. can tolerate FW and SW up to 74-114 parts per thousand (ppt), whereas most freshwater species are unable to survive in salinities above 29
ppt. Whitehead (2010) proposed that most of the freshwater *Fundulus* sp. can only tolerate waters up to a salinity below 25 ppt.

Gulf killifish, *F. grandis* (Baird and Girard, 1853) are widely distributed in the marshes along the Gulf of Mexico, where they face periodic changes in environmental salinity, temperature, and oxygen. In their natural habitat, *F. grandis* can tolerate salinity from 0.05 ppt (FW) to 76.1 ppt (i.e., 2-fold full strength SW; reviewed by Griffith, 1974), although a recent study proposes an upper limit of more than 100 ppt water in a Florida population (Nordlie and Haney, 1998). Because of their euryhalinity, long reproductive cycle from March until August (Phelps *et al.*, 2010), ease of collection in the field, and maintenance in the lab, and availability of cellular and molecular tools, *F. grandis* is an excellent model organism for studying the physiology of osmotic tolerance.

1.2. Scope of dissertation

The goal of this dissertation was to investigate the cellular and molecular basis of osmoregulation in developing and adult *F. grandis* acclimated to salinities ranging from FW (0.1 ppt water) to SW (32 ppt water). Previous studies found that the seawater type MRCs in the gill filament can be observed during acclimation in 5 ppt and 1 ppt water; freshwater type MRCs were found in the gills of fish acclimated in 0.1 ppt water (Guan *et al.*, in prep). Fish stop drinking when they are acclimated at a salinity approaching FW, suggesting that the transition between seawater and freshwater physiology occurs at a very low salinity (Copeland, 1947, 1950; Philpott and Copeland, 1963). Fish acclimated in 5 ppt water were used as control animals for hypoosmotic challenge experiments because at this salinity the fish gill still and intestine exhibit a marine fish phenotype. By acclimating fish to 5 ppt rather than to higher
salinities, we hoped to limit the magnitude of the stress of a hypoosmotic challenge needed to evoke phenotypic changes to *F. grandis*.

This dissertation includes studies on the effects of osmotic challenges on the mRNA and protein levels, and cellular distribution of the ion transporting proteins, NKA, NKCC, and CFTR in: 1) embryonic and larval *F. grandis*; and 2) intestine of adult *F. grandis*. These data are part of a larger set of studies that were conducted in our laboratory to assess the functionality of these proteins in multiple organs that are associated with osmoregulation.

1.2.1. In developing *F. grandis*

The capacity for osmoregulation is developed during the early ontogeny of tissues in teleosts, and is likely associated with the formation of physiological systems in the integument, gills, kidneys, and gastrointestinal tract. The major osmoregulatory site may change during development and the pattern of this ontogeny may vary among species and among environmental salinities (Varsamos et al., 2005). During embryonic stages, mechanisms of active ion regulation in teleosts develop initially in the integument and the gastrointestinal tract, and then develop in the gills and kidneys (Hiroi and McCormick, 2012; Kaneko et al., 2002; Shi et al., 2008; Sucre et al., 2013; Sucre et al., 2011; Varsamos et al., 2005). Different MRC types, as identified by the expression and colocalization of NKA, NKCC, CFTR, and other transport proteins, are found on the embryo integument (Hiroi and McCormick, 2012; Kaneko et al., 2002) and on the gills of larval/juvenile fish (Bodinier et al., 2009b; Lorin-Nebel et al., 2006). However, the functions of the intestine during embryonic and larval development, as examined by the distribution and regulation of NKA, NKCC and CFTR, have only been reported in *D. labrax* (Sucre et al., 2013). Furthermore, little is known about the function of CFTR in the teleost kidney (Bodinier et al., 2009c).
Chapter 2 investigated the effect of rearing in various salinities on the morphology, distribution and regulation of NKA, NKCC, and CFTR during embryonic and larval stages in *F. grandis*. This chapter provides new insights on the effects of salinity on the development of osmoregulatory tissues in the euryhaline *F. grandis*. In the first set of experiments, embryos were exposed throughout embryogenesis to 0.1, 5 and 32 ppt water, and sampled prior to hatch. In a second experiment, embryos were air incubated until hatch, and then hatched simultaneously in 0.1, 5, and 32 ppt water, where they were incubated from 6 hours post hatch (hph) until 6 weeks post hatch (wph). The morphology, distribution and regulation of NKA, NKCC, and CFTR, and whole body protein level of these proteins during osmotic challenge were used to assess how environmental salinity influenced the osmoregulatory tissues during embryonic and larval development.

1.2.2. Intestine of adult *F. grandis*

Chapters 3 and 4 focused on assessing the role of NKA, NKCC, and CFTR in anterior and posterior intestine during osmotic challenges. Possible functions of these transport proteins have been elucidated in the intestine of marine teleosts, although no suitable cellular model of the freshwater intestine has been established (Grosell, 2006; Grosell and Taylor, 2007; Takei and Yuge, 2007).

In Chapter 3, the cellular distribution, and the mRNA and protein levels of the ion-transport proteins, NKA, NKCC, CFTR, in the anterior and posterior intestine were investigated to test the possible functions of the intestine during osmotic challenge. Fish were transferred from 5 ppt to 0.1, 1, and 5 ppt water, and sampled at 6 h, 3 d, and 7 d post transfer. In another experiment, fish were acclimated to 0.1 ppt water then transferred to 0.1 ppt and 32 ppt water, and sampled at 6 h, 3d and 7 d after transfer. NKA and CFTR mRNA and protein level, as well
as cellular localization of these proteins were measured in anterior intestine and posterior intestine during osmotic challenge to examine if the possible functions of enterocytes change during acclimation to different salinities.

In Chapter 4, the regulation of NKA, NKCC, CFTR in the anterior intestine was investigated using isolated membranes from the anterior intestine mounted on an Ussing Chamber. Pharmacological blockers of NKA (ouabain), NKCC (bumetanide), and CFTR (CFTR-inh172) were used to inhibit the activity of these membrane binding proteins. Short circuit current ($I_{sc}$) and transmembrane resistance ($R_t$) were recorded during the pharmacological inhibition of these proteins to test the putative role of these transporters in active ion transport in the anterior intestine of *F. grandis* during osmotic challenge. Finally, apical application of reduced Na$^+$ and/or Cl$^-$ solutions were used to assess the individual and combined roles of these ions in active ion transport in the intestine. Isolated membranes of anterior intestine of fish acclimated to salinities ranging from FW to SW were mounted on Ussing chambers, and $I_{sc}$ and $R_t$ measured during application of the ion reduced solutions.
CHAPTER TWO - EFFECT OF OSMOTIC CHALLENGE ON THE PROTEIN LEVEL AND CELLULAR DISTRIBUTION OF NKA, NKCC, AND CFTR IN EMBRYONIC AND LARVAL GULF KILLIFISH, *FUNDULUS GRANDIS*

2.1. Introduction

Although the gill, kidney, and intestine are the major organs involved in osmoregulation in adult fish (Evans, 2008; Evans *et al.*, 2005; Krogh, 1939), tissues such as the skin may play more prominent roles in ion transport and water balance during early development. Early teleost development is a period of rapid change in the physiology of transporting epithelia critical to osmoregulation. The embryonic and early larval integument, which consists of a single layer of cells, is the main site for ion and acid-base transport, gas exchange, and nitrogenous waste excretion before the onset of gill function (Dunson and Travis, 1994; Hiroi *et al.*, 1998; Kunz, 2004; Pelis *et al.*, 2001). Increased folding and vascularization of the gills and intestine lead to a progressive shift away from the use of the skin in mediating these physiological processes (Hiroi *et al.*, 1998; Kaneko *et al.*, 2002; Kunz, 2004; Sucre *et al.*, 2011; Varsamos *et al.*, 2005). In addition, the ontogeny of the intestines and kidney will support other physiological processes such as nutrient absorption or waste excretion needed for growth (Grosell *et al.*, 2011; Kunz, 2004). As such, an integrative approach needs to be considered when investigating the role of transporting epithelia in fish osmoregulation during development.

Mitochondrion rich cells (MRCs), characterized by their abundance of basolateral Na⁺/K⁺ ATPase (NKA), mediate the bulk of active ion transport in epithelia (Tang and Lee, 2011; Tang *et al.*, 2010b). In addition to NKA, other transport proteins, including Na⁺/K⁺/Cl⁻ cotransporter (NKCC) and chloride channel cystic fibrosis transmembrane conductance regulator (CFTR), have been implicated in active transepithelial secretion or absorption. Although MRCs contain some of the same transport proteins, the cellular distribution in specific
membranes may confer differences in the directionality of active transport (Evans et al., 2005; Kaneko et al., 2002; Katoh et al., 2001). For instance, various MRC types have been observed in the integument (Hiroi and McCormick, 2012; Hiroi et al., 2008; Kaneko et al., 2002) and in the gills (Allen et al., 2009; Hiroi et al., 1998; Hiroi and McCormick, 2012; Kaneko et al., 2002; Rojo and Gonzalez, 1999) of yolk-sac larvae and in all osmoregulatory organs of free-feeding larvae based on the cellular distribution and protein abundance of ion transporting proteins such as NKA, NKCC, and CFTR.

NKA is localized on the basolateral membrane of all teleost epithelia and will energize either active ion absorption or active ion secretion depending on the passive-mediated transport of proteins expressed in an epithelium (Choi et al., 2010; Evans et al., 2005; Fisher et al., 2013; Grosell, 2006; Hiroi and McCormick, 2012; Lin et al., 2004; Sucre et al., 2013; Sucre et al., 2011; Zadunaisky, 1996). NKCC will utilize the favorable electrochemical gradient of Na$^+$, established by NKA, to facilitate the entry of Na$^+$, K$^+$ and 2 Cl$^-$ into a cell. NKCC can exist in several isoforms, each which confer different roles in transepithelial Na$^+$ and/or Cl$^-$ transport. NKCC1 is localized to the basolateral membrane, where it together with NKA cycles Na$^+$ across the membrane, with the goal of driving Cl$^-$ into a cell (Field et al., 1978; Frizzell et al., 1979; Katoh et al., 2008; Lorin-Nebel et al., 2006; Marshall et al., 2002b; McCormick et al., 2003; Musch et al., 1982; Takei and Yuge, 2007). Under this scenario, Cl$^-$ transporting proteins such as CFTR, localized on the apical membrane, will help to mediate transepithelial Cl$^-$ movement (Christensen et al., 2012; Evans and Claiborne, 2006; Hiroi and McCormick, 2012; Hiroi et al., 2008; Inokuchi et al., 2008). NKCC2 localized to the apical membrane provides one of several mechanisms for transepithelial Na$^+$ absorption. Na$^+$ is actively transported from epithelia into the extracellular fluid by the basolateral NKA. This action generates the low intracellular Na$^+$
concentration required drive apical NKCC2 (Grosell et al., 2011; Takei and Yuge, 2007). The cellular localization of these proteins on transporting epithelia may insights in the ontogeny and function of osmoregulatory function in development *F. grandis*.

The goal of this study was to assess the ontogeny of osmoregulatory tissues during embryonic and larval development in the euryhaline Gulf killifish, *Fundulus grandis*, during acute hypoosmotic and hyperosmotic challenges. Salinity exposure may affect the ontogeny of osmoregulatory tissues, or it may influence the relative importance of each tissue towards whole animal osmoregulation (reviewed by Varsamos, 2005). Furthermore, the ontogeny of osmotic regulatory organs during early life stages may affect the long-term tolerance of an animal to osmotic challenges. In this study, the effects of salinity on the ontogeny of osmoregulation were tested during late embryonic and early larval stages to understand the early life fitness consequences of developing *F. grandis* reared in different salinities. More specifically, the study investigated the ontogeny of osmoregulatory organs, as well as the cellular distribution and protein abundance of NKA, NKCC, and CFTR during late embryonic and early larval stages reared at salinities ranging from fresh water (FW) to sea water (SW).

### 2.2. Material and methods

#### 2.2.1. Animal husbandry

Animal care holding and experimental use for this study was approved by the LSU Institutional Animal Care and Use Committee (10-076).

Adult *F. grandis*, originally obtained from Cocodrie, LA, were reared in outdoor tanks at the Aquaculture Research Station (LSU AgCenter) and used as a brood stock for embryo production. Spawning mats were deployed on the sides of brood tanks where eggs could be deposited by females and fertilized by males *ad libitum*. The embryos were collected within 2
days of fertilization and transferred within minutes to the Department of Biological Sciences, LSU, between foam mats moistened with 5 ppt water (Coulon et al., 2012), and held at room temperature (approximately 21-23 ºC) awaiting further processing. Embryos at stages 19-22 of embryonic development (Armstrong and Child, 1965) were selected for use in experiments. Stage 19 embryos were characterized as having a well-defined embryonic axis and stage 22 was characterized by the lack of pigments on their yolk sac and body. All the embryos were kept air-incubated (Coulon et al., 2012), except for a subset of animals that were water immersed immediately as described below.

2.2.1.1. Exposure of the embryos

A subset of embryos between developmental stages 19-22 were randomly divided into 3 groups, and transferred into 0.1, 5, or 32 ppt water at a density of 30 embryos per container. These embryos were reared in water alongside the air-incubated animals as described below. Ten embryos from each salinity were sampled at stage 30 (S30) of development, chorions were removed, and embryos processed for Hematoxylin & Eosin (H & E) staining and immunohistochemistry analysis. S30 embryos have retinal pigmentation with a discernable outline of the lens and a straight tail. At this stage, embryos were undergoing organodifferentiation but did not possess well-differentiated heart chambers (Armstrong and Child, 1965).

2.2.1.2. Exposure of larval fish

In order to facilitate simultaneous hatching of embryos in water of different salinities, embryos were air-incubated immediately upon collection, and then hatched in water at 0.1, 5, and 32 ppt when all embryos had reached stage 34 of development. Air-incubated embryos were hatched by immersion in containers with 0.1, 5, and 32 ppt water; any animals that were
unhatched within 15 min of immersion were removed immediately. This protocol allowed approximately 95% of the embryos to hatch within 10 min of water immersion. Hatchlings were transferred to a recirculating system and maintained on a 12 h light: 12 h dark cycle at a temperature of 21.2 ± 0.68 ºC. Water salinities were measured everyday using an YSI 85 meter (YSI, Yellow Springs, OH, USA). Upon hatch, larvae (300 larvae per salinity) were fed artemia twice a day for the first 21 d post hatch, and then fed fish pellets (Cargill 6243-1, Minneapolis, MN, USA).

Following hatch upon transfer to water of different salinities, fish were sampled between 6 hours post hatch (hph) to 6 weeks post hatch (wph), as described below. Ten larvae per salinity per time point were sampled for histology at 6 hph, and at 1 and 4 wph. Furthermore, six samples were collected for protein analysis per salinity per time point at 6 hph, 3 days post hatch (dph), 1, 2, 4, and 6 wph for quantification of NKA, NKCC, and CFTR protein levels using an enzyme-linked immunosorbent assay (ELISA). Each protein sample contained the whole bodies of several fish (5 fish pooled at 6 hph, 3 fish at 3 day post hatch (dph) and 1wph, 2 fish at 2 and 4 wph, and 1 fish at 6 wph).

2.2.2. Protein analysis

Tissue samples were homogenized in SEI buffer (300 mM sucrose, 20 mM EDTA, 100 mM imidazole, pH 7.3) containing protease inhibitors (Roche Applied Science, Indianapolis, IN, USA). After 1.5 h on ice, homogenates were centrifuged at 2000 g for 6 min at 4 ºC. Pellets were resuspended in 2.4 mM sodium deoxycholate (Invitrogen, Carlsbad, CA, USA) in SEI solution with protease inhibitors and centrifuged at 2000 g for 6 min at 4 ºC, then stored at -20 ºC. The total protein concentration of each supernatant was determined by the absorbance at 280 nm using a NanoDrop ND-1000 spectrophotometer V3.3 (Thermo Scientific, Wilmington,
The total protein concentration was adjusted to 1 mg/ml prior to relative NKA, NKCC, and CFTR protein analysis (calibrated using BSA as a standard).

Proteins were quantified using an ELISA adopted from an indirect ELISA protocol from ABCAM (Cambridge, MA, USA). Whole-body homogenates (10 µl per sample) containing 100 µg/ml protein per sample were added to a coating buffer (30 mM Na$_2$CO$_3$, 71 mM NaHCO$_3$, 90 µl per sample). Microtiter plates (Greiner Bio-One, Monroe, NC, USA) were incubated overnight at 4 °C, then washed with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$) before adding 200 µl of 5% skim-milk-PBS into each well. After a 60 min incubation, 100 µl primary antibody was added to each well: polyclonal rabbit anti-human NKA (1.5 µg/ml, Santa Cruz Biotechnology, Dallas, TX, USA), monoclonal mouse anti-human CFTR (1.5 µg/ml, R&D Systems, Minneapolis, MN, USA), or polyclonal goat anti-human NKCC1 (3 µg/ml, Santa Cruz Biotechnology), diluted in 0.5% skim-milk-PBS, and incubated at room temperature for 2 h. Afterwards, 100 µl secondary antibody: peroxidase-conjugated goat anti-mouse IgG (1:5000, Jackson ImmunoResearch, West Grove, PA, USA), peroxidase-conjugated goat anti-rabbit IgG (1:10000, Jackson ImmunoResearch), or peroxidase-conjugated donkey anti-goat IgG (1:5000, Santa Cruz Biotechnology) were used to conjugate with antibodies against NKA, CFTR, and NKCC, respectively. These secondary antibodies were diluted in 0.5% skim-milk-PBS, and incubated for an additional 2 h at room temperature. Color reactions were developed by adding 50 µl One-Step TM Ultra TMB-ELISA (Thermo Fisher Scientific, Rockford, IL, USA). The plate was incubated at room temperature for 10 to 25 min. Reactions were stopped by adding 50 µl of 2 M sulfuric acid. The absorbance was measured at 450 nm (Bio-Rad Microplate reader 680 XR, Bio-Rad Laboratories, Inc.,
Hercules, CA, USA), and standardized using protein samples consisting of a pooled protein mixture of each treatment.

2.2.3. Hematoxylin & Eosin staining

Ten fish per salinity at each time point were collected and fixed in Bouin’s solution for 48 h. Samples were dehydrated in an ascending gradient of ethanol (i.e., 70% until the solution was clear of Bouin’s fixative, 95% twice for 45 min each, and 100% twice for 45 min each). Samples were put into butanol (Sigma-Aldrich, St. Louis, MO, USA) for two washes (one overnight and one for 2 h) and Histochoice Clearing Agent (Amresco, Solon, OH, USA) for two washes (each for 2 h), and then embedded in Paraplast (Sigma-Aldrich) after 4 washes in Paraplast at 65 °C. Tissue blocks were cut into 4-6 µm-thick sections on a Leitz 1212 microtome. Tissue slices were put on poly-L-lysine (Sigma-Aldrich) coated slides. After deparaffinization in Histochoice Clearing Agent (Amresco) and rehydration, slides were immersed in PBS and stained with Harris modified Hematoxylin (Fisher Scientific, Pittsburg, PA, USA). Sections were dehydrated with an ascending ethanol series, stained with Eosin Y (Fisher Scientific), washed in 95% ethanol, dehydrated in 100% ethanol, and cleared in Histochoice for 30 s per wash. Sections were coverslipped on mounting medium (Polymount Polysciences, Warrington, PA, USA), and slides air-dried for 12 h to 1 d. Slides were imaged using a Nikon Microphot FXA microscope (Nikon, Melville, NY, USA) with a Spot RT230-Slider camera (Spot Imaging Solutions, Diagnostic Instruments, Sterling Heights, Michigan, USA) and/or a Leica MZ75 dissection scope (Leica Camera, Allendale, NJ, USA) with a Spot Insight camera (Spot Imaging Solutions, Diagnostic Instruments). Montage images of the H&E-stained sections were adjusted for brightness and color balanced using Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA).
2.2.4. Immunohistochemistry

After being deparaffinized in Histochoice Clearing Agent (Amresco®, OH, USA) and rehydrated in descending percentages of ethanol, slides were immersed in PBS, and microwaved for 5 min twice to reveal the antigen binding sites (Bancroft and Gamble, 2002). Slides were washed for 10 min in PBS-Tween (0.01% Tween 20, 150 mM NaCl in 10 mM PBS), blocked in 5% skim milk in PBS at room temperature for 20 min, and then washed three times in PBS for 5 min per wash. The primary antibodies, polyclonal rabbit anti-human NKA (8 μg/ml), monoclonal mouse anti-human CFTR (10 μg/ml), or polyclonal goat anti-human NKCC (14 μg/ml), were diluted in 0.5% skim milk in PBS, then applied to the slides. Slides were kept in a dark moist chamber at room temperature for 2 h. Control slides were treated using the same conditions but without primary antibodies added to the incubation mixture. Secondary antibodies, which included AlexaFluor 546-conjugated anti-rabbit IgG (12 μg/ml), AlexaFluor 488-conjugated anti-mouse IgG (12 μg/ml), AlexaFluor 633-conjugated anti-goat IgG (14 μg/ml, Invitrogen), were applied after washing slides 3 times in PBS for 5 min per wash. Slides were incubated with secondary antibodies and then mounted in Fluoro-Gel II with DAPI (Electron Microscopy Sciences, Hatfield, PA, USA) and observed using a Leica DM ExA2 Microscope (Leica Microsystems Inc.) with specific filters for fluorescence (Texas Red, CY5, GFP, and DAPI filters). Images were captured using a SensiCam QE high performance camera (PCO-TECH, Kelheim, Germany) using SlideBook software (Intelligent Imaging Innovations, Denver, CO, USA). For IHC images, Illustrator (Adobe Systems Inc.) was used to add larger font scale bars and notations.
2.2.5. Statistics

The statistical comparisons of relative protein levels were performed using analysis of variance (ANOVA) with a *post hoc* tukey test using SAS 9.3 Software (SAS Institute, Cary, NC, USA). A significance level of $\alpha = 0.05$ was used.

2.3. Results

There was no significant difference in the whole body weight (Table 2.1), nor in total body length (Table 2.2), in larvae reared in 0.1, 5, or 32 ppt water at any time point between 6 hph and 4 wph. There were no noticeable differences in hatching success or in overt swimming behavior or feeding in larvae reared at different salinities.

Table 2.1. Whole body wet weight (in mg) of larval *F. grandis* after 6 hours post hatch (hph), 1 and 4 weeks post hatch (wph) following exposure to 0.1, 5 and 32 ppt water. Values are means ± SE. There is no significant difference at significant level of $\alpha = 0.05$ in whole body wet weight among salinity treatments.

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Whole Body Weight (mg)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>6 hph</td>
</tr>
<tr>
<td>0.1 ppt</td>
<td>3.4 ± 0.10 (n = 27)</td>
</tr>
<tr>
<td>5 ppt</td>
<td>3.1 ± 0.10 (n = 27)</td>
</tr>
<tr>
<td>32 ppt</td>
<td>3.0 ± 0.16 (n = 24)</td>
</tr>
</tbody>
</table>

Table 2.2. Total length (in mm) of larval *F. grandis* after 6 hours post hatch (hph), 1 and 4 weeks post hatch (wph) following exposure to 0.1, 5 and 32 ppt water. Values are means ± SE. There is no significant difference at significant level of $\alpha = 0.05$ in total body length among salinity treatments.

<table>
<thead>
<tr>
<th>Salinity</th>
<th>Whole Body Length (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 hph</td>
</tr>
<tr>
<td>0.1 ppt</td>
<td>7.8 ± 0.86 (n = 10)</td>
</tr>
<tr>
<td>5 ppt</td>
<td>7.8 ± 0.27 (n = 10)</td>
</tr>
<tr>
<td>32 ppt</td>
<td>7.9 ± 0.23 (n = 9)</td>
</tr>
</tbody>
</table>
2.3.1. H&E staining

Body structures and major osmoregulatory organs at S30 of embryonic development were not appreciably different among embryos or larvae (up to 6 wph) reared at 0.1, 5, and 32 ppt water (Fig. 2.1-4). As such, only a representative H&E stained image from a 5 ppt water acclimated embryo is shown in the results (Fig. 2.1-4). Embryos at S30 were characterized by the onset of eye pigmentation, a discernable outline of the lens, and by the straightening of the caudal region. S30 embryos had large amounts of yolk (Fig. 2.1A), the presence of pharyngeal arches (Fig. 2.B, D), and pronephric ducts in the head kidney (Fig. 2.1C). The intestine (Fig. 2.2D-F) was initially a simple tube with no folding. At 6 hph, larvae still possessed a yolk sac with a diameter of approximately 0.5 mm (5 - 10% of its total body length), and primordial gills had emerged, replacing the pharyngeal arches seen in S30, although filaments were short and lamellae poorly developed (Fig. 2.2A, B). The folding pattern on the lumenal side of the intestinal epithelium was slightly more developed in larvae at 6 hph than in embryos at S30 (Fig. 2.2C, D); however, discrimination between the “anterior” and “posterior” or “caudal” regions of the intestine was yet unable to be made with certainty. The intestine was divided into an anterior and caudal portion after 1 wph (Fig. 2.1, 3A). The pronephric ducts in the head kidney of larvae at 6 hph were noted (Fig. 2.2D). After 1 wph, the yolk sac had been completely absorbed and the body length increased by about 40% compared to the 6 hph larvae (Fig. 2.3A). Despite these changes, the gills and pronephric ducts in the head kidney were not different from those in 6 hph larvae (Fig. 2.3B, C). The intestine appeared elongated in 1 wph larvae compared to the 6 hph yolk-sac stage larvae (Fig. 2.3A) and were divided into an anterior (adjacent to the liver and heart, Fig. 2.3D) and caudal intestine (towards the caudal fin, Fig. 2.3E). By 4 wph, larvae possessed gills that appeared to have longer filaments and lamellae compared to those of
larvae at earlier stages of development (Fig. 2.4A, B). The anterior (Fig. 2.4D) and caudal (Fig. 2.4E) intestine had folds with greater development and differentiation compared to those in larvae at earlier stages.

Figure 2.1. Histomorphology of representative, lateral sagittal sections of *F. grandis* embryos at stage 30 (Armstrong and Child, 1965) acclimated in 5 ppt water. Tissues were stained with Hematoxylin & Eosin. The embryo at this stage has a yolk sac (Yolk), digestive tract (DT), head (H), pharyngeal arches (PA), pronephric tubules (PN) (A, D). Higher magnification images (B-F) of the pharyngeal areas lacking lamellae (arrows; B), pronephric tubules (arrowheads; C), and the simple tube consisting of one or two cell layers and surrounding muscle (D-E). Lines represent scale bars.

2.3.2. Relative NKA, NKCC, and CFTR protein levels

Whole body NKA protein levels in larvae reared to 0.1 ppt water increased 2.2-fold between 6 hph and 4 wph but decreased by 110% between 4 wph and 6 wph (*P* = 0.001, *N* = 36).
Figure 2.2. Histomorphology of representative, lateral sagittal sections of *F. grandis* larvae at 6 hours post hatch acclimated in 5 ppt water. Tissues were stained with Hematoxylin & Eosin. A montage of a larva is included (A) containing gills, liver, head kidney, intestine, and small amounts of yolk. Higher magnification images (B-D) of the gills containing filaments (F) and lamellae (La) (B), an intestine with lumen (L) surrounded by epithelium (C), and pronephric tubules in the head kidney (arrow heads; D). Lines represent scale bars.

The total body NKA level increased by approximately 160% in larvae at 5 ppt between 6 hph and 4 wph ($P < 0.0001$, $N = 35$) but stayed constant thereafter until 6 wph. In contrast, larvae exposed to 32 ppt water had a total larval NKA level that plateaued after only 3 dph ($P = 0.002$, $N = 35$), but then stayed constant until 6 wph. Significant differences between salinities
Figure 2.3. Histomorphology of representative, lateral saggital sections of *F. grandis* larvae at 1 week post hatch acclimated in 5 ppt water. Tissues were stained with Hematoxylin & Eosin. A montage of a larvae is included (A) containing gills, liver, head kidney, and a differentiated anterior intestine (AI) and caudal intestine (CI). Higher magnification images (B-D) of the gills containing filaments (F) and lamellae (La) (B), pronephric tubules in the head kidney (arrow heads; C), and the intestine (D, E), which has developed both in length and in diameter. The AI (D) and CI (E) are shown with their lumen (L). Lines represent scale bars.

existed at 4 and 6 wph. In 4 wph larvae, whole body NKA protein level was elevated in 0.1 ppt acclimated larvae compared to 32 ppt acclimated larvae (*P* = 0.026, *N* = 18). In contrast, NKA protein in whole body homogenates of 0.1 ppt acclimated larvae was lower than in 5 ppt acclimated larvae at 6 wph (*P* = 0.002, *N* = 18) (Fig. 2.5A). Two-way ANOVA results indicated that there was no statistical interaction between time and salinity (*P* = 0.216, *N* = 106).

Whole body NKCC protein levels varied significantly during development (0.1 ppt: *P* = 0.0001, *N* = 35; 5 ppt: *P* = 0.004, *N* = 35; and 32 ppt water: *P* = 0.0003, *N* = 35). During the 0.1
Figure 2.4. Histomorphology of representative, lateral sagittal sections of *F. grandis* larvae at 4 week post hatch acclimated in 5 ppt water. Tissues were stained with Hematoxylin & Eosin. A montage of a larvae is included (A) containing gills, liver, head kidney, and a differentiated anterior intestine (AI) and caudal intestine (CI). Higher magnification images (B-D) of the gills containing filaments (F) and lamellae (La) (B), pronephric tubules in the head kidney (arrow heads; C), and the intestine (D, E), which has developed both in length and in diameter. The AI (D) and CI (E) are shown with their lumen (L), and differentiated goblet cells (GC) and enterocytes (EC). Lines represent scale bars.

In 32 ppt water exposed larvae, the lowest levels of total body NKCC occurred between 3 dph and 4 wph. The whole body NKCC protein level in 0.1 ppt water acclimated larvae was significantly higher than in 32 ppt water acclimated larvae at 4 wph (*P* = 0.048, *N* = 18). At 6
NKCC protein levels were higher in 0.1 and 32 ppt water than in 5 ppt water \((P = 0.0004, N = 18, \text{Fig. 2.5B})\). Only at these two time points were there any differences in NKCC protein levels in larvae reared at different salinities. Two-way ANOVA analysis demonstrated a significant interaction between developmental time and salinity \((P < 0.0001, N = 104)\).

The whole body CFTR protein level of larvae acclimated in 0.1 ppt water increased progressively between 6 hph and 4 wph, but then remained stable until at least 6 wph \((P < 0.0001, N = 35)\). The whole body CFTR protein level of larvae acclimated in 5 ppt water increased until 2 wph, then stayed at a similar level during 4 and 6 wph \((P < 0.0001, N = 34)\). In 32 ppt acclimated fish, there was a transient increase in the whole body CFTR protein level at 3 dph, before increasing progressively between 1 to 2 wph, then stabilizing through 6 wph \((P < 0.0001, N = 35)\). Significant differences in total body CFTR of larvae reared at different salinities existed at 3 dph \((P = 0.0035, N = 18)\) and 1 wph \((P = 0.0241, N = 17)\). At 3 dph, relative CFTR protein level was increased in 32 ppt water acclimated fish compared to those reared to 0.1 ppt and 5 ppt water. At 1 wph, a higher whole body CFTR protein level was observed in 0.1 ppt acclimated fish compared to those in 5 ppt and 32 ppt water acclimated fish (Fig. 2.5C). Two-way ANOVA results suggested that there was also a significant interaction between salinity and time \((P = 0.0097, N = 104)\).

2.3.3. Immunohistochemistry

NKA, NKCC, and CFTR were all expressed in the gills, intestine, kidney, and skin of late stage embryos and larvae of *F. grandis* reared at salinities ranging from 0.1 ppt to 32 ppt water. In embryos at S30, these proteins were localized in the gut, pronephric tubules, and skin, but not on the pharyngeal arches (Fig. 2.6A1-C1).
Figure 2.5. Relative protein abundance of NKA (A), NKCC (B), and CFTR (C) from the whole body homogenates of *F. grandis* larvae at 6 hours (h), 3 days (d), 1, 2, 4, and 6 weeks (w) after hatch in 0.1, 5, and 32 ppt water. Values are means ± SE (n = 5 ~ 6 for each mean value). The mean expression level of the 5 ppt acclimated fish at 6 hph is set arbitrarily to 1, and the relative protein levels are ratios of the mean values at each point compared to the mean of the 5 ppt controls at 6 hph. Different letters represent significant differences between different time points for any given salinity; * and # signs represent significant differences in the relative protein or mRNA levels in the whole body homogenates from larvae acclimated in different salinities at the same time point (ABCD denote differences during exposure to 0.1 ppt water, PQRS denote differences during exposure to 5 ppt water, and XYZW denote differences during exposure to 32 ppt water).
Primordial gill filaments and lamellae were formed in *F. grandis* larvae by 6 hph (Fig. 2.2A). NKA was expressed on the basolateral membrane of presumptive MRCs of the filamental epithelium regardless of the salinity at all the time points (Fig. 2.6). CFTR was localized diffusely throughout the cell, but also showed a discernable basolateral localization on the MRCs of the filaments and in other cells on gill lamellae of 0.1 and 5 ppt acclimated 6 hph larvae (Fig. 2.6A2, B2). In contrast, NKCC was localized to the basolateral membrane of MRCs on the filament in 0.1 ppt (Fig. 2.6A2) and 5 ppt (Fig. 2.6B2) acclimated larvae. In larvae at 6 hph in 32 ppt water, CFTR was poorly expressed in the gill, although NKCC was observed basolaterally in the MRCs as well as in other cells on the gill filament (Fig. 2.6C2). In the gills from 1 wph larvae reared in 0.1 ppt water, CFTR was weakly localized to the MRCs on the filament and also on the lamellae (Figure 6A3); in larvae from 5 ppt and 32 ppt water, the CFTR fluorescence was localized on several cells on the filament, but not in the MRCs (Fig. 2.6B3, C3). NKCC showed basolateral staining on the MRCs only in larvae acclimated to 32 ppt water for 1 wph (Fig. 2.6C3). In 4 wph larvae that were reared in 0.1 ppt water, CFTR was distributed diffusely on the MRCs and on the lamellae (Fig. 2.6A4). CFTR was localized on the apical side of some MRCs of the gill filament in 5 ppt and 32 ppt acclimated larvae (Fig. 2.6B4, C4). Positive NKCC fluorescence was localized on the basolateral side of the MRCs in 5 ppt and 32 ppt acclimated fish, and NKCC was localized on the basolateral membrane of non-MRCs on the gill filament during 0.1 ppt acclimation (Fig. 2.6B4, C4).

Basal localization of NKA, apical and subapical localization of CFTR, weak fluorescence of NKCC in the pronephric duct was found in embryos and larvae acclimated in all three salinities. (Fig. 2.7).
In the digestive tract from S30 embryos and 6 hph larvae, NKA was localized on the basolateral side of the enterocytes. In the gut of S30 embryos, CFTR was localized on the apical side of the cells during acclimation in all salinities, but was also observed on the subapical and

![Image of immunofluorescence localization of NKA (red), NKCC (blue) and CFTR (green) in the gills of embryos at stage 30 (S30; A1-C1), and larvae at 6 hours post hatch (hph; A2-C2), 1 (A3-C3) and 4 weeks post hatch (wph; A4-C4) acclimated in 0.1 ppt (A1-A4); 5 ppt (B1-B4), and 32 ppt water (C1-C4). Pharyngeal arches (arrows, A1-C1), and filaments (F), lamellae (L), and opercula (O) are shown in figures (A2-C4). All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. The arrows in A1 to C1 illustrate the pharyngeal arches. F = Filament; L = lamellae; O = opercula. Lines represent scale bars. The Venn diagram provides colors of single and colocalized proteins.]

Figure 2.6. Immunofluorescence localization of NKA (red), NKCC (blue) and CFTR (green) in the gills of embryos at stage 30 (S30; A1-C1), and larvae at 6 hours post hatch (hph; A2-C2), 1 (A3-C3) and 4 weeks post hatch (wph; A4-C4) acclimated in 0.1 ppt (A1-A4); 5 ppt (B1-B4), and 32 ppt water (C1-C4). Pharyngeal arches (arrows, A1-C1), and filaments (F), lamellae (L), and opercula (O) are shown in figures (A2-C4). All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. The arrows in A1 to C1 illustrate the pharyngeal arches. F = Filament; L = lamellae; O = opercula. Lines represent scale bars. The Venn diagram provides colors of single and colocalized proteins.
Figure 2.7. Immunofluorescence localization of NKA (red), NKCC (blue) and CFTR (green) in the head kidney pronephric tubules of embryos at stage 30 (S30; A1-C1), and larvae at 6 hours post hatch (hph; A2-C2), 1 (A3-C3) and 4 weeks post hatch (wph; A4-C4) acclimated in 0.1 ppt (A1-A4), 5 ppt (B1-B4), and 32 ppt water (C1-C4). All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. The arrows are pointed to the pronephric tubules. Lines represent scale bars. The Venn diagram provides colors of single and colocalized proteins.

basolateral regions of the cells in 5 ppt and 32 ppt acclimated embryos (Fig. 2.8A1-C1). NKCC protein was only seen on the subapical portion of the cells of embryos reared in 5 ppt and 32 ppt water (Fig. 2.8B1, C1). In 6 hph larvae reared in 0.1 ppt and 32 ppt water, CFTR was localized subapically and basolaterally on the enterocytes; NKCC had a punctate distribution on the basolateral membrane and was localized apically on the tips of some cells on epithelial foldings (Fig. 2.8A2-C2).
Figure 2.8. Immunofluorescence localization of NKA (red), NKCC (blue) and CFTR (green) in the digestive tract of embryos at stage 30 (S30, A1-C1) and larvae at 6 hours post hatch (hph; B2-C2) acclimated in 0.1 ppt (A1, A2), 5 ppt (B1, B2), and 32 ppt water (C1, C2). All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. Images illustrate the lumen (Lu) and serosal (Se) sides of the intestine and the surrounding muscle (M). Lines represent scale bars. The Venn diagram provides colors of single and colocalized proteins.

NKA was always localized basolaterally. In the anterior intestine of 1 wph larvae, CFTR was localized subapically and basolaterally on most of the enterocytes; NKCC had punctate staining on the basolateral portion of enterocytes (Fig. 2.9A1-C1). NKCC fluorescence was also seen on the bush border of the enterocytes on the tips of folds in the anterior intestine from 1 wph larvae. In 4 wph larvae, CFTR was mostly localized to the apical and subapical portion of the enterocytes, CFTR fluorescence was also colocalized with NKA on the basal portion of the cells (Fig. 2.9A2-C2). In larvae acclimated to 0.1 ppt for 4 wph, NKCC was present on vesicles in the intestine and sometimes on the apical surface along the apex of folds on the intestine. In contrast larvae acclimated to 5 ppt water, NKCC had punctate staining on the basal portion of the cells (Fig. 2.9A2, B2). In 4 wph larvae that were acclimated in 32 ppt water, NKCC had punctate staining on the basal side and also on the subapical regions of cells (Fig. 2.9C2).
Figure 2.9. Immunofluorescence localization of NKA (red), NKCC (blue) and CFTR (green) in the anterior part of the intestine in *F. grandis* larvae at 1 (A1-C1) and 4 weeks post hatch (wph; A2-C2) acclimated in 0.1 ppt (A1, A2), 5 ppt (B1, B2), and 32 ppt water (C1, C2). All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. Images illustrate the lumen (Lu) and serosal (Se) sides of the intestine and the surrounding muscle (M). Lines represent scale bars. The Venn diagram provides colors of single and colocalized proteins.

In the caudal intestine, NKA was always on the basolateral side of the epithelium. In 1 wph larvae acclimated to 0.1 ppt water, CFTR was localized to the brush border of the enterocytes but showed very weak staining in 5 and 32 ppt acclimated fish; NKCC showed punctate staining on the basal side in larvae from 0.1 and 5 ppt water, but showed staining on the vesicles in the intestine in 32 ppt acclimated larvae. In 4 wph larvae that were reared in 0.1 ppt water, CFTR was distributed to the brush border of the caudal intestinal epithelium with punctate staining of NKCC on the basal side. Four wph larvae that were acclimated in 5 ppt water showed apical and basal localization of CFTR and a very weak amount of NKCC on the caudal intestinal epithelium. In 32 ppt acclimated larvae at 4 wph, CFTR was localized to the brush border and the basolateral membrane of the enterocytes; NKCC was localized basally and subapically on the cells (Fig. 2.10).
During late embryonic development, NKA was distributed to the basolateral side of MRCs on the yolk sac and the skin of the embryos, especially near the skin at the tail (Fig. 2.11A). The lack of NKA fluorescence on the skin indicated that MRCs were rarely localized to its surface in 4 wph larvae (Fig. 2.11B).

2.4. Discussion

This study investigated the histology of osmoregulatory tissues and the expression of transport proteins on tissues of the Gulf killifish during embryonic or larval exposures to salinities ranging from FW to SW. These fish are amongst the most euryhaline species known, with the ability to undergo frequent and episodic variations in salinity (Griffith, 1974). Adults *F. grandis* are capable of withstanding acute challenges to salt ranging from FW to at least two
times the strength SW (Griffith, 1974). Although the physiological and genomic basis of this extreme euryhalinity in adult fish has been the focus of several recent studies (Whitehead, 2010; Whitehead et al., 2011; Whitehead et al., 2012), comparably less is known of the ontogeny of osmoregulatory systems in these animals during early development (Brown et al., 2012; Brown et al., 2011; Fisher et al., 2013; Patterson et al., 2012).

*Fundulus* embryos can develop in air and hatch upon water immersion when fully developed. In nature, *Fundulus* embryos will often spend much of their embryonic development attached to emergent marsh grasses, but hatch quickly when fully developed upon water immersion during extreme high tides (i.e., spring tides) (Taylor, 1999). In this study, this characteristic permitted the synchronization of hatch of air-incubated embryos in waters of different salinities, allowing the differentiation between the effects of salinity during embryonic versus larval stages of development.
2.4.1. Growth of larval *F. grandis*

The fitness of developing fish is influenced by osmotic perturbations, presumably by increasing the energetic costs of osmoregulation. However, in the current study, *F. grandis* exhibited no differences in mass or total length, nor was there any fish mortality by the end of the 6 week larval exposures to salinities ranging from FW to SW (Table 2.1, 2.2). Although no effects of rearing salinity on embryonic growth were recorded, a previous study found that *F. grandis* embryos exhibited differential growth during exposures ranging from 0.4 to 30 ppt water (Brown *et al.*, 2012). Similarly, osmotic challenges have been reported to affect fertilization success and larval survival in the common killifish, *Fundulus heteroclitus* (Able and Palmer, 1988), hatching success in the profret, *Pampus punctatissimus* (Shi *et al.*, 2008), and larval survival in the African catfish, *Heterobranchus longifilis* (Fashina-Bombata and Busari, 2003), mangrove red snapper, *Lutjanus argentimaculatus* (Estudillo *et al.*, 2000), and Pacific herring, *Clupea pallasi* (Alderdice *et al.*, 1979). Larval growth in juvenile black bream, *Acanthopagrus butcheri* (Partridge and Jenkins, 2002) and killifish, *Aphanius chantrei* (Yildirim and Karacuha, 2008) is perturbed by hyperosmotic exposures. Furthermore, the growth of juvenile *F. grandis* is significantly lower in 0.5 ppt water compared to 8 ppt and 12 ppt water (Patterson *et al.*, 2012). In the current study, the lack of influence of salinity on larval growth is surprising, but could possibility be explained by specific experimental conditions, including differences in fish density or feeding rates during exposures. Regardless, these findings attest the extreme euryhalinity of the species even during early development.

Differences in rearing conditions during early life may alter developmental trajectories and influence the long-term fitness of animals. Although the current experimental design allowed initiation of simultaneous larval exposures to different salinities, it created the
confounding problem that embryos used in embryonic challenges were reared entirely in water, whereas animals used for larval challenges were raised almost entirely in air. Perschbacher et al. (1995) suggested there were no significant differences between the growth rates of *F. grandis* incubated in air versus water, although survivorship post hatch was compromised by protracted air exposure. Tingaud-Sequeira *et al.* (2009) demonstrated that air-exposed *F. heteroclitus* embryos developed more quickly and had altered expression of aquaporins on their enveloping epithelial cell layer to resist desiccation. However, beyond this, little is known of how differences in water salinity during embryonic development influences long term tolerance to osmotic challenges or to the emergence of osmoregulatory systems.

2.4.2. Whole body NKA, NKCC, and CFTR protein levels during development

Relative protein levels of NKA, NKCC, and CFTR in whole body homogenates were detected as early as 6 hph; a result that was supported by immunohistochemical detection of the proteins. During early larval stages, whole body proteins were likely associated with the yolk, although the autofluorescence of the mass precluded a definitive assessment of protein abundance at this developmental stage. Furthermore, the nature of the attachment of the yolk to the body wall makes removing the yolk from the whole body unfeasible. Fortunately, yolk was fully absorbed in *F. grandis* larvae by 1 wph making the problem of autofluorescence only a short-term complication. Transport proteins had a discernable immunolocalization to developing epithelial membranes even during late embryogenesis, suggesting a role of the cutaneous surface in osmoregulation. Whole body NKA and CFTR protein levels increased in abundance during larval development, but in the case of NKA, decreased in abundance relative to the total protein pool in the 0.1 ppt acclimated larvae towards the latter stages of exposure, or remained stable after 1 wph in the 5 and 32 ppt acclimated animals. This apparent reduction in
NKA protein is likely more an effect of dilution of the NKA signal relative to the total protein pool, which may have increased to a greater extent during larval development.

Variations of whole body CFTR protein levels during development were observed at the beginning of yolk depletion, and differences of whole body CFTR protein level between salinities were only observed at 6 hph and 1 wph, during yolk absorption.

Whole body NKCC protein varied in abundance during development but showed no clear temporal or salinity-dependent patterns of expression, although this could be potentially due to the inability of the C-14 NKCC antibody to differentiate between NKCC isoforms. The same antibody was used previously to detect NKCC in European sea bass, *Dicentrarchus labrax* (Sucre *et al.*, 2013). In the current study, there was evidence that the antibody targeted both NKCC2 (apical localization) and NKCC1 (basolateral localization). As such, both NKCC1 and NKCC2 could be changing in abundance with salinity, thus obscuring any interpretation. Differences of whole body NKCC protein between salinities were observed at 3 dph, 4 and 6 wph, which may suggest divergent regulation of NKCC in osmoregulatory organs.

Although no other study has investigated the effects of salinity on whole body NKA, NKCC, or CFTR protein levels during development, existing studies suggest that relative mRNA expression is strong influence by salinity (Sucre *et al.*, 2013).

2.4.3. Integument during development

The skin serves as an important barrier to water and ion movement, however during early life the cutaneous surface can be an important osmoregulatory tissue (Hiroi *et al.*, 1998; Kaneko *et al.*, 2002; Katoh *et al.*, 2001; Kunz, 2004). The skin has also been implicated in gas exchange, acid-base regulation, and nitrogenous waste excretion in developing fish (Bodinier *et al.*, 2009a; Cucchi *et al.*, 2012; Hiroi *et al.*, 1998; Kaneko *et al.*, 2002; Sucre *et al.*, 2011).
In the current study, *F. grandis* embryos expressed NKA-rich cells, presumably ionocytes, on the external surfaces of their bodies and yolk sacs. Strong auto fluorescence of the yolk and erythrocytes in embryos made the assessment of CFTR and NKCC localization difficult. In the embryos of Mozambique tilapia, *Oreochromis mossambicus*, MRCs on the skin have been identified with cell specific expression of CFTR and Na\(^+/\)Cl\(^-\) cotransporter (NCC) that varies with salinity (Hiroi and McCormick, 2012; Hiroi *et al*., 2008). Freshwater MRCs on the skin were observed with apical NCC/NKCC localization; while seawater MRCs on the skin expressed apical CFTR and basal NKCC expression, indicating an ion absorption function during freshwater acclimation and an ion secretion function during seawater acclimation (Hiroi and McCormick, 2012; Hiroi *et al*., 2008; Kaneko *et al*., 2002).

2.4.4. Digestive tract during development

The digestive tract is derived from the endoderm and its formation begins at gastrulation (Oppenheimer, 1936; reviewed by Kunz, 2004). The gut tube is first formed anteriorly in medaka, *Oryzias latipes* (Kobayashi *et al*., 2006), or both anteriorly and posteriorly, in zebrafish, *Danio rerio* (Wallace and Pack, 2003).

In the current study, there were few gross morphological differences in the guts of fish collected in late embryogenesis in all salinity treatments. The gastrointestinal tube in late embryogenesis was a simple tube with a small luminal space with little folding on the apical membrane. The guts of larvae at 6 hph were all similar regardless of environmental salinity, with little apical folding but a greatly enhanced luminal space. Increased epithelial folding was also observed in other species, such as Atlantic halibut, *Hippoglossus hippoglossus* (Kamisaka *et al*., 2001). In *D. rerio* and *D. labrax*, the intestinal tube increases in length and has more developed folding following mouth opening (Oppenheimer, 1936; reviewed by Kunz, 2004).
*D. labrax*, the digestive tube is closed at both ends at hatch with cells at various stages of differentiation (Wilson and Castro, 2011; Govoni et al., 1986). Compared to *D. labrax*, in which the mouth opens after the jaw appears at 4 dph (Cucchi et al., 2012), *Fundulus species* have a relatively early onset of mouth opening, occurring almost coincident with hatch. Interestingly, jaw formation allows for synthesis of a hatching enzyme by cells in the buccal and gill cavities in pre-hatch *F. heteroclitus* (Armstrong and Child, 1965). As early as 1 wph, the gastrointestinal tract showed discernable segmentation along the longitudinal axis of the tube (Fig. 2.3A). The anterior section of the intestine had an elaborate folding pattern that was fairly consistent in killifish regardless of rearing salinity (Fig. 2.9). In contrast, the caudal sections of the gut were typically less complex in their architecture, especially in the guts of the 5 and 32 ppt acclimated fish (Fig. 2.10). The intestinal epithelium of 4 wph larvae at these higher salinities were highly folded, and expressed differentiated enterocytes and goblet cells in both the anterior and posterior portions of the gastrointestinal tract (Fig. 2.3D, E). Goblet cells, which are the major sites of mucous secretion, were localized predominantly on the tips of the epithelial folds. In contrast, enterocytes, which are the major cells types for nutrient absorption and water/ion regulation (Wilson and Castro, 2011), were found throughout the gut (Fig. 2.3D, E).

The cellular localization of CFTR and NKCC in the gastrointestinal tract of *F. grandis* was highly dependent on the longitudinal position along the gut, the larval stage of development and rearing salinity. CFTR is involved in the transcellular efflux of Cl⁻, although it has also been implicated in HCO₃⁻ secretion and as a regulator of the activity of other transport proteins, such as the Cl⁻/HCO₃⁻ exchanger (Singh et al., 2008; Takei and Yuge, 2007). The latter two proposed functions are intriguing considering the importance of HCO₃⁻ secretion in precipitation of imbibed calcium and magnesium; a mechanism that promotes osmotic water absorption in
marine fish (Grosell, 2006; Grosell et al., 2011; Singh et al., 2010). In the developing killifish gut, CFTR expression on the apical membrane increased along the longitudinal axis of the gut, especially in larvae reared at 0.1 ppt for 1 wph, or at all salinities for 4 wph. Differences in function along the caudal portion of the intestine of larvae at 1 wph but not at 4 wph may be influenced by acclimation salinity. Apical CFTR was only observed in the intestine of fish acclimated to 0.1 ppt but not 5 or 32 ppt, indicating Cl⁻ or HCO₃⁻ secretion may not exist in 1 wph larvae acclimated in brackish to marine salinities. Similarly, apical localization of CFTR has also been found in the posterior intestine of the adult F. grandis (Meng et al., submitted), in larval/juvenile D. labrax (Bodinier et al., 2009c), and eelpout, Zoarces viviparus (Takei and Loretz, 2011), facilitating Cl⁻ or HCO₃⁻ secretion into the lumen. HCO₃⁻ in the lumen of seawater acclimated fish may react with Ca²⁺ and Mg²⁺ to produce CaCO₃ and MgCO₃ precipitation, which facilitates further HCO₃⁻ secretion and water absorption. In the freshwater acclimated fish, apical CFTR mediated HCO₃⁻ secretion may be involved in acid base regulation. Expression of CFTR protein to different membranes in anterior and posterior intestine in F. grandis at the larval stages suggest distinct transporter function.

The constant localization of basolateral CFTR on the anterior intestinal epithelium indicates a function in Na⁺ and Cl⁻ absorption in larvae regardless of rearing salinity (Grosell, 2006; Marshall, 2002). Similar basolateral localization of CFTR is reported in the intestine of D. labrax (Bodinier et al., 2009b; Sucre et al., 2013; Sucre et al., 2011).

2.4.5. Gills during development

The gill epithelium represents the major route of solute secretion in marine teleosts and the site of active ion uptake in freshwater fish. Interestingly, there is evidence that the gill initially functions in osmoregulation rather than in gas exchange (Fu et al., 2010). Only as the
area of the cutaneous surface becomes limiting and vascularization of the gill enhanced is its preeminent role in external respiration achieved (Evans et al., 2005). In the present study, the gill epithelium of embryos presented negligible development of filaments and lamellae. Larvae as young as 6 hph already developed gill primordial with short lamellae and filaments covered by epithelia expressing key transport proteins. Gills developed progressively over time appearing to be fully developed based on morphology later in larval development.

The gills of freshwater acclimated larvae exhibited a diffuse pattern of CFTR expression in the MRCs (indicated by colocalization with NKA) on the gill filament and on non-MRCs on the epithelium surrounding the gill arches and lamellae (Bodinier et al., 2009b; Evans et al., 2005; Sucre et al., 2013; Tang and Lee, 2011). NKCC localization was observed in non-MRCs, and sometimes on the basolateral side of the MRCs. The lack of apical localization for NKCCs/NCC is similar to that seen in MRCs of freshwater-acclimated rainbow trout, Oncorhynchus mykiss (Katoh et al., 2008), but different from that in freshwater acclimated D. labrax (Lorin-Nebel et al., 2006) and F. heteroclitus (Hiroi and McCormick, 2012). The basolateral localization of CFTR suggest a possible role in Cl⁻ absorption, although the lack of active Cl⁻ uptake in the gill of freshwater-acclimated Fundulus sp. makes the role of NKCC in the freshwater gill unclear and worth further investigation (Evans, 2011; Patrick et al., 1997; Patrick and Wood, 1999; Scott et al., 2005).

In adult SW fish gill, it is known that NKA establishes a strong electrochemical gradient for Na⁺ and indirectly establishes a negative membrane potential. This electrochemical gradient fuels Na⁺ entry via the NKCC (presumably NKCC1); the ensuing elevation in intracellular Cl⁻ drives its passive secretion into SW via CFTR (Evans et al., 2005). Transepithelial Na⁺
secretion is thought to be driven by the passive efflux via leaky junctions between MRCs and accessory cells (Evans et al., 2005; Evans et al., 1999).

In 4 wph larvae, apical localization of CFTR with basolateral distribution of NKA and NKCC in the MRCs suggests a Cl\(^-\) secretion function in the gills during 5 and 32 ppt acclimation. Lack of apical NKCC localization in MRCs of gills during early larval development indicates that there was no Cl\(^-\) absorptive function in the gills at these stages when acclimated to FW. Other organs such as anterior intestine and the skin may play a role to compensate the passive ion loss to the ambient environment during freshwater acclimation.

2.4.6. Kidney during development

Freshwater teleosts tend to absorb ions and produce dilute urine by reabsorbing ions through the kidney to keep a constant plasma osmolality, while seawater teleosts produce only a limited amount of urine. The typical adult teleostean kidney consists of a nephric tubule, consisting of a proximal tubule (in the pronephron or head kidney), a distal tubule, and a collecting duct (Hickman and Trump, 1969). The embryonic nephron of teleosts consists of pronephric and mesonephric tubules (Kunz, 2004), although only pronephric tubules were investigated in the current study. Pronephric tubules of the kidney (or proximal tubule in adult fish) were formed by the late stages of embryogenesis (S30) in _F. grandis_. Its expression of NKA on the basolateral membrane of the epithelium and weak expression of CFTR on the apical membrane indicate a possible role in active Cl\(^-\) transport. In contrast, NKCC expression was not observed in kidney of larvae acclimated at any salinity. The same basal distribution of renal NKA has been reported in the proximal, distal, and collecting tubules of milkfish, _Chanos chanos_ (Tang et al., 2010b), spotted green pufferfish _Tetraodon nigroviridis_ (Lin et al., 2004), _F. heteroclitus_, and _O. mykiss_ (Katoh et al., 2008) in diverse environmental salinities.
NKCCs/NCC (detected using T4 antibody) protein is only found basolaterally in the proximal tubules of *F. heteroclitus*, however, NKCCs/NCC is localized to the apical membrane of the distal and collecting tubules in *F. heteroclitus* and *O. mykiss* (Katoh *et al.*, 2008). Apical localization of NKCC is also found in the collecting tube and urinary bladder of freshwater but not in seawater acclimated *D. labrax* (Lorin-Nebel *et al.*, 2006), suggesting a function in ion/water reabsorption in freshwater acclimated fish but not in seawater acclimated fish. In the present study, CFTR has been localized to the apical and basolateral membranes of the pronephric tubules during acclimation in water at all salinities, which indicates that both Cl$^-$ absorptive and secretive functions may occur in the head kidney of larval *F. grandis*.

In conclusion, the relative protein levels of NKA and CFTR level in whole body homogenates increased during development, while relative protein levels of NKCC in whole body homogenates fluctuated during development. The salinity effects on relative protein levels of NKA, NKCC, and CFTR were only shown at several stages. The effects of osmotic challenges on the gross morphology of late stage embryos and larval Gulf killifish were not observed, however, immunohistochemistry results indicated differential functions in the intestine and gills of larval *F. grandis*. These results suggested that the incubation salinities could affect the osmoregulatory tissue function during larval stages.
3.1. Introduction

Marine fish regulate major ions in blood plasma at concentrations substantially lower than the surrounding sea water (SW). This disequilibrium is maintained by the active secretion of monovalent ions at the gills and divalent ions at the kidney to compensate the passive influx of solutes from SW at transporting epithelia (Evans et al., 2005; Evans et al., 1999; Watanabe and Takei, 2011a, b). The high osmolarity of the surrounding water relative to the extracellular fluids also leads to osmotic water loss to the environment from tissues such as the gills. To compensate, marine teleosts drink SW from which they actively absorb Na\(^+\) and Cl\(^-\) to facilitate water uptake (Smith, 1932; Smith et al., 1930). Freshwater teleosts have the opposing problem of losing ions from the animal but gaining water passively from the environment across external surfaces. Extracellular osmolyte balance is regulated by fish in hypoosmotic environments by actively absorbing ions at the gills and kidneys; the latter process leading to the production of a dilute urine (Evans, 2008; Evans, 2011; Evans et al., 2005; Wong and Woo, 2006). The combined effects of a large production of urine and the cessation of drinking in freshwater fish maintain a net negative water flux to overcome the passive entry of water at the gills (Evans, 2008; Krogh, 1939).

In all teleost fish, osmoregulation is maintained by the active transport of Na\(^+\) and Cl\(^-\) across epithelia facilitated by ion transport proteins such as NKA, NKCC, and possibly CFTR (reviewed in Evans and Claiborne, 2006). In the intestine of freshwater and seawater fish, NKA reduces the intracellular concentration of Na\(^+\) and indirectly helps establish an inside negative membrane potential, creating an electrochemical gradient for Na\(^+\) entry into enterocytes.
Symporters such as the NKCC can utilize this favorable electrochemical gradient of Na⁺ to facilitate the entry of Na⁺, K⁺, and Cl⁻ into enterocytes (Grosell \textit{et al.}, 2011), supporting either transepithelial solute absorption or secretion depending on the cellular localization of the protein (Field \textit{et al.}, 1980; Frizzell \textit{et al.}, 1979; Halm \textit{et al.}, 1985; Musch \textit{et al.}, 1982). Isoforms of NKCC have been found in the intestine of European eel, \textit{Anguilla anguilla}, including absorptive NKCC2a and secretive NKCC1a and NKCC1b (Cutler and Cramb, 2002, 2008).

Apical NKCC2 together with basolateral NKA form one of several routes for the transepithelial absorption of Na⁺. Cl⁻ transported by NKCC may move into the plasma by Cl⁻ transporting proteins on the basolateral membrane, such as CFTR (Bodinier \textit{et al.}, 2009a; Lorin-Nebel \textit{et al.}, 2006; Marshall \textit{et al.}, 2002a), or secreted back into the lumen via apical CFTR (Takei and Yuge, 2007). Alternatively, basolateral NKCC1 may couple with apical Na⁺ and/or Cl⁻ transporting proteins to facilitate the secretion of these ions into the gut lumen, or to function in cell volume regulation (Cutler and Cramb, 2002; Hoffmann \textit{et al.}, 2007).

In mammals, CFTR localized to the apical membrane has been shown to regulate the activity of the Cl⁻/HCO₃⁻ exchanger in the apical membrane of intestine (Singh \textit{et al.}, 2008). Similarly, CFTR in the gut of seawater acclimated teleosts may secrete Cl⁻ or HCO₃⁻ into the gastrointestinal lumen, or regulate the Cl⁻/HCO₃⁻ exchanger (Takei and Yuge, 2007), the major path for HCO₃⁻ secretion in marine teleosts (Grosell, 2006; Taylor and Grosell, 2006). When HCO₃⁻ is secreted into the lumen, the Cl⁻ secreted by the CFTR could potentially cycle back into the enterocyte via the Cl⁻/HCO₃⁻ exchanger, whereas luminal HCO₃⁻ can react with Ca²⁺ and Mg²⁺ to form CaCO₃ and MgCO₃ precipitates (Faggio \textit{et al.}, 2011; Kurita \textit{et al.}, 2008; Mekuchi \textit{et al.}, 2013; Takei and Yuge, 2007; Whittamore, 2012; Whittamore \textit{et al.}, 2010; Yuge and Takei, 2007). The latter reaction decreases the osmolality of the intestinal fluid in the lumen.
thus facilitating water absorption in the posterior intestine (Carvalho et al., 2012; Whittamore, 2012; reviewed by Takei and Loretz, 2011).

The role of the fish intestine in osmoregulation has been primarily studied in marine teleosts (Grosell, 2006; Grosell and Taylor, 2007; Marshall et al., 2002a; Tresguerres et al., 2010). Although intestinal NKA, NKCC, and CFTR have been implicated in marine osmoregulation, their physiological functions in fresh water (FW) are still not fully elucidated (Marshall, 2011; Marshall et al., 2002a; Marshall and Singer, 2002). Furthermore, there is incomplete information on how the functions of many of these integral proteins are differentially regulated in different segments of the intestine during osmotic challenges. It is expected that the transcription and translation, and the cellular localization of these proteins in the epithelium will be affected by osmotic challenge (Evans, 2008; Evans, 2011; Evans et al., 2005; Karnaky, 1980; Karnaky et al., 1976; Marshall et al., 1999; Zadunaisky, 1996). The major goal of this study was to assess the effects of salinity ranging from FW to SW on the mRNA and protein expression and the cellular distribution of NKA, NKCC, and CFTR in the intestine of the euryhaline Gulf killfish, Fundulus grandis. This study provides a detailed assessment of the distribution of these proteins along the longitudinal axis of the intestine, and describes their regulation in the context of existing models of ion transport in the gastrointestinal tract of seawater and freshwater acclimated fish.

3.2. Materials and methods

3.2.1. Animal husbandry

Adult F. grandis (N = 150 fish, total wet weight 7.92 ± 0.20 g, total length 8.70 ± 0.06 cm), supplied by Gulf Coast Minnows (Thibodaux, LA), were acclimated to 5 ppt water in a 1600-liter fiberglass tank for at least 1 month. Water total ammonia nitrogen, nitrite, and nitrate
were monitored twice a week using either an API saltwater or an API freshwater master test kit (API®). Partial water replacements were performed at least twice a week to keep nitrogenous waste below the detection limit of the commercial kits. The aquarium was plumbed to a recirculation system with mechanical filtration, biological filtration, and ultraviolet sterilization. Water salinity, temperature, and dissolved oxygen were monitored using an YSI 85 meter (Richly Hydrological Company, Columbus, OH, USA). Mean water temperature was 23.2 ± 0.96 °C, and fish were on a 12 h light: 12 h dark cycle using fluorescent lighting controlled by an automatic timer. Dissolved oxygen was kept near air-saturation using a low-pressure aerator. Fish were fed with commercial fish pellet (45% protein, 12% lipid, Cargill, Minneapolis, MN, USA) twice daily at 2% body weight per day.

At least one week prior to experiments, fish were transferred to glass aquaria containing either 5 ppt (n = 90) or 0.1 ppt (n = 60) water. Fish acclimated to 5 ppt water were abruptly transferred to 0.1, 1, or 5 ppt water (n = 30 per salinity) at the start of exposures. Alternatively, fish acclimated for at least one week to 0.1 ppt water were abruptly transferred to 0.1 ppt or 32 ppt water (n = 30 per salinity) at the start of experiments. These exposures were all conducted in 113-liter glass aquaria with tank side filters (n = 30 fish per aquarium). Following transfer to hypoosmotic or hyperosmotic conditions, fish were randomly sampled and killed by decapitation at 6 h, 3 d, and 7 d post transfer (n = 9 per time). The entire intestine was removed and separated into two sections termed the anterior and posterior intestine. The anterior intestine was defined as the region after the enlarged pseudogaster and before the 2nd fold of the whole gut, and the posterior intestine, immediately caudal to the anterior intestine to 0.5 cm before the rectum, indicated by a thickened circular muscle layer. Tissues were collected from fish
exposed to each salinity for 6 h, 3 d and 7 d for analyses of mRNA and protein abundance (n = 7) or immunohistochemistry (n = 2).

3.2.2. Total NKA, NKCC1, and CFTR mRNA levels

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. DNA contamination was removed from total RNA using RNase-free DNase (Invitrogen). Total RNA concentrations were determined by UV absorbance at 260 and 280 nm using a spectrophotometer (Nanodrop ND-1000 V3.3; Thermo Scientific, Wilmington, DE, USA) and reverse transcribed (M-MLV reverse transcriptase and oligo dT primers; Invitrogen) for quantitative PCR analyses (ABI® 7000 system and SYBR® Green PCR Core Reagents; ABI-Life Technologies, Grand Island, NY, USA) of NKA, NKCC1, and CFTR mRNA abundance. Elongation factor (EF1α) was used as a housekeeping gene in qPCR reactions. Specific primers (Sigma-Aldrich, St. Louis, MO, USA), as described by Scott et al. (2005) or as designed using an online primer design software Primer 3 (V. 0.4.0 http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm, Table 1), were used in qPCR at concentrations of 0.1 µM for NKA and NKCC amplification and 0.2 µM for CFTR amplification after forward and reverse primer concentration optimization. Quantitative PCR was performed using the following conditions: enzyme activation (50 °C, 2 min), 1 cycle of denaturation (95 °C, 10 min), 40 cycles of cDNA amplification (95 °C, 15 s; 60 °C, 1 min), and a final dissociation (i.e., melting curve) (60 °C increased to 95 °C at 1 °C per min). Relative mRNA levels were calculated using the 2^ΔΔCt comparative method (Livak and Schmittgen, 2001; Pfaffl, 2001). PCR efficiencies for primer sets were calculated between 90% and 100% using a serial dilution of a cDNA mixture.
Table 3.1. Primer sequences used in q-PCR. EF1α and CFTR sequences were from Scott, (2005). NKCC and NKA sequences were designed based on *F. heteroclitus* NKCC1 mRNA, partial cds (GenBank accession no. AY533706.1), and *F. heteroclitus* NKA alpha subunit isoform 1 mRNA, complete cds (GenBank accession no. AY057072.1). All primers were synthesized at Sigma-Aldrich® Co. LLC.

<table>
<thead>
<tr>
<th>Sequences</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td><strong>EF1α</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>GGGAAAGGGCTCCTTTCAAGT</td>
</tr>
<tr>
<td>Reverse</td>
<td>ACGTCGGCCTTCAGCTT</td>
</tr>
<tr>
<td><strong>NKA</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>ACTGCCAAGGCCATTGCTAA</td>
</tr>
<tr>
<td>Reverse</td>
<td>AACGACGCAAGCTTTGGCAT</td>
</tr>
<tr>
<td><strong>CFTR</strong></td>
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</tr>
<tr>
<td>Forward</td>
<td>AATCGAGCAGTTCCCAGACAAG</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGCTGTGTTGCCCCATTGC</td>
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<tr>
<td><strong>NKCC1</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
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</tr>
<tr>
<td>Reverse</td>
<td>GAAACTGCAACCCCCAAGTA</td>
</tr>
</tbody>
</table>

3.2.3. Protein isolation

Anterior and posterior intestine were homogenized using a pellet pestle™ in 1.5 ml microcentrifuge tubes (Fisherbrand™, Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) in ice-cold SEI buffer (300 mM sucrose, 20 mM EDTA, 100 mM imidazole at pH 7.3 containing protease inhibitors (PI) (Complete Protease Inhibitor Cocktail Tablets, Roche Applied Science, Roche Applied Science, Madison, WI, USA). After 1.5 h on ice, homogenates were centrifuged at 2000 g for 6 min at 4 °C. Pellets were resuspended in 2.4 mM sodium deoxycholate (Invitrogen) in SEI-PI buffer and centrifuged at 2000 g for 6 min at 4 °C, and supernatants stored at -20 °C. The total protein concentration of each supernatant was determined using a NanoDrop spectrophotometer and the total protein concentration of each sample was adjusted to 1 mg/ml.

3.2.4. Western blot analyses of NKA, NKCC, and CFTR

The specificity of antibodies against killifish NKA, NKCC, and CFTR was assessed using Western blot analysis. In brief, total protein isolated from the intestines of *F. grandis* was separated by polyacrylamide gel electrophoresis (4% stacking gel and 7.5% separating gel) at
120 V. Proteins were transferred to nitrocellulose membranes (Hybond™ ECL™ nitrocellulose membrane; Amersham - GE Healthcare Biosciences Pharmacia, Pittsburgh, NJ, USA) at 100 V for 90 min (Mini-PROTEAN Electrophoresis System, Bio-Rad Laboratories Inc., Hercules, CA, USA). Membranes were blocked with 5% skimmed milk in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$) for 1 h at 37 °C, and incubated with primary antibodies. Primary antibodies for NKA, NKCC1, and CFTR included polyclonal rabbit anti-human NKA (10 µg/ml; Santa Cruz Biotechnology, Dallas, TX, USA), polyclonal goat anti-human NKCC1 (14 µg/ml, Santa Cruz Biotechnology), and monoclonal mouse anti-human CFTR (12 µg/ml, R&D Systems, Minneapolis, MN, USA), respectively, dissolved in 0.5% skim milk and PBS. After an overnight incubation at 4 °C, membranes were washed and incubated for 1 h at 37 °C with secondary antibodies, including peroxidase-conjugated donkey anti-goat IgG (1:5000; Santa Cruz Biotechnology); peroxidase-conjugated goat anti-rabbit IgG (1:10000; Jackson ImmunoResearch, West Grove, PA, USA); and peroxidase-conjugated goat anti-mouse IgG (1:5000; Jackson ImmunoResearch) in 0.5% skim milk in PBS, respectively. Membranes were visualized by chemiluminescence using the West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions, and film was developed in a Mini-Medical Series automatic film processor (ImageWorks, Elmsford, NY, USA).

3.2.5. Dot blot analyses of NKA, NKCC1, and CFTR

The relative abundance of NKA, NKCC1, and CFTR in intestines was analyzed by dot blot analysis. Briefly, 2 µg protein per replicate was added to Hybond™ ECL™ nitrocellulose membrane and allowed to bind for 30 min. For each sample, 3 replicate dots were added to 3 separate membranes (i.e., 9 dots per sample); each membrane was treated separately for
determination of NKA, NKCC, or CFTR protein levels. Membranes were incubated with primary and secondary antibodies, and visualized using the same conditions as described for Western blots. The intensity of each spot was measured using Adobe® Photoshop® CS5 Extended software (Adobe Systems Incorporated, San Jose, CA, USA). The mean intensity of the protein dots from fish acclimated to 5 ppt water and sampled at 6 h was arbitrarily set to 1, and referred to as the reference. The relative protein levels for all other treatments and time points were expressed as a ratio of the mean pixel intensity of each dot relative to mean pixel intensity of the control treatment at 6 h after transfer.

3.2.6. Immunohistochemistry

Intestines from 2 fish per salinity per time point were fixed in Bouin’s solution for 48 h. Samples were dehydrated in an ascending concentration gradient of ethanol (70% until clearing of the Bouin’s solution, 2-45 min washes in 95% ethanol, and 2-45 min washes in 100% ethanol), and washed overnight in butanol (Sigma-Aldrich). Tissues were rinsed for 2 h in 100% butanol, washed twice for 2 h per wash in Histochoice Clearing Agent (Amresco®, Solon, OH, USA), washed 4 times in Paraplast (Sigma-Aldrich) at 65 °C for 2 h per wash, and embedded in Paraplast. Tissue blocks were cut using a Leitz microtome into 4 µm sections, which were mounted on poly-L-lysine (Sigma-Aldrich)-coated slides, deparaffinized in Histochoice Clearing Agent, and rehydrated in descending concentrations of ethanol. Antigen retrieval was conducted by microwaving in PBS for 10 min (Bancroft and Gamble, 2002). Slides were washed for 10 min in PBS-Tween (0.01% Tween 20, 150 mM NaCl in PBS), and blocked in 5% skim milk in PBS at room temperature for 20 min then washed three times in PBS for 5 min per wash. Slides were incubated for 2 h with a mixture of the NKA, CFTR, and NKCC antibodies (8 µg/ml polyclonal rabbit anti-human NKA; 10 µg/ml monoclonal mouse
anti-human CFTR; 14 μg/ml polyclonal goat anti-human NKCC1) dissolved in 0.5% skim milk in PBS, respectively. Control slides were treated using the same conditions, but with no primary antibodies added to the incubation medium. A mixture of 3 secondary antibodies, including 12 μg/ml AlexaFluor 546-conjugated anti-rabbit IgG, 12 μg/ml AlexaFluor 488-conjugated anti-mouse IgG, 14 μg/ml AlexaFluor 633-conjugated anti-goat IgG (Invitrogen) in 0.5% skim milk in PBS was applied. Slides were then mounted in 2 to 3 drops of Fluoro-Gel II with DAPI (Electron Microscopy Sciences, Hatfield, PA, USA), incubated in dark for 10 min, and assessed for NKA, CFTR, and NKCC localization using a Leica DM ExA2 Microscope (Leica Microsystems Inc. Buffalo Grove, IL, USA) with specific filters for fluorescence (i.e., Texas Red, GFP, CY5, and DAPI filters). Images were captured using a SensiCam QE high performance camera (PCO-TECH Inc., Kelheim, Germany) and processed using a deconvolution algorithm (Slidebook, v.4.2; Intelligent Imaging Innovations, Inc., Denver, CO, USA).

Another set of slides was used to observe the localization of CFTR using confocal microscopy. These slides were prepared, rehydrated, washed and blocked the same way as described previously for immunohistochemistry. One hundred μl of monoclonal mouse anti-human CFTR dissolved in 0.5% skim milk in PBS was applied to each slide at 14 μg/ml. Slides were kept in a humid chamber for 2 h before applying 100 μl of Dylight 488 conjugated anti-mouse IgG (1/2000; Jackson ImmunoResearch) and then kept in a dark humid chamber for 1 h. Slides were mounted in 10% Mowiol® 4-88 (Sigma-Aldrich) in 4% n-propyl gallate (Sigma-Aldrich). Tissues were imaged for CFTR localization using a Leica TCS SP8 STED scanning laser confocal microscope (Leica Microsystems). Images were captured and exported using LAS AF software (Leica Microsystems Inc.).
3.2.7. Statistical analysis

Analysis of variance (ANOVA) was used to assess statistical differences between relative mRNA and protein levels in the anterior or posterior intestine of fish during hypoosmotic challenges over time. Statistically significant differences in relative mRNA and protein levels between salinities in the anterior or posterior intestine during hyperosmotic challenge were tested by Student’s t-test. Values were considered significantly different at $\alpha = 0.05$. Statistical differences were tested using Tukey’s post hoc test after significant treatment effects were established in the ANOVA analyses. All statistical analyses were performed using SAS software (SAS Institute, NC, USA).

3.3. Results

*F. grandis* did not exhibit any overt changes in behavior or feeding in response to hypoosmotic or hyperosmotic challenges, nor did any fish die during the 7 d exposure periods.

3.3.1. Western blot

Western blot analysis was used to assess the specificity of heterologous antibodies and to quantify the molecular weights of NKA, NKCC, and CFTR in the anterior intestine of killifish (Fig. 1). The antibody against $\alpha$-NKA stained intensely to a band at approximately 102 kDa, the NKCC1 antibody bound intensely to one band at 49 kDa and weakly to two bands at 130 and 177 kDa, and the CFTR antibody stained strongly to bands at 132 and 113 kDa. The negative control membranes showed no discernable staining of protein bands (data not shown).

3.3.2. Influence of hypoosmotic exposure on the anterior intestine

3.3.2.1 mRNA and protein levels

The NKA, CFTR, and NKCC1 mRNA levels in the anterior intestine of killifish acclimated in 5 ppt water were not significantly different from those of fish transferred acutely
to 0.1 ppt or 1 ppt water (Fig. 3.2A1-C1). The only significant differences in CFTR and NKCC1 mRNA levels of anterior intestines were observed between tissues collected from killifish exposed to 0.1 ppt and 1 ppt water for 3 d (Fig. 3.2B1, B2).

Figure 3.1. Western blots of NKA, NKCC and CFTR proteins isolated from the anterior intestine of *F. grandis*. The left lane shows the molecular weight markers of an internal protein standard. The antibody against NKAα subunit stained one strong band at 102 kDa, the NKCC1 antibody bound to one strong band at 49 kDa and two weak bands at 130 kDa and 177 kDa, and the CFTR antibody stained strongly to bands at 132 kDa and 113 kDa.

No significant differences in the relative NKA, CFTR, or NKCC protein levels were observed in fish acclimated in 5 ppt water (Fig. 3.2A2-C2). The relative NKA protein expression of anterior intestine decreased to 47% and 37% of the relative level of the 5 ppt-acclimated fish after 6 h and 3 d exposure to 0.1 ppt water, respectively, whereas it decreased to 50% and 55% of the level in 5 ppt-exposed fish following 6 h and 3 d exposure to 1 ppt water, respectively. However, following 7 d exposure to 0.1 or 1 ppt water, the NKA protein level in the anterior intestine returned to the level measured in 5 ppt acclimated fish (Fig. 3.2A2). Relative CFTR protein expression was not significantly different among treatments at any time of exposure (Fig. 3.2B2). In comparison, fish acclimated to 0.1 ppt water for 6 h had a relative NKCC protein level in the anterior intestine approximately 51% lower than that in 5 ppt-acclimated fish. Following 3 d acclimation to 1 ppt water, the relative NKCC protein value of
the anterior intestine decreased to 34% of the value in fish acclimated to 5 ppt water, although there was no significant difference in relative NKCC protein level in fish exposed to these salinities by day 7 (Fig. 3.2C2).

Figure 3.2. Relative mRNA and protein level of NKA (A1, A2), CFTR (B1, B2) and NKCC (C1, C2) in the anterior intestine of *F. grandis* following acclimation to 0.1 ppt (white bars), 1 ppt (patterned bars), and 5 ppt water (black bars) for 6 hours (h), 3 days (d) and 7 d. Values are means ± SE (n = 5~7 for each mean value). Relative protein levels are the ratio of intensity for each treatment to the mean intensity of the 5 ppt (6 h) control. Treatments not sharing capital letters are statistically different in relative protein or mRNA level compared across different salinity treatments at any given time point, whereas those not sharing lower case letters represent significant differences at different time points for any given salinity.
3.3.2.2. Cellular distribution

Control slides incubated with secondary antibodies alone exhibited no immunostaining, nor did they have any appreciable autofluorescence (data not shown).

NKA protein was localized to the basolateral membrane of the anterior intestine of killifish regardless of salinity and time course of exposure (Fig. 3.3A2-C2). CFTR was distributed primarily to the subapical and lateral regions of the anterior intestine in fish acclimated to 5 ppt and 1 ppt water throughout the 7 d exposures (Fig. 3.3B3-C3). This cellular distribution was validated using confocal microscopy (Fig. 3.3D2-D3). In killifish transferred to 0.1 ppt water, CFTR was strongly expressed on the brush border of the anterior intestine as early as 6 h post transfer from 5 ppt water (data not shown); this cellular distribution was maintained for at least 7 d post exposure (Fig. 3.3A3, D1). Most of the enterocytes and some of the goblet cells of the anterior intestine expressed CFTR. NKCC showed a complex cellular distribution in 5 ppt acclimated fish, with it distributed predominantly to the apical and subapical regions of the enterocytes, but also exhibiting a punctate expression pattern on the basolateral membrane. Only the latter staining pattern persisted in fish transferred to 0.1 ppt and 1 ppt water (Fig. 3.3A4, B4).

3.3.3. Influence of extreme hypoosmotic exposure on the posterior intestine

3.3.3.1. mRNA and protein levels

Unlike the minor changes observed in the anterior intestine with salinity, relative NKA, CFTR, and NKCC1 mRNA levels in the posterior intestine were significantly increased in killifish acclimated in 0.1 ppt water for 3 d (Fig. 3.4A1-C1) relative to the 5 ppt acclimated controls. The relative mRNA level of NKA was increased 1.6-fold (Fig. 3.4A1), the CFTR mRNA level was increased 0.7-fold (Fig. 3.4B1), and the NKCC1 mRNA level was increased
1.6-fold in posterior intestine of killifish acclimated for 3 d to 0.1 ppt water compared to tissues collected from fish in 5 ppt water (Fig. 3.4A1-C1).

There was no significant change in NKA, CFTR, or NKCC protein levels in the posterior intestine of 5 ppt acclimated fish over the 7 d exposure period. In contrast, fish in 0.1
ppt water for 7 d exhibited a 6-fold increase in NKA protein (Fig. 3.4A2) and a 0.7-fold increase in CFTR protein in their posterior intestine (Fig. 3.4B2) relative to the 5 ppt control fish. In comparison, the level of NKCC protein in the posterior intestine was lowered by exposure to 0.1 ppt water (relative to that in the 5 ppt water control, Fig. 3.4C2), despite an increase in NKCC1 mRNA level in the posterior intestine of these fish.

3.3.2. Cellular distribution

NKA was localized to the basolateral membrane of the posterior intestine during the entire time course of osmotic challenges (Fig. 3.5A2-C2). CFTR was observed mostly on the brush border of the enterocytes of the posterior intestine in killifish exposed to 0.1 ppt, 1 ppt, or 5 ppt water, although weak fluorescence was also seen in sub-apical and basolateral portions of the enterocytes (Fig. 3.5A3-C3, D1-D3). NKCC had a punctate staining pattern on the basolateral membrane of the posterior intestine at all times following transfer to 0.1 ppt, 1 ppt, and 5 ppt water (Fig. 3.5A4-C4), but also showed sub-apical localization on the posterior intestine of killifish acclimated to 5 ppt water (Fig. 3.5C4).

3.3.4. Influence of extreme hyperosmotic exposure on the anterior intestine

3.3.4.1 mRNA and protein levels

The relative mRNA levels of NKA, CFTR, and NKCC1 in the anterior intestine of killifish did not significantly change during the entire acclimation period to 0.1 ppt water (Fig. 3.6A1-C1). Following transfer to 32 ppt water, the relative mRNA levels of NKA in the anterior intestine increased between 1.6 to 2-fold relative to the 0.1 ppt acclimated fish over the 7 d post-transfer period (Fig. 3.6A1). The relative mRNA level of CFTR in the anterior intestine of the killifish was increased 2.7-fold after 7 d of acclimation to 32 ppt water (Fig. 3.6B1), whereas the relative NKCC1 mRNA level in the anterior intestine of the killifish was only significantly
elevated (i.e., 2-fold) following 6 h transfer to 32 ppt water relative to the 0.1 ppt acclimated control fish (Fig. 3.6C1).

![Figure 3.4](image_url)

Figure 3.4. Relative mRNA and protein levels of NKA (A1, A2), CFTR (B1, B2) and NKCC (C1, C2) in the posterior intestine of *F. grandis* following acclimation to 0.1 ppt (white bars), 1 ppt (patterned bars), and 5 ppt water (black bars) for 6 hours (h), 3 days (d) and 7 d. Values are means ± SE (n = 5~7 for each mean value). Relative protein levels are the ratio of intensity for each treatment to the mean intensity of the 5 ppt (6 h) controls. Treatments not sharing capital letters are statistically different in relative protein or mRNA level compared across different salinity treatments at any given time point, whereas those not sharing lower case letters represent significant differences at different time points for any given salinity.
Figure 3.5. Representative deconvolutional (A-C) or confocal (D) images of the immunolocalization of NKA (red, A2-C2), CFTR (green, A3-C3), NKCC (blue, A4-C4), or the merged images of NKA, CFTR, and NKCC (A1-C1) in the posterior intestine of *F. grandis* following acclimation to 0.1 ppt (A1-A4), 1 ppt (B1-B4), and 5 ppt (C1-C4) for a period of 6 hours to 7 days. All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. D1-D3 are images of CFTR localization by confocal microscopy and differential interference contrast of the anterior intestine (arrows show brush border staining). M = muscle, G = goblet cell, * = enterocyte, Ap = apical, Ba = basal side of the intestinal epithelium. Scale bars represent 50 µm. The Venn diagram shows single or multiple localization of proteins.

No significant differences in the relative NKA, CFTR, or NKCC protein levels of anterior intestine of killifish acclimated to 0.1 ppt water were observed during the time course of acclimation (Fig. 3.6A2, B2). Relative NKA protein level in the anterior intestine of killifish
after 3 d acclimation to 32 ppt water was decreased by 25% compared to the 0.1 ppt acclimated control (Fig. 3.6A2). Relative CFTR protein level in the anterior intestine showed an approximate increase of 1.5-fold in 32 ppt acclimated fish compared to the 0.1 ppt exposed controls over time (Fig. 3.6B2).

3.3.4.2. Cellular distribution

NKA was localized to the basolateral membrane of the intestinal epithelium of the anterior intestine of killifish at all times during exposure to 0.1 ppt and 32 ppt water (Fig. 3.7A2, B2). In fish exposed to 0.1 ppt water, CFTR was strongly distributed to the brush border of epithelial folds and also to the subapical and lateral regions of the anterior intestine (Fig. 3.7A3). However, brush border CFTR fluorescence was no longer observed in the anterior intestine of fish after only 6 h post transfer to 32 ppt water (Fig. 3.7B3). NKCC showed punctate distribution on the basolateral membrane of the anterior intestine during the whole time course of acclimation to 0.1 ppt water (Fig. 3.7A4, only one time point shown), but localized to the brush border of the “apical crypts” in the anterior intestine of fish that were exposed to 32 ppt water from 6 h to at least 7 d post-transfer (Fig. 3.7B4, B4’).

3.3.5. Influence of extreme hyperosmotic exposure on the posterior intestine

3.3.5.1. mRNA and protein levels

The relative mRNA levels of NKA, CFTR, and NKCC1 in the posterior intestine of killifish did not significantly change during the time course of acclimation to 0.1 ppt water (Fig. 3.8A1-C1). Upon transfer to 32 ppt water, the relative mRNA level of NKA in the posterior intestine was 2-fold and 2.5-fold of the level observed in the 0.1 ppt water exposed controls after 6 h and 7 d exposure, respectively (Fig. 3.8A1). The relative mRNA level of CFTR in the posterior intestine decreased to 18% and 55% of the 0.1 ppt control levels in the fish acclimated
to 32 ppt water for 3 and 7 d, respectively (Fig. 3.8B1). NKCC1 mRNA level showed no significant difference at any time during exposure to 0.1 or 32 ppt water (Fig. 3.8C1).

Figure 3.6. Relative mRNA and protein levels of NKA (A1, A2), CFTR (B1, B2) and NKCC (C1, C2) in the anterior intestine of *F. grandis* following acclimation to 0.1 ppt (white bars) and 32 ppt water (black bars) for 6 hours (h), 3 days (d) and 7 d. Values are means ± SE (n = 5~7 for each mean value). Relative protein levels are the ratio of intensity for each treatment to the mean intensity of the 0.1 ppt (6 h) controls. Treatments not sharing capital letters are statistically different in relative protein or mRNA level compared across different salinity treatments at any given time point, whereas those not sharing lower case letters represent significant differences at different time points for any given salinity.
Figure 3.7. Representative deconvolutional images of the immunolocalization of NKA (red, A2, B2), CFTR (green, A3, B3), and NKCC (blue, A4, B4), or the merged images of NKA, CFTR, and NKCC (A1, B1) in the anterior intestine of *F. grandis* following acclimation to 0.1 ppt (A1-A4) and 32 ppt (B1-B4) for a period of 6 hours and 7 days. The insert (B4') illustrates the rectangular area in B4. All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. M = muscle, G = goblet cell, * = enterocyte, Ap = apical, Ba = basal side of the intestinal epithelium. Scale bars represent 50 µm. The Venn diagram shows single or multiple localization of proteins.

The relative protein levels of NKA, CFTR, and NKCC in the anterior intestine did not vary over time during acclimation to 0.1 ppt water (Fig. 3.8A2-C2). Following 3 d of transfer to 32 ppt water, the relative level of NKA protein was decreased by 19% compared to the level measured in killifish exposed to 0.1 ppt water (Fig. 3.8A2). In contrast, the relative CFTR and
NKCC protein levels of anterior intestine of fish exposed to either 0.1 ppt or 32 ppt did not vary over time (Fig. 3.8B2, C2).

3.3.5.2. Cellular distribution

NKA was localized at all times to the basolateral membrane of the posterior intestine of killifish exposed to 0.1 ppt or 32 ppt water (Fig. 3.9A2, B2). In both 0.1 ppt and 32 ppt exposed killifish, strong CFTR immunofluorescence was observed on the brush border of the epithelial folds of the posterior intestine. CFTR was also localized in the sub-apical regions of some of the enterocytes and the goblet cells (Fig. 3.9A3, B3). In fish exposed to 32 ppt water, NKCC was localized to the subapical regions of the enterocytes and to the basolateral membrane of the posterior intestine, although the latter staining was only punctate in distribution. In contrast, NKCC had only punctate distribution on the basolateral membrane of the epithelial folds of the posterior intestine of fish acclimated to 0.1 ppt water (Fig. 3.9A4, B4).

3.4. Discussion

*F. grandis* is a euryhaline fish capable of tolerating environmental salinities ranging from 0.05 ppt to 76.1 ppt (reviewed by Griffith, 1974). Over the time course of salinity challenges, killifish are able to undergo short-term compensatory responses and long term-acclimation to their transporting epithelia to help restore extracellular osmotic regulation.

The general premise of the study is that membrane-bound transport proteins are necessary for the translocation of ions across plasma membranes (Nilius and Droogmans, 2001; Wills *et al.*, 1996). Another well-founded principle of transepithelial ion transport is that distinct proteins are targeted to opposing membranes, creating a pathway for solute movement across the apical and basolateral membranes (Evans and Claiborne, 2006). This protein asymmetry is maintained by tight junction complexes that prevent migration along the plasma membrane,
Figure 3.8. Relative mRNA and protein levels of NKA (A1, A2), CFTR (B1, B2) and NKCC (C1, C2) in the posterior intestine of *F. grandis* following acclimation to 0.1 ppt (white bars) and 32 ppt water (black bars) for 6 hours (h), 3 days (d) and 7 d. Values are means ± SE (n = 5~7 for each mean value). Relative protein levels are the ratio of intensity for each treatment and the mean intensity of the 0.1 ppt (6 h) controls. Treatments not sharing capital letters are statistically different in relative protein or mRNA level compared across different salinity treatments at any given time point, whereas those not sharing lower case letters represent significant differences at different time points for any given salinity.

thus promoting cellular polarity by limiting the diffusion of proteins between opposing sides of an epithelium (Cereijido and Anderson, 2001; Tsukita *et al.*, 2001). Although membrane occupancy by a protein is insufficient on its own to confer functionality in transepithelial transport, knowledge of the cellular distribution of transport proteins in an epithelium can
provide valuable insights into the mechanisms of ion transport. In the current study, protein distribution on an epithelium was used as a proxy of the transport capacity of the intestinal epithelium of the Gulf killifish.

![Figure 3.9](image)

Figure 3.9. Representative deconvolutional images of the immunolocalization of NKA (red, A2, B2), CFTR (green, A3, B3), and NKCC (blue, A4, B4), or the merged images of NKA, CFTR, and NKCC (A1, B1) in the posterior intestine of *F. grandis* following acclimation to 0.1 ppt (A1-A4) and 32 ppt (B1-B4) for a period of 6 hours and 7 days. All sections were stained with DAPI (grey) to provide contrast. Pseudo colors were assigned to different fluorescent signals. M = muscle, G = goblet cell, * = enterocyte, Ap = apical, Ba = basal side of the intestinal epithelium. Scale bars represent 50 µm. The Venn diagram shows single or multiple localization of proteins.

3.4.1. NKA

Western blot analyses using a polyclonal NKAα (H-300) antibody against the α subunit of NKA produced a single band at 102 kDa, similar in molecular weight to the NKAα subunit.
previously described in the intestine of juvenile marble goby, \textit{Oxyeleotris marmorata} (Chew \textit{et al.}, 2010) and in gill mitochondrion rich cells of Atlantic salmon, \textit{Salmo salar}. In the common killifish, \textit{Fundulus heteroclitus}, a protein band at about 120 kDa, and several fainter bands with molecular weights of 101 kDa, 72 kDa, and 68 kDa in gills were observed (Marshall \textit{et al.}, 2002b). These studies suggested that different isoforms of NKA may exist in some species and that variations in isoform abundance among tissues may mediate unique physiological processes.

NKA protein level was differentially affected by osmotic challenges in both the anterior and posterior intestine of the Gulf killifish. NKA protein level was decreased in the anterior intestine, but increased in the posterior intestine, indicating a difference in regulation of the protein during hypoosmotic challenge. Hyperosmotic challenge increased the NKA mRNA level in both the anterior and posterior intestine. NKA protein level was decreased temporarily due to hyperosmotic challenge in both portions of the intestine. The expression of NKA$\alpha$ protein was elevated following seawater transfer in the intestine of European sea bass, \textit{Dicentrarchus labrax}, four-eyed sleeper, \textit{Bostrychus sinensis}, and \textit{A. anguilla} (Cutler \textit{et al.}, 2000; Jensen \textit{et al.}, 1998; Peh \textit{et al.}, 2009). Divergent effects of salinity on the NKA mRNA level were also shown between the anterior and posterior regions of the intestine from \textit{F. grandis}.

3.4.2. CFTR

Western blot analysis identified two CFTR protein bands with approximate molecular weights of 132 kDa and 113 kDa in the anterior intestine of \textit{F. grandis}. Different sized subunits of CFTR with molecular weights at 175 kDa band, and two lighter bands at 90.3 kDa and 94.2 kDa, are reported in posterior intestine of \textit{F. heteroclitus} (Marshall \textit{et al.}, 2002a). In the gills of \textit{F. heteroclitus}, CFTR antibody identified bands with molecular weights of 151.3 kDa, 99.3 kDa,
and 89.3 kDa (Marshall et al., 2002b). These variations of molecular weights indicate that the expression of CFTR subunits may vary between species or among tissues.

CFTR mRNA and protein levels were relatively consistent in the anterior intestine during hypoosmotic challenge, demonstrating a temporary increase in mRNA or protein levels during freshwater acclimation. These results support the translocalization of CFTR from the basolateral to apical membranes, or the degradation of basolateral CFTR and reassembly of CFTR at the apical membrane of the anterior intestine as a mechanism for recycling of CFTR in the enterocytes rather than the synthesis of new CFTR (Bomberger et al., 2010; Golin-Bisello et al., 2005; Silvis et al., 2009). CFTR mRNA abundance was increased in the anterior intestine but decreased in the posterior intestine by seawater acclimation, indicating different modes of regulation between the two regions of the intestine. The increased CFTR protein level during hyperosmotic challenge coincided with the elevated basolateral CFTR localization, suggesting a role of the anterior intestine in ion absorption during seawater exposure. Patterns of CFTR mRNA level change in response to osmotic challenge also vary among species. CFTR mRNA level remains unchanged in the intestine of F. heteroclitus during hypoosmotic challenge (Scott et al., 2006). CFTR mRNA level decreases in the rectum, but increases in the anterior intestine of gilthead sea bream, Sparus aurata, following hypoosmotic challenge (Gregorio et al., 2013). Furthermore, CFTR protein levels decrease in the posterior intestine of juvenile D. labrax during freshwater acclimation (Bodinier et al., 2009b). Although not confirmed in the transporting epithelia of fish, the potential for post-translational regulation of CFTR is known to exist in mammalian airway epithelial cells and enterocytes (Bomberger et al., 2010; Golin-Bisello et al., 2005), and could explain much of the regulation of the protein. The effects of
salinity on CFTR mRNA and protein level are different between anterior and posterior intestine, suggesting a differential regulation of CFTR between the two regions of the intestine.

3.4.3. NKCC

The NKCC1 (C-14) antibody used in this study is raised against a conserved epitope of the human NKCC1 (GenBank accession no. P55011). Although sequence alignment found no similarity between the epitope sequence and sequences for NKCC2, it does not preclude the possibility that this antibody may have bound to teleost NKCC2. The same antibody was used in the embryos and larval *D. labrax*, detecting a basolateral localization of NKCC (NKCC1) in the gut (Sucre *et al.*, 2013).

Western blot analysis of NKCC in the anterior intestine showed bands at 49 kDa and two weak bands at 130 kDa and 177 kDa. The 177 kDa band may represent the phosphorylated NKCC1 as reported in the gills of *F. heteroclitus* (Flemmer *et al.*, 2010). Studies using the commercial, T4 antibody, found that NKCC/NCC proteins were separated into multiple bands including the most abundant fraction at 225 kDa, and two weak bands at 120 kDa and 110 kDa in the gills of adult *D. labrax*, (Lorin-Nebel *et al.*, 2006), or fractions at 150 kDa, 93 kDa, and 88 kDa in *F. heteroclitus* gill (Marshall *et al.*, 2002b). These results show that the NKCC expression is tissue specific and highly complex.

A temporary increase in NKCC mRNA level was observed in both the anterior and posterior intestine during hypoosmotic challenge. However, a decrease in NKCC protein abundance was observed during both hypo- and hyperosmotic challenges. No significant difference in NKCC protein level was observed during hyperosmotic challenge, NKCC1 mRNA level was only temporarily increased during hyperosmotic challenge. The apparent disconnect between NKCC1 mRNA and NKCC protein regulation in both segments of the intestine during
hypoosmotic challenges could suggest that posttranscriptional and/or posttranslational mechanisms of regulating teleost NKCC1 exist during perturbations of environmental salinity. However, we cannot exclude the possibility that the NKCC1 antibody also detected NKCC2 in protein assays. The hyperosmotic challenge increased the level of NKCC1 mRNA transiently and had no effect on the NKCC protein level. These results may indicate that there are multiple protein isoforms that show distinct responses to salinity. NKCC1 mRNA level is increased during hypoosmotic challenge in the anterior intestine of *S. aurata*, but shows a decrease in the rectum; while NKCC2 mRNA level is increased in the anterior intestine during hyperosmotic challenge (Gregorio *et al.*, 2013). The level of NKCC2a mRNA is increased with seawater acclimation in the intestine of *A. anguilla*, but NKCC1 did not show significant change (Cutler and Cramb, 2008). NKCC protein and mRNA levels were divergently regulated in different regions of the intestine, each showing a distinct regulation of NKCC during osmotic challenges.

3.4.4. Functions of anterior and posterior intestine during osmotic challenge

Previous studies have shown that variations in protein localization along the longitudinal and latitudinal axes of the intestine exist, and that these differences probably lead to unique transport characteristics for the anterior and posterior segments of the intestine (Gregorio *et al.*, 2013; Grosell *et al.*, 2011). In the present study, there was a clear zonation of protein distribution along the gut, particularly in the case of CFTR. Although CFTR was typically localized to the brush border on the subapical regions of the enterocytes along the entire gut of *F. grandis* during exposure to FW, there was a trend of greater abundance in the posterior regions of the intestine. Similarly, CFTR distributes to the brush border of the rectum of seawater acclimated embryos and larvae (Sucre *et al.*, 2013) or to the posterior intestine and rectum of freshwater acclimated larvae of *D. labrax* (Bodinier *et al.*, 2009a). In contrast, CFTR
is localized to the brush border of the posterior intestine in juvenile *D. labrax* exposed to FW (Bodinier *et al.*, 2009b).

During seawater acclimation, the apical NKCC (presumably NKCC2) and basolateral CFTR in the anterior intestine of *F. grandis* may function in Na\(^+\) and Cl\(^-\) absorption (Grosell, 2006; Grosell *et al.*, 2011). In the posterior intestine during seawater acclimation, CFTR on the brush border may mediate carbonate precipitation in the lumen to facilitate water absorption; a mechanism proposed in the intestine of several marine teleosts (Takei and Yuge, 2007; Yuge and Takei, 2007). CFTR on the brush border may activate the apical anion exchanger Cl\(^-\)/HCO\(_3^-\) exchanger (Slc26a6A and Slc26a6B) family (Singh *et al.*, 2010; Singh *et al.*, 2008). Anion Cl\(^-\)/HCO\(_3^-\) exchanger exists along the entire intestine considering the high titratable alkalinity in the intestinal fluids throughout the gut (Grosell and Genz, 2006; Grosell *et al.*, 2001; Grosell *et al.*, 2005; Wood *et al.*, 2010). One possible mechanism has Cl\(^-\) being secreted by CFTR into the lumen, and then it being used as a substrate for the Cl\(^-\)/HCO\(_3^-\). The secreted HCO\(_3^-\) can react with Ca\(^{2+}\) and Mg\(^{2+}\) to form CaCO\(_3\) and MgCO\(_3\) precipitates (Faggio *et al.*, 2011; Kurita *et al.*, 2008; Mekuchi *et al.*, 2013; Whittamore, 2012; Whittamore *et al.*, 2010). In this study, killifish transferred acutely to 32 ppt water quickly formed white precipitates (presumably CaCO\(_3\) and MgCO\(_3\), data not shown) along the entire intestine except the rectum. The formation of the precipitate creates a gradient for further HCO\(_3^-\) secretion by the family of Cl\(^-\)/HCO\(_3^-\) exchangers (Slc26a6A and Slc26a6B) expressed on the luminal side of the intestine (Grosell *et al.*, 2011; Takei and Yuge, 2007). Chloride transport via CFTR creates a continual source of the ion, which otherwise might become limiting due to the additional absorption of Cl\(^-\) via the apical NKCC2 (Grosell *et al.*, 2009). Bicarbonate precipitation of divalent ions is seen as an important
mechanism for decreasing the osmotic pressure of the intestinal fluid and stimulating water absorption across the marine gut (Faggio et al., 2011; Kurita et al., 2008; Whittamore, 2012).

Apical CFTR activation of the Slc26A6 family appears critical for HCO$_3^-$ secretion in mouse duodenum (Singh et al., 2010). Apical CFTR may also secrete HCO$_3^-$ rather than Cl$^-$ into the lumen (Guba et al., 1996). In this case, the process can be driven by the relatively low luminal pH in mammals under certain physiological states (Singh et al., 2008). A similar role for CFTR is not likely in Fundulus species owing to absence of a stomach, and the fact digestion involves base secretion solely, lacking any mechanism for concentrated acid secretion (Babkin, 1928; Wood et al., 2010). Thus any putative role in HCO$_3^-$ secretion is likely more associated with an aforementioned role in carbonate precipitation of divalent ions (Grosell, 2006; Grosell et al., 2011; Takei and Yuge, 2007).

Basolateral CFTR and apical NKCC (presumably NKCC2) localization in the anterior intestine from 1 and 5 ppt acclimated fish suggests an ion absorption function, similar to anterior intestine during seawater acclimation. Similar to what is observed in seawater acclimated fish, the posterior intestine of 1 ppt and 5 ppt acclimated fish may also favor a HCO$_3^-$ secretory function. However, both portions of the intestine during 1 ppt and 5 ppt acclimation do not produce carbonate precipitates in the gut lumen.

3.4.5. NKA, NKCC, and CFTR localization

As typically seen in all transporting epithelia, NKA protein is localized on the basolateral membrane of killifish intestine; CFTR protein is observed on the brush border in most cases, but also found on the basolateral membrane in certain cells in the anterior intestine during acclimation in higher salinities. However, the presence of diffuse or subapical CFTR may represent nascent protein that has not yet been processed for trafficking to the membrane or
that is undergoing degradation. In contrast, NKCC was expressed in numerous cells on the apical membrane or on isolated cells on the basolateral membrane, as well as localized subapically in the enterocytes.

In addition to differential targeting of proteins to epithelial surfaces, post translational regulation of NKCC and CFTR proteins during osmotic stress may occur, potentially decreasing the cost of transcriptional regulation during osmotic challenges (Bodinier et al., 2009b). The mechanisms including second messenger-induced phosphorylation, that control transport activity without enhancing the endocytotic activity of protein internalization (Catterall, 2000; Cohen, 1989). In mammals, CFTR trafficking and activation on the enterocytes is regulated by cAMP and cGMP-dependent phosphorylation, including protein kinase A (PKA), protein kinase C (PKC) and AMP-activated kinase (AMPK) (Golin-Bisello et al., 2005; Hallows et al., 2003).

In the branchial MRCs of *F. heteroclitus*, osmotically mediated cAMP independent activation of apical CFTR by focal adhesion kinase (FAK) was proposed by Marshall et al. (2009). Phosphorylation of NKCC1 on the basolateral membrane of the branchial epithelium of *F. heteroclitus* has been suggested to be activated by cAMP-protein kinase A (cAMP-PKA) during acute hyperosmotic acclimation (Flemmer et al., 2010). In opercular MRCs of *F. heteroclitus* during hypotonic shock, dephosphorylation of FAK leads to NKCC1 deactivation in the basolateral membrane (Marshall et al., 2008). The basolaterally localized NKCC may also be involved in volume-sensitive ion regulation (Hoffmann et al., 2007). Future studies should focus on the role of these NKCC or CFTR activating pathways in the regulation of solute transport along the fish intestine during osmotic challenges.

In conclusion, in the intestine of euryhaline *F. grandis*, the mRNA and protein levels of NKA, NKCC, and CFTR are affected by hypo- or hyperosmotic challenges. The localization of
NKA, NKCC, and CFTR suggest that the anterior and posterior intestine of 0.1 ppt acclimated killifish may function in $\text{HCO}_3^-$ or $\text{Cl}^-$ secretion; the anterior intestine from 32 ppt acclimated fish may facilitate both $\text{Cl}^-$ and water absorption; and the posterior intestine during 32 ppt acclimation may facilitate $\text{HCO}_3^-$ or $\text{Cl}^-$ secretion.
CHAPTER FOUR - PHARMACOLOGICAL INHIBITION OF NKA, NKCC AND CFTR IN THE ANTERIOR INTESTINE OF THE GULF KILLIFISH FUNDULUS GRANDIS

4.1. Introduction

Most euryhaline teleost fish are obligatory osmoregulators that must keep a constant extracellular fluid osmolality despite variations in environmental salinity (Krogh, 1965). During seawater exposure, teleosts must stimulate active secretion at the gill to compensate for the passive and active uptake of salts from imbibed water in the gastrointestinal tract. Studies have shown that the plasma osmolality, and plasma Na$^+$ or Cl$^-$ concentrations of euryhaline fish may change rapidly after abrupt salinity challenge (Al-Jandal and Wilson, 2011; Scott et al., 2006; Whitehead et al., 2011; Whitehead et al., 2012). In the common killifish, Fundulus heteroclitus, the osmolality and Na$^+$ concentration of blood plasma decrease rapidly during hypoosmotic challenges (after 6 hours transfer from 32 ppt to 0.1 ppt water) (Whitehead et al., 2011), however homeostatic mechanisms allow the fish to restore plasma osmolality quickly. This study focuses on the possible compensatory responses of ion transport proteins in the anterior intestine of F. grandis during osmotic challenges.

The gut has a myriad of transport proteins that function to stimulate active Na$^+$ and Cl$^-$ uptake, including the apically localized Na$^+$/K$^+$/2Cl$^-$ cotransporter (NKCC) and basolaterally localized Na$^+$/K$^+$ ATPase (NKA). Other transport proteins, including Na$^+$/Cl$^-$ cotransporter (NCC), Cl$^-$/HCO$_3^-$ exchanger and cystic fibrosis transmembrane conductance regulator (CFTR), have been implicated in active ion transport and stimulation of osmosis in the marine gut (Cutler and Cramb, 2002; Field et al., 1978; Grosell, 2006; Kurita et al., 2008; Marshall et al., 2002a). Electrophysiological studies have suggested that transport of Cl$^-$ across the intestine is mainly active, while Na$^+$ transport can be active via ion transporting proteins such as NKA and NKCC or passive through paracellular pathways (Evans, 2008; Gill et al., 2011; Li and Naren,
Active ion transport in intestine can be measured using short circuit current ($I_{sc}$) and transmembrane permeability can be indicated by transepithelial resistance ($R_t$) across isolated intestine mounted on an Ussing chambers (Krogh, 1937; Ussing, 1951). Inhibition of basolateral NKA, apical CFTR, and both apical and basolateral NKCC have all been shown to significantly decrease $I_{sc}$ across the teleost intestine (Lionetto et al., 2001; Faggio et al., 2011; Thiagarajah and Verkman, 2003), a response presumably associated with decreased active Cl$^{-}$ absorption. Furthermore, Cl$^{-}$ absorption, as well as water absorption, may be reduced following inhibition of NKA and CFTR (Trischitta et al., 2004; Kim et al., 2008).

In the euryhaline Gulf killifish, *Fundulus grandis* (Baird and Girard, 1853), NKCC is localized on the apical membrane of the anterior intestine during acclimation to brackish water and during hyperosmotic challenge to facilitate ion absorption. In hypoosmotic conditions, NKCC has a punctate distribution on the basolateral membrane (see Chapter 3). Although CFTR is expressed on the brush border of the anterior intestine in freshwater acclimated killifish, it is also expressed in the seawater intestine. In the marine gut, apical CFTR may stimulate bicarbonate secretion through the Cl$^{-}$/HCO$_3^-$ exchange, by directly secreting Cl$^{-}$ into the lumen to allow the ion to cycle back to the cytoplasm through the Cl$^{-}$/HCO$_3^-$ exchanger. Alternatively, CFTR may directly secrete HCO$_3^-$ or regulate other transporters such as the Cl$^{-}$/HCO$_3^-$ exchanger, while itself moving no ions. In fresh water, CFTR may be localized to the apical membranes in the anterior and posterior sections of the intestine, but occasionally, distributed weakly to isolated cells in the posterior intestine. These diverse mechanisms indicate CFTR and NKCC, as well as other associated transport mechanisms are diverse and complex, and may have varied functions during osmotic challenges.
The objectives of this study were to assess the transport functions of NKA, NKCC, and CFTR in the anterior intestine of *F. grandis* acclimated to salinities ranging from FW to SW. *I*<sub>sc</sub> and *R*<sub>t</sub> were measured in isolated anterior intestine of fish acclimated to these salinities during inhibition of these transport proteins, and during exposure to Na<sup>+</sup> and Cl<sup>-</sup> reduced conditions. This study hypothesized that: a) inhibition of these transport proteins would decrease active ion transport, but that inhibition of CFTR or NKCC would have its greatest effect in fish acclimated to 0.1 ppt acclimated, and that NKA inhibition would alter active ion transport across all salinities to a similar extent; b) application of Na<sup>+</sup> free solution on the apical surface would reduce the serosal negative *I*<sub>sc</sub> and that application of Cl<sup>-</sup> free solution on the apical surface would increase the serosal *I*<sub>sc</sub> negativity; and c) basal ouabain inhibition would decrease *I*<sub>sc</sub> (making the *I*<sub>sc</sub> more negative) to a larger extent after the *I*<sub>sc</sub> reaching new steady state following apical Na<sup>+</sup> substitution compared to that following the apical Cl<sup>-</sup> substitution.

4.2. Material and Methods

4.2.1. Acclimation and tissue preparation

Adult *F. grandis* (N = 120, total wet weight = 11.49 ± 0.68 g) were acclimated at least one week to 5 ppt water in four 113-liter glass tanks plumbed into a single fish holding system with biological and mechanical filtration, and UV sterilization.

At the start of hypoosmotic challenges, fish were abruptly transferred to separate 38-liter glass tanks containing water at either 5 ppt (control; n = 2 per batch for a total of 40) or 0.1 ppt water (hypoosmotic challenge; n = 10 per batch for a total of 40) for at least 7 days (d). In another series of experiments, fish were transferred from 5 ppt to 0.1 ppt water, then acclimated to 0.1 ppt water for at least 7 d. Following acclimation, fish were acutely transferred from 0.1 ppt water to 32 ppt water (hyperosmotic challenge; n = 10 per batch for a total of 40) for at least
7 d. Due to the nature of the isolated membrane preparations, it was necessary to stagger the start times of the acclimation period for fish. In general, batches of 10 fish were acclimated at one time in individual tanks as described above, and used within 3 days of transfer. Water quality in tanks was maintained with individual biological filters and air pumps.

Fish were killed by decapitation, after which the entire gastrointestinal tract removed from animals, and approximately 1 cm of anterior intestine isolated for mounting on Ussing chambers. The anterior intestine (the region after the enlarged pseudogaster and before the second fold of the whole gut) was dissected from fish and mounted to a set of plexiglass supports (Physiological Systems, San Diego, CA) with the membrane spanning an aperture of 0.1 cm$^2$ (P2303, Physiological Systems) or 0.2 cm$^2$ (P2307, Physiological Systems). Once an intestine was mounted, plexiglass supports were inserted in individual Ussing Chambers (Physiological Instruments) and incubated for at least 45-60 min under symmetrical conditions as described below. Both sides of the intestine were aerated with 97% O$_2$:3% CO$_2$ using a gas mixing flowmeter (Cameron Instruments GF-3/MP). Each side of the membrane had a single Ag-AgCl voltage electrode and a current electrode in 2.5% agar in 3 M KCl connected to the half-chamber. These electrodes were used to measure $I_{sc}$ and $R_t$ in real time as described below.

4.2.2. Symmetric conditions

The intestine was bathed in modified Cortland’s saline (151 mM NaCl, 3 mM KCl, 10 mM CaCl$_2$, 8.8 mM MgSO$_4$, 4.6 mM Na$_2$HPO$_4$, 0.4 mM K$_2$HPO$_4$, 7.1 mM HEPES-free acid, 45 mM urea, 17.8 mM NaHCO$_3$, 340 mOsm, pH 7.8, Tresguerres et al., 2010b). The basolateral side was supplemented with glucose (3 mM) and the apical side supplemented with mannitol (3 mM). Each side of the intestine contained 2 ml of the respective solutions. The experiments were started after the $I_{sc}$ across the intestine stabilized (about 45 to 60 min). $I_{sc}$ and $R_t$ were...
measured under symmetrical conditions prior to replacement of solutions with reduced concentrations of ions.

4.2.3. Asymmetric conditions

Intestine was bathed in Cortland’s saline under symmetrical conditions until stabilization of $I_{sc}$. The apical solution was replaced with 2 mL of one of the following solutions, including:

- a) Na$^+$ reduced Cortland’s saline (168 mM N-methyl-D-glucamine, 3 mM KCl, 10 mM CaCl$_2$, 8.8 mM MgSO$_4$, 4.6 mM Na$_2$HPO$_4$, 0.4 mM K$_2$HPO$_4$, 7.1 mM HEPES-free acid, 45 mM urea);
- b) Cl$^-$ reduced Cortland’s saline solution (151 mM sodium gluconate, 3 mM KCl, 10 mM CaCl$_2$, 8.8 mM MgSO$_4$, 4.6 mM Na$_2$HPO$_4$, 0.4 mM K$_2$HPO$_4$, 7.1 mM HEPES-free acid, 45 mM urea); or
- c) mannitol substituted Cortland’s saline (285 mM D-mannitol, 3 mM KCl, 10 mM CaCl$_2$, 8.8 mM MgSO$_4$, 4.6 mM Na$_2$HPO$_4$, 0.4 mM K$_2$HPO$_4$, 7.1 mM HEPES-free acid, 45 mM urea). Two ml Cortland’s saline with 3 mM glucose was added to the basolateral solution. $I_{sc}$ and $R_t$ were measured after stabilization to the new steady state (within 40 min after adding the ion reduced solution).

4.2.4. Ion transport protein inhibition

Pharmacological inhibitors against NKA, NKCC, and CFTR were dissolved in dimethyl sulfoxide (DMSO) at 1000-times the final concentration and added to the serosal and/or apical solutions at a 1:1000 dilution (i.e., 2 µL inhibitor to 2 mL saline). Final concentrations of these inhibitors were chosen from previous studies. The NKA inhibitor, ouabain (final concentration = 0.2 mM; Grosell and Genz, 2006), NKCC inhibitor, bumetanide (final concentration = 0.1 mM; Clarke, 2009), and CFTR inhibitor, CFTR inh-172 (final concentration = 20 µM; Ma et al., 2002) were added to the apical or serosal solutions. An equal volume of DMSO was added to the opposite side of the membrane that inhibitor was added to control for the possible effects.
of volume change. All pharmacological inhibitors and DMSO were obtained from Sigma-Aldrich® Co. (St. Louis, MO, USA). The $I_{sc}$ and $R_t$ were measured once signals stabilized.

4.2.5. Data acquisition

$I_{sc}$ across the anterior intestine was recorded continuously; currents were all expressed relative to the apical side of the membrane. A pulse (V) of 2 mV was applied to the membrane every minute to calculate the transepithelial resistance ($R_t$). Signals were obtained using a data acquisition system (PowerLab using LabChart 7 software; ADInstrument Inc., Colorado Springs, CO, USA). $I_{sc}$ was expressed as µamp per cm$^2$ and normalized to the surface area of the aperture of the plexiglass support (see Section 4.2.1). $R_t$ was calculated by applying Ohm’s Law ($R=V/\Delta I_{sc}$), where V represents the pulse voltage in volts and $\Delta I_{sc}$ represents the change in $I_{sc}$ due to the transient voltage pulse.

4.2.6. Data analyses and statistical comparisons

Data were expressed as absolute change in $I_{sc}$ ($|\Delta I_{sc}|$), percent change in $I_{sc}$ ($%\Delta I_{sc}$), or percent change in $R_t$ ($%\Delta R_t$) according to equations 4.1, 4.2, and 4.3, respectively.

$$|\Delta I_{sc}| = |I_{sc}\text{after} - I_{sc}\text{before}|$$  \hspace{1cm} \text{Equation 4.1}

where $(I_{sc})_{after}$ is the stable $I_{sc}$ value after addition of ion reduced solution or pharmacological blocker, and $(I_{sc})_{before}$ is the stable $I_{sc}$ value under symmetrical conditions before experimental manipulation.

$$%\Delta I_{sc} = \left(\frac{(I_{sc})_{after} - (I_{sc})_{before}}{(I_{sc})_{before}}\right) \times 100\%$$  \hspace{1cm} \text{Equation 4.2}

where $(I_{sc})_{after}$ and $(I_{sc})_{before}$ are values as described for equation 1.

$$%\Delta R_t = \left(\frac{(\Delta R_t)_{after} - (\Delta R_t)_{before}}{(\Delta R_t)_{before}}\right) \times 100\%$$  \hspace{1cm} \text{Equation 4.3}

where $(\Delta R_t)_{after}$ is the stable $R_t$ after addition of ion reduced solution or pharmacological blocker, and $(R_t)_{before}$ is the stable $R_t$ under symmetrical conditions before experimental manipulation.
Statistically significant differences $|\Delta I_{sc}|$, $\%\Delta I_{sc}$, or $\%\Delta R_t$ among salinities were tested by analysis of variance (ANOVA). Statistically significant differences between the initial calibration in symmetrical saline and treatment challenges were tested by a Students t-test. Values were considered significantly different at $\alpha = 0.05$. Statistical differences were tested using Tukey’s post hoc test after significant treatment effects were established in the ANOVA analyses. All statistical analyses were performed using SAS software (SAS Institute Inc., NC, USA).

4.3. Results

When a biological membrane is maintained under symmetrical conditions, a positive $I_{sc}$ represents the active transport of a net negative charge from the serosal side to the apical side, or alternatively, it represents an active transport of a net positive charge from the apical side to the serosal side of the membrane. A negative $I_{sc}$ represents the active transport of a positive charge from the serosal to apical sides of the membrane, or an active transport of a negative charge from the apical to serosal sides of a membrane.

Under symmetrical conditions, the anterior intestine maintained a negative $I_{sc}$ that ranged between -3.85 and 1.17 $\mu$amp/cm$^2$. The mean $I_{sc}$ produced by the anterior intestine under symmetric isotonic conditions was similar in all salinities. In contrast, mean $R_t$ of the anterior intestine was significantly higher in fish acclimated to 0.1 and 32 ppt water compared to 5 ppt water (Table 4.1).

NKA inhibition by serosal application of ouabain decreased the $\Delta I_{sc}$ of the intestine by $-54.6 \pm 17.8\%$ to $-94.9 \pm 48.9\%$. In comparison, there was no effect of ouabain on $\%\Delta R_t$ or $\Delta R_t$ between salinity treatments (Fig. 4.1A, D). Similarly, inhibition of CFTR by apical application of CFTR inh-172 decreased $\Delta I_{sc}$ in intestine between $-15.6 \pm 6.6$ to $-68.4 \pm 15.3\%$. CFTR
inhibition was significantly more potent in reducing $I_{sc}$ in the anterior intestine from 0.1 ppt acclimated fish compared to 5 ppt acclimated fish. No significant difference in $\Delta R_t$ was measured in anterior intestine isolated from fish acclimated to all salinities (Fig. 4.1C, F). NKCC inhibition by apical application of bumetanide elicited no effects on $\Delta I_{sc}$, nor on $\Delta R_t$ among salinity treatments (Fig. 4.1B, E). Strangely, $I_{sc}$ was positive under control conditions, and decreased in its serosal side positive $I_{sc}$ with apical application of bumetanide.

Table 4.1. The $I_{sc}$ and $R_t$ of isolated anterior intestine from *F. grandis* acclimated to 0.1, 5, or 32 ppt water for at least 7 days. Values represent means ± SE. There was no significant difference in $I_{sc}$ among salinity treatments. The transmembrane resistance of the anterior intestine from fish following acclimation of 5 ppt water was lower than that from fish during 0.1 and 32 ppt acclimation. Treatments not sharing same letters are statistically different in resistance ($P = 0.0132$).

<table>
<thead>
<tr>
<th>Salinity</th>
<th>$I_{sc}$ (µAmp·cm$^{-2}$)</th>
<th>Resistance (Ω·cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ppt</td>
<td>1.2 ± 4.38 (n = 21)</td>
<td>119.9 ± 8.31$^a$ (n = 18)</td>
</tr>
<tr>
<td>5 ppt</td>
<td>-2.3 ± 3.73 (n = 40)</td>
<td>88.1 ± 7.19$^b$ (n = 31)</td>
</tr>
<tr>
<td>32 ppt</td>
<td>-3.8 ± 5.37 (n = 23)</td>
<td>113.4 ± 8.26$^a$ (n = 23)</td>
</tr>
</tbody>
</table>

Reduction of apical Na$^+$, Cl$^-$, or reduction of both apical Na$^+$ and Cl$^-$, resulted in a significant change in the $I_{sc}$ across the anterior intestine (Fig. 4.2A-C). Applying either Na$^+$ reduced Cortland’s saline or mannitol modified Cortland’s saline made the $I_{sc}$ across the anterior intestine highly positive (Fig. 4.2A, C). There was no significant difference observed in the changes of $I_{sc}$ across the intestine among fish that were acclimated in different salinities (Fig. 4.2D, F). Application of Cl$^-$ reduced Cortland’s saline on the apical side led to large increased negativity in $I_{sc}$ (Fig. 4.2B). The change in $I_{sc}$ across the membrane from control fish was significantly higher than in hypoosmotic challenged fish (Fig. 4.2F).
Figure 4.1. $I_{sc}$ (A,B,C), percent $I_{sc}$ change (%Δ$I_{sc}$, black bar), and percent $R_t$ change (%Δ$R_t$, grey bar D,E,F) in fish acclimated in 0.1, 5, or 32 ppt water for at least 7 days, and subjected to 0.2 mM ouabain on the serosal side (A, D); 0.1 mM bumetanide on the mucosal side (B, E); or 20 µM CFTR inh-172 on the mucosal side (C, F). Panels A, B, and C are representative traces. Values in panels D, E, and F are mean ± SE (n = 5~7 for each mean value). Treatments not sharing capital letters are statistically different in %Δ$I_{sc}$, whereas those not sharing lower case letters represent significant differences in %Δ$R_t$.

The $|ΔI_{sc}|$ in the anterior intestine of killifish acclimated to different salinities caused by apical Na$^+$ reduced Cortland’s saline is higher than the $I_{sc}$ changes caused by exposure to Cl$^-$ reduced or mannitol modified Cortland’s saline (all three $P$ values were < 0.01, Fig. 4.3A). The value of $I_{sc}$ change ($ΔI_{sc}$) due to low apical Na$^+$ is similar in magnitude to the $ΔI_{sc}$ produced by
Figure 4.2. $I_{sc}$ change ($\Delta I_{sc}$, A, B, C, black bar in D, E, F) and percent $R_t$ change ($\% \Delta R_t$, grey bay, D,E,F) after applying Na$^+$ reduced (A, D) or Cl$^-\text{reduced Cortland’s saline (B, E), or mannitol modified Cortland’s saline (C, F) on the apical side of the anterior intestine of } F.\text{ grandis after acclimation to 0.1, 5, or 32 ppt water for at least 7 days. Values are mean ± SE (n = 5~7 for each mean value). Treatments not sharing capital letters are statistically different in } \Delta I_{sc}, \text{ whereas those not sharing lower case letters represent significant differences in } \% \Delta R_t.$

Apical application of Cl$^-$ reduced or mannitol supplemented Cortland’s saline. The $\Delta R_t$ caused by apical application of mannitol modified Cortland’s saline was significantly higher than that measured during exposure to Na$^+$ or Cl$^-$ reduced Cortland’s saline (Fig. 4.3B).
The effects of addition of serosal ouabain to anterior intestine following exposure apical ion-reduced exposures was performed to assess residual levels of active ion transport in the membrane following apical ion depletion. Serosal ouabain to intestine pre-treated with apical Na\(^+\)-free solution produced a 39 ± 5 % reduction in \(I_{sc}\) from the steady state \(I_{sc}\) obtained following apical administration of the Na\(^+\) reduced solution (Fig. 4.4A, D). Serosal ouabain had little effect (<10% change) on the new steady state \(I_{sc}\) achieved by apical administration of the Cl\(^-\) reduced solution (Fig. 4.4B, E). In comparison, serosal ouabain elicited a 51± 13% reduction in negative \(I_{sc}\) from the steady state \(I_{sc}\) achieved by apical application of mannitol modified Cortland’s saline (Fig. 4.4C, F).

![Graphs showing \(I_{sc}\) change and \%Δ\(R_t\) change](image)

Figure 4.3. Comparison of absolute value of \(I_{sc}\) change (\(|ΔI_{sc}|\), top panel) and percent resistance change (\(%ΔR_t\), bottom panel) after applying Na\(^+\) or Cl\(^-\) reduced Cortland’s saline, and mannitol modified Cortland’s saline on the apical side of the anterior intestines from F. grandis after exposure to 0.1, 5 or 32 ppt. Values are mean ± SE (n = 5~7 for each mean value). Treatments not sharing letters are statistically different in \(|ΔI_{sc}|\), or \(%ΔR_t\).
4.4. Discussion

The present study used the short circuit technique to assess the effects of protein inhibition and apical ion substitution on the $I_{sc}$ and $R_t$ in isolated anterior intestine of Gulf killifish to have a better understanding of the possible functions of NKA, NKCC, and CFTR in active ion transport in the anterior intestine of *F. grandis* subjected to acute osmotic challenge.

4.4.1. Effects of inhibition of NKA, NKCC, and CFTR on active ion transport in the anterior intestine

![Graphs A-F](image_url)

Figure 4.4. Effect of basal ouabain application on the $I_{sc}$ (A, B, C), percent $I_{sc}$, and $R_t$ change (%Δ$I_{sc}$, black bar, %Δ$R_t$, grey bar, D, E, F) after the establishment of a new steady state in $I_{sc}$ was reached after application of Na$^+$ or Cl$^-$ reduced Cortland’s saline, or mannitol modified Cortland’s saline on the mucosal side of the anterior intestine of *F. grandis* acclimated to 5 ppt water. Panels A, B, and C are representative traces. Values in panels D, E, and F are mean ± SE (n = 5–7 for each mean value). Treatments not sharing capital letters are statistically different in %Δ$I_{sc}$, whereas those not sharing lower case letters represent significant differences in %Δ$R_t$. 
In the intestine of seawater acclimated fish, Na\(^+\) and Cl\(^-\) can be absorbed from the lumen through apical NKCC and serosal NKA causing an osmotic gradient for water reabsorption, however, apical localized CFTR may also contribute in osmoregulation (Grosell \textit{et al.}, 2011; Takei and Yuge, 2007). Freshwater fish may utilize transporters, such as apical Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3^-\) exchangers, as well as serosal NKCC and NKA, to absorb ions to counteract the diffusive loss of ions at the gills (Evans \textit{et al.}, 2005). Other serosal Cl\(^-\) channels may also exist (Movileanu \textit{et al.}, 1998). The intestine of both seawater and freshwater acclimated fish rely on active transport through these membrane-binding proteins to mediate transepithelial transport. These proteins are susceptible to pharmacological inhibition and the active transport can be monitored continuously using electrophysiological approaches such as the short circuit technique on biological membranes obtained from fish.

Despite the highly disparate transport systems used in the intestine of freshwater and seawater fish, both rely on the electromotive force derived by NKA. NKA is usually localized on the serosal membrane of the intestinal epithelium, helping to create a favorable electrochemical gradient and reduced intracellular concentration of Na\(^+\) to stimulate the facilitated transport of solute by other ion transport proteins (Grosell \textit{et al.}, 2011; Takei and Yuge, 2007). Previous studies have shown that application of basal ouabain was found to almost totally abolish the serosal negative \(I_{sc}\) in the intestine of goby fish, \textit{Gobius niger} and \textit{D. labrax} without changing \(R_t\) (Trischitta \textit{et al.}, 2004; Faggio \textit{et al.}, 2011). In the present study, serosal NKA inhibition using ouabain also elicited a similar extent in \(I_{sc}\) reduction and a marginal \(R_t\) increase across the anterior intestine regardless of acclimation salinity of adult fish. This decrease is presumably associated with the reduction in active transepithelial Na\(^+\) or Cl\(^-\) transport across the anterior intestine due to the inhibition of serosal NKA. The results are
consistent with our hypothesis that basal NKA inhibition did not differentially affect $\% \Delta I_{sc}$ and $\% \Delta R_i$ in the anterior intestine of fish acclimated to salinities ranging from 0.1 to 32 ppt. These results indicate that serosal NKA is essential in both active transepithelial ion absorption and secretion (Colin et al., 1985; Grosell and Taylor, 2007).

NKCC is localized on the apical membrane in seawater acclimated killifish, or to the basolateral membrane in freshwater acclimated $F$. grandis (see Chapter 3). NKCC has distinct isoforms localized to different membranes of epithelia. NKCC1 mainly localizes on the basolateral membrane, whereas NKCC2 usually localizes on the apical membrane (Cutler and Cramb, 2002, 2008). In seawater acclimated teleosts, NKCC2 on the apical membrane of the intestine utilizes the electromotive force of $Na^+$ to transport $Cl^-$ from the lumen into enterocytes against the electrochemical gradient of that ion. Inhibition of NKCC2 by apical application of bumetanide has been shown to attenuate the serosal negative $I_{sc}$ (Lionetto et al., 2001). $Cl^-$ was found absorbed through bumetanide-sensitive NKCC2 cotransport on the luminal membrane (Schettino and Lionetto, 2003), leading to a relative positive change in $I_{sc}$ due to reduction of active $Cl^-$ transport. Apical or serosal application of bumetanide significantly caused an 80% reduction of $I_{sc}$ across the intestine of $G$. niger without changing the $R_i$ (Trischitta et al., 2004). In comparison, freshwater acclimated teleosts such as eel, $Anguilla anguilla$ also absorb $Cl^-$ through the NKCC, albeit in small amounts (Schettino and Lionetto, 2003). Interestingly, both $Fundulus$ species and $A$. anguilla have highly attenuated active $Cl^-$ uptake at the gill, and must rely on $Cl^-$ absorption at the gut for long-term maintenance of plasma $Cl^-$ homeostasis (Patrick et al., 1997; Patrick and Wood, 1999; Schettino and Lionetto, 2003; Scott et al., 2006). Both $Na^+$ and $Cl^-$ fluxes, as well as $I_{sc}$, were lower in freshwater compared to seawater acclimated flounder, $Platichthys flesus$ (Gibson et al., 1987). Apical bumetanide application showed no
significant differences in $\%\Delta I_{sc}$ and $\%\Delta R_I$ among killifish acclimated to the three salinities. This is different from our original hypothesis that apical bumetanide application to anterior intestine would have a much greater effect on $\%\Delta I_{sc}$ in seawater acclimated fish than it would in freshwater acclimated killifish intestine, due to a possible apical localization of NKCC (NKCC2) in the intestine during seawater acclimation, but a basolateral localization of NKCC (NKCC1) during freshwater acclimation.

CFTR was localized at the brush border of the anterior intestine of killifish acclimated to 0.1 ppt water but not to 5 or 32 ppt water (Chapter 3). Apical administration of the CFTR inhibitor, CFTR-172, has been shown to inhibit CFTR activity in various transporting epithelia (Laverty et al., 2012; Pondugula et al., 2013; Stahl et al., 2012), leading to alterations in active ion transport measured using the $I_{sc}$ technique. In the current study, $\%\Delta I_{sc}$ caused by apical CFTR inhibition in 0.1 ppt acclimated fish was significantly higher than the $\%\Delta I_{sc}$ in 5 ppt acclimated fish. The results supported a greater role of CFTR in active ion transport in freshwater acclimated $F.\ grandis$, potentially associated with $\text{Cl}^-$ or $\text{HCO}_3^-$ secretion. The $\%\Delta I_{sc}$ of anterior intestine from fish acclimated to 0.1 ppt water was not significantly different from $\%\Delta I_{sc}$ in 32 ppt acclimated fish. This result might be due to the fact that 32 ppt acclimated fish were originally acclimated to 0.1 ppt water before transferring to 32 ppt water. This result was partially consistent with our hypothesis that $\%\Delta I_{sc}$ caused by apical CFTR inhibition in the intestinal epithelium are different between fish acclimated in 0.1 ppt water and fish acclimated in 5 ppt water. However the significant difference in $\%\Delta I_{sc}$ was not observed in the anterior intestine between fish acclimated in 0.1 versus 32 ppt water.
4.4.2. Effects of apical ion substitution on the $I_{sc}$ and $R_t$ in the anterior intestine

The isolated anterior intestine of fish acclimated to these salinities were exposed to Na$^+$ and Cl$^-$ reduced conditions to test the effect of apical Na$^+$ or Cl$^-$ substitution on the $\Delta I_{sc}$ and $\%\Delta R_t$ in the intestine from fish that were acclimated in different salinities.

Low apical Cl$^-$ concentration stimulated a decrease in $I_{sc}$ (making the $I_{sc}$ more negative), indicating a decrease of Cl$^-$ secretion or an increase of Na$^+$ absorption across the epithelium. For 0.1 ppt acclimated fish, the extent of the decrease in the active ion transport across the intestinal epithelium was significantly lower than that from 5 ppt water acclimated fish. This result is consistent with the cellular localization of CFTR in the anterior intestine under in vivo conditions. CFTR was found to be localized to the apical membrane of anterior intestine of 0.1 ppt acclimated $F.\ grandis$, supporting a possible function in Cl$^-$ or HCO$_3^-$ secretion. In contrast, apical CFTR fluorescence was absent in the anterior intestine from $F.\ grandis$ acclimated to 5 and 32 ppt water.

4.4.3. Effects of basal ouabain inhibition on the $I_{sc}$ and $R_t$ in the anterior intestine following ion replacement experiments

This set of experiments tested the effects of serosal NKA inhibition on $I_{sc}$ in epithelia maintained in reduced Na$^+$ or Cl$^-$ concentrations on the apical side of the epithelium. These experiments tested the effect of basal NKA inhibition on the $I_{sc}$ and $R_t$ after the acute hypotonic challenge.

After the new steady state $I_{sc}$ had been reached following apical administration of Na$^+$ reduced solution or mannitol substituted Cortland’s saline, serosal ouabain application caused a significant decrease in the $\%\Delta I_{sc}$. In contrast, after the new steady state $I_{sc}$ had been reached following apical administration of Cl$^-$ reduced solution $I_{sc}$ was more negative in the beginning but went back to the original level within 30 min after basal ouabain application. Application of
basal ouabain has been found to decreases the HCO$_3^-$ secretion in the seawater acclimated toadfish (Grosell and Genz, 2006), which may gradually decrease Cl$^-$ absorption from the lumen, showing a more positive $I_{sc}$ after ouabain application. These results are consistent with our hypothesis that the basal ouabain application only inhibits Cl$^-$ excretion but not Na$^+$ excretion across the anterior intestine.

In conclusion, inhibition of apical CFTR reduced active ion transport most significantly in fish acclimated to 0.1 ppt water, indicating that the activity of apical CFTR was the highest during freshwater acclimation. Reduction of apical [Cl$^-$] concentration increased the serosal negative $I_{sc}$ in the anterior intestine from fish reared in all salinities, although an attenuated response was observed in the epithelium from freshwater acclimated fish. Apical [Na$^+$] and [Cl$^-$] reduction both caused the increase of $R_t$ across the intestinal epithelia. The results of basal ouabain application indicated that the basal NKA inhibition only reduced the active Cl$^-$ secretion after the $I_{sc}$ reaching new steady state following apical Na$^+$ or Cl$^-$ substitution.
CHAPTER FIVE - CONCLUSION

Osmoregulation in fish is maintained by the active transport of Na\(^+\) and Cl\(^-\) across epithelia facilitated by membrane bound ion transport proteins such as Na\(^+\)/K\(^+\) ATPase (NKA), Na\(^+\)/K\(^+\)/2Cl\(^-\) cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR). These three proteins have been found in gills, intestine, and kidney of various teleost species, and their cellular distribution and activity directly related to active ion transport. Basolateral localized NKA provides the electrochemical gradient across the plasma membrane. Apical localized NKCC (NKCC2) and basolateral localized CFTR facilitate Cl\(^-\) absorption; while basolateral localized NKCC (NKCC1) and apical localized CFTR may mediate Cl\(^-\) secretion in the intestine (Evans and Claiborne, 2006).

The euryhaline Gulf killifish, *Fundulus grandis*, was used as a model species to investigate the cellular distributions and protein expression of NKA, NKCC, and CFTR proteins during osmotic challenge at late embryonic and early larval stages. The cellular distribution, and mRNA and protein expression of these ion transport proteins were also assessed in the intestines of adult *F. grandis* acclimated to salinities ranging from FW to SW. Most studies have focused on the effects of salinity on the regulation of these proteins in the gills of adult fish or on the integument of embryos or larval fish. The purpose of this dissertation was to provide an integrative view of the ontogeny of major osmoregulatory tissues during development, and ion secreting/absorbing functions of the intestine in the adults during osmotic challenges.

This dissertation sought to answer several questions related to the influence of hypoosmotic and hyperosmotic challenges on the intestine of adult *F. grandis*, as well as the development of osmoregulatory tissues of *F. grandis* during the late embryonic and early larval stages. More specifically, we asked:
a) What is the influence of salinity on the emergence of osmoregulatory organs and on the expression of NKA, NKCC, and CFTR proteins in developing *F. grandis*? This question was addressed by exposing embryos or larvae to salinities ranging from FW to SW, then examining fish over time for differences in the gross morphology of osmoregulatory tissues. We then examined osmoregulatory tissues for their cellular distribution and total whole body abundance of ion transport proteins during osmotic challenges.

b) What is the influence of acute hypoosmotic and hyperosmotic challenges on the osmoregulatory physiology of the anterior and posterior intestine of adult *F. grandis*? This question was addressed by acutely transferring adult *F. grandis* to salinities ranging from FW to SW, then assessing the cellular distribution, and mRNA and protein levels of NKA, NKCC, and CFTR in the anterior and posterior intestine.

c) What is the effect of osmotic challenges on the mechanism of active ion transport in the anterior intestine? This question was addressed by acclimating adult *F. grandis* to salinities ranging from FW to SW, then assessing the magnitude active ion transport and transepithelial resistance of isolated anterior intestine mounted on Ussing Chambers using the short circuit technique. Pharmacological inhibitors and reduced ion solutions were also used in conjunction with the $I_{sc}$ technique to investigate the relative contribution of NKA, NKCC, and CFTR in active ion transport in the intestine.

5.1. The effects of different rearing salinities on the ontogeny of the osmoregulatory tissues during late embryonic and early larval stages

This study utilized a unique methodological approach to investigate the effects of salinity on embryonic and larval *F. grandis* development. Embryo exposures were conducted by exposing embryos shortly after fertilization to salinities ranging from FW to SW, then assessing biological effects at stage 30 of development (Armstrong and Child, 1965). In contrast, fish
from the same clutch were reared as embryos in air until fully developed, then hatched simultaneously to salinities ranging from FW to SW. This method allowed us to commence larval exposures to these various salinities almost simultaneously. In both cases, no noticeable differences on gross morphology were observed in embryos or larvae regardless of rearing salinity. All embryos at stage 30 had a gill primordium containing no filaments nor lamellae, a rudimentary digestive tract consisting of a simple tube, and pronephric tubules. Similarly, few overt morphological differences existed between larvae exposed from hatch to at least 6 wph to salinities ranging from FW to SW. Larval *F. grandis* had developed, what appeared to be, fully functional gills by 4 wph, and the gastrointestinal tract progressively became elongated and developed increased mucosal folding. Pronephric tubules and skin showed no discernible morphological differences during development. These observations suggested an increased involvement of these tissues in larval osmoregulation (Fig. 2.1-2.4).

Embyros and larvae exposed to salinities ranging from FW to SW were also sampled for assessment of the cellular localization and whole body protein abundance of NKA, NKCC, and CFTR. Differences in the cellular localization of CFTR and NKCC in gill and intestine were observed during development. The integument, head kidney, and digestive tubes were presumed to be important osmoregulatory organs in late embryogenesis based on their strong tissue NKA protein expression. In comparison, pharyngeal arches were believed not to be active sites of osmoregulation in embryos based on the negligible expression of NKA proteins (Fig. 2.6). The localization of NKCC to the basolateral membrane and CFTR to the apical membrane suggested a possible role of larval gill in Cl⁻ secretion by 4 weeks post hatch (wph) when acclimated in 5 and 32 ppt water, but not when reared in 0.1 ppt water (Fig. 2.10). This pattern of NKCC and CFTR protein localization was not observed in larvae at earlier stages of development. In the
anterior intestine, the eventual localization of NKCC to the apical membrane and CFTR to the basolateral membrane supported a possible role of the tissue in Cl\(^-\) absorption; one that was observed in all stages of larval development regardless of acclimation salinity. In the caudal portion of the intestine of larvae at 1 wph, the apical localization of CFTR suggested a putative role in Cl\(^-\) or HCO\(_3^\)\(^-\) secretion at the gut of fish acclimated to 0.1 ppt water, but not when fish were acclimated to 5 and 32 ppt water. After 4 wph, the caudal intestine from 5 and 32 ppt acclimated larvae also developed a Cl\(^-\) or HCO\(_3^\)\(^-\) secretion function indicated by the strong expression of CFTR on the apical membrane; an expression pattern that supported several possible diverse functions in ion transport. Furthermore, this study suggested that rearing salinity could affect the osmoregulatory tissue function during larval stages, but that immunohistochemistry and not gross morphology was required to assess these physiological differences.

5.2. Possible functions of intestine in the adult *F. grandis* during osmotic challenge

Prior studies suggested that there was no mechanism of active Cl\(^-\) uptake in the gills during freshwater acclimation in *Fundulus* species (Patrick *et al.*, 1997; Patrick and Wood, 1999). However, during continued exposure to FW, plasma Cl\(^-\) concentration would eventually recover back to a pretransfer level, or stabilize at a new steady state (Scott *et al.*, 2006); this despite the absence of active branchial Cl\(^-\) uptake. Studies have suggested that reduction of paracellular Cl\(^-\) loss is an important compensatory response in *Fundulus* species to surviving an extreme hypoosmotic challenge. However, regulation of Cl\(^-\) ion loss is unable to entirely explain the recovery of plasma Cl\(^-\) homeostasis following freshwater exposure. Based on these findings, *Fundulus* species must depend on other tissues, such as the intestine, as a site for active Cl\(^-\) uptake to maintain plasma chemistry during prolonged hypoosmotic challenge. The
present study studied the expression of NKCC and CFTR, two proteins often implicated in Cl\(^-\) transport in epithelia. In 0.1 ppt acclimated fish, the apical CFTR and basolateral NKCC in the anterior intestine support a role of Cl\(^-\) or HCO\(_3\)\(^-\) secretion. Meanwhile, at 5 and 32 ppt, the anterior intestine had a basolateral CFTR and apical NKCC expression pattern, which collectively supported a function in Cl\(^-\) absorption (Fig. 3.3, 3.7, 5.1). In contrast, CFTR and NKCC were expressed on the apical and basolateral membranes of the posterior intestine, respectively, regardless of the acclimation salinity. These data also support a role of the posterior intestine in Cl\(^-\) or HCO\(_3\)\(^-\) secretion (Fig. 3.5, 3.9, 5.1).

The regulation of NKA, NKCC, and CFTR in isolated anterior intestine during osmotic challenges was then assessed using \(I_{sc}\) technique of intestine mounted on Ussing Chambers. Mucosal application of the CFTR inhibitor, CFTR-inh172, had a greater inhibitory effect on active ion transport in the anterior intestine of 0.1 ppt acclimated fish than in 5 ppt acclimated fish. In contrast, basal inhibition of NKA by ouabain decreased the active ion transport to a similar magnitude in the anterior intestine of fish reared at all salinities. Apical inhibition of NKCC had a similar effect as that elicited by basal NKA inhibition. No significant difference was observed in the transmembrane resistance in the anterior intestine of fish acclimated in different salinities (Fig. 4.1). Interestingly, reduction of mucosal Cl\(^-\) had a limited effect on active ion transport in the anterior intestine of fish acclimated to 0.1 ppt water (Fig. 4.2). After the establishment of the new steady state \(I_{sc}\) following apical application of Na\(^+\) reduced Cortland’s saline, application of serosal ouabain significantly decreased the active ion transport across the anterior intestine (Fig. 4.4) indicating that basal NKA inhibition was most effective in reducing active Cl\(^-\) secretion.
A.

Intestine of seawater acclimated *F. grandis*

![Diagram of ion transport in seawater acclimated intestine.]

B.

Intestine of freshwater acclimated *F. grandis*

![Diagram of ion transport in freshwater acclimated intestine.]

Figure 5.1. Putative ion transport of the intestine of seawater and freshwater acclimated *F. grandis*. The anterior intestine of seawater acclimated fish may function in Cl⁻ absorption via an apical NKCC and basolateral CFTR (Grosell, 2011). The posterior intestine of marine fish may function in apical CaCO₃ and MgCO₃ precipitation and water absorption (Takei and Yuge, 2007). The whole intestine during freshwater acclimation may have a Cl⁻ or HCO₃⁻ secretory function facilitated by a basal NKCC and apical CFTR, together with an apical Cl⁻/HCO₃⁻ exchanger. Note however that CFTR was also weakly expressed on the basolateral membrane of the anterior intestine.

5.3. The influence of osmotic challenges on NKA, NKCC, and CFTR mRNA and protein expression in osmoregulatory tissues

The lack of influence of salinity on NKA activity on active transport, as assessed by *I*ₘₑₚ, were in stark contrast to the influence of salinity on NKA mRNA and protein abundance. In adult *F. grandis*, NKA protein level was decreased in the anterior intestine, but increased in the posterior intestine during hypoosmotic challenge. In contrast, the NKA protein level was
decreased temporarily in both portions of the intestine due to hyperosmotic challenge. NKA mRNA level in both the anterior and posterior intestine were increased by hyperosmotic exposure. Apical inhibition of CFTR showed greater effects on the active ion transport in the anterior intestine during hypoosmotic challenge. In contrast, CFTR mRNA and protein levels were relatively consistent in the anterior intestine during hypoosmotic challenge, confirming that the CFTR were localized mainly on the apical membrane during freshwater acclimation, and on the basolateral membrane of the adult *F. grandis* anterior intestine. During hyperosmotic challenge CFTR mRNA abundance was increased in the anterior intestine but decreased in the posterior intestine. A temporary increase in NKCC mRNA level was observed in both the anterior and posterior intestine during hypoosmotic challenge. A decrease in NKCC protein abundance was observed during both hypo- and hyperosmotic challenges. No significant difference in NKCC protein level was observed during hyperosmotic challenge. NKCC1 mRNA level was only temporarily increased during hyperosmotic challenge. The differential regulation of mRNA and protein for NKA, NKCC and CFTR suggest that a post-translational regulatory mechanism may be influenced in a salinity-dependent fashion. There is also the possibility that the NKCC1 antibody used here also detected NKCC2 in protein assays, whereas the NKCC1 primers used for mRNA detection were isoform specific.

In larval *F. grandis*, the whole body protein levels of NKA showed significantly higher expression during acclimation to 0.1 ppt water compared to 32 ppt water exposure until 4 and 6 wph. Whole body NKCC protein level was significantly higher during 0.1 ppt water acclimation compared to that during 32 ppt acclimation at 4 wph. Whole body NKCC protein level was significantly higher during 0.1 and 32 ppt water acclimation compared to that during 5 ppt water acclimation at 6 wph. Whole body CFTR protein level was significantly higher in 32 ppt
acclimated fish compared to that in 0.1 and 5 ppt acclimated fish at 3 dph and 1 wph. However, whole body CFTR protein level was significantly higher in 0.1 ppt acclimated fish compared to that in 32 ppt acclimated fish at 1 wph. These results suggest that osmotic regulatory tissues may express these ion transport proteins differently during development, or that the emergence of osmoregulatory tissues occurs at different rates.

5.4. Summary

In conclusion, this dissertation provides a better understanding of the ontogeny of osmoregulatory organs during embryonic and early larval stages, and the possible functions of intestine in ion and acid/base regulation during osmotic challenges in adult fish. We describe large differences of salinity on the cellular distribution, and mRNA and protein abundance of NKCC and CFTR, and propose that CFTR may play important roles in fish osmoregulation. However, there is also evidence that post-translational regulation of these transport proteins is likely critical in the fish intestine, and should be the basis of future studies. Furthermore, future studies should also study the functions of other ion transport proteins that inhibit or activate NKA, NKCC, or CFTR, such as apical Cl⁻/HCO₃⁻ exchanger and Na⁺/H⁺ exchanger to gain a more integrative assessment of osmoregulation in the fish intestine of Fundulus species.
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Figure A1. Immunolocalization of NKA, NKCC and CFTR at 6 hr (A1-D1), 3 d (B2-D2), and 7 d (A3-D3) in gills of *F. grandis* in 0.1 ppt (A1-A3), 1 ppt (B1-B3), 5 ppt (C1-C3), and 32 ppt (D1-D3) water. F = filament, L = lamella. Scale bars indicate 10 μm.
Figure A2. Immunolocalization of NKA, NKCC and CFTR at 6 hr (A1-D1), 3 d (B2-D2), and 7 d (A3-D3) in kidney of *F. grandis* in 0.1 ppt (A1-A3), 1 ppt (B1-B3), 5 ppt (C1-C3), and 32 ppt (D1-D3) water. CD = collecting duct, DT = distal tubule, G = glomeruli PT = proximal tubule. Scale bars indicate 10 μm.
Figure A3. Relative mRNA levels of NKA (A, D), NKCC (B, E) and CFTR (C, F) in the gills (A, C, E) and the kidney (B, D, F) of *F. grandis* in 0.1, 1, 5, and 32 ppt after 6 hours, 3 and 7 days. Values are means ± SE (n = 5~7 for each mean value). Treatments not sharing capital letters are statistically different in relative protein or mRNA level compared across different salinity treatments at any given time point, whereas those not sharing lower case letters represent significant differences at different time points for any given salinity.
Figure A4. Relative protein levels of NKA (A, D), NKCC (B, E) and CFTR (C, F) in the gills (A, C, E) and the kidney (B, D, F) of *F.grandis* in 0.1, 1, 5, and 32 ppt after 6 hours, 3 and 7 days. Values are means ± SE (n = 5~7 for each mean value). Relative protein levels are the ratio of intensity for each treatment and the mean intensity of the 5 ppt (6 hours) controls. Treatments not sharing capital letters are statistically different in relative protein or mRNA level compared across different salinity treatments at any given time point, whereas those not sharing lower case letters represent significant differences at different time points for any given salinity.
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