Osmoregulation and acid-base tolerance in fish

Shujun Zhang
Louisiana State University and Agricultural and Mechanical College, szhang7@tigers.lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_dissertations

Recommended Citation
https://digitalcommons.lsu.edu/gradschool_dissertations/3362
OSMOREGULATION AND ACID-BASE TOLERANCE IN FISH

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biological Sciences

by
Shujun Zhang
B.S., Huazhong University of Science & Technology, 2003
M.S., Huazhong University of Science & Technology, 2005
August 2012
ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Fernando Galvez, for his devotion of time on mentoring my research, financial support, and his guidance on the way to enjoy research. I appreciate his sincere and warm-hearted help which makes my life much happier.

I would like to thank my committee members, Dr. Andrew Whitehead, Dr. Steve C. Hand, Dr. Evanna Gleason, Dr. Gentry Glen T for their approval of my research project, sharing their laboratory facilities, their advice, and their stimulating discussion in my research.

I would like to thank Dr. Charlotte M Bodinier, Benjamin Dubansky, Ying Guan, Yanling Meng, Christine Savolainen, Charles Brown, and my friends in the Department of Biological Sciences. Their friendship and help in the experiments made my time in the lab much easier.

My special thanks to my brother and sister, Zheng and Jing, for their never-ending support, no matter if I am in high or low.

Lastly, my sincere thanks go to my parents, Dong and Feng, for their love and devotion during my study; without their support I would not have finished my Ph.D. Research.
TABLE OF CONTENTS

ACKNOWLEDGMENTS..............................................................................................................II

LIST OF TABLES..........................................................................................................................V

LIST OF FIGURES..........................................................................................................................VI

ABSTRACT.......................................................................................................................................VIII

CHAPTER 1 LITERATURE REVIEW

General Principles of Osmoregulation in Teleost Fish.........................1
Ion Transport Models in the Gills of Freshwater (FW)-Acclimated Fish.................................................................................3
Ion Transport Models in the Gills of Seawater (SW)-Acclimated Fish..................................................................................10

*Fundulus* as the Premier Model in Environmental Biology and Fish Physiology.......................................................................12

Significance of Paracellular Pathways in Osmoregulation .............15

General Principles of Acid-base Tolerance in Teleost Fish...........18

Brief Outline of Main Points of Each Chapter.................................20

CHAPTER 2 ALTERATIONS IN CLAUDIN EXPRESSIONS IN THE GILLS OF *FUNDULUS GRANDIS* FOLLOWING ABRUPT SALINITY TRANSFER

Introduction.................................................................................................................................23

Materials and Methods..............................................................................................................26

Results........................................................................................................................................38

Discussion.....................................................................................................................................48

CHAPTER 3 ACID-BASE TOLERANCES AMONG *FUNDULUS* SPECIES

Introduction.................................................................................................................................59

Materials and Methods..............................................................................................................62

Results........................................................................................................................................68

Discussion.....................................................................................................................................73
LIST OF TABLES

Table 2.1 Ion concentration (µM) in the waters used in the experiments……28

Table 2.2 Sequences of primers used for quantitative PCR……………………37
LIST OF FIGURES

Figure 1.1: Traditional model for NaCl uptake by FW-adapted teleost fish gills

Figure 1.2: Traditional model for NaCl secretion in SW-acclimated fish gills

Figure 1.3: A typical model showing the structure of tight junction proteins in paracellular pathways between two adjacent cells

Figure 2.1: Plasma sodium (A) (N=6) and chloride (B) (N=6) levels in Fundulus grandis

Figure 2.2: Total (net) flux and unidirectional fluxes of Na\(^+\) (A) and Cl\(^-\) (B) in Fundulus grandis after transfer from 5 ppt water to 0.1 ppt water (N=6) versus time

Figure 2.3: Gill SEM images of Fundulus grandis

Figure 2.4: Total gill permeability rate of PEG-4000 in Fundulus grandis

Figure 2.5: Junctions between MRCs and adjacent cells in Fundulus grandis 3 days after the transfer from 5 ppt water to 5, 2, 1, 0.5, 0.1 ppt water

Figure 2.6: The effects of hypoosmotic exposure (0.1 ppt and 0.5 ppt exposure) on the abundance of Cldn3, Cldn5, Cldn23, Cldn28, Cldn7 and Cldn26 in gills of Fundulus grandis

Figure 2.7: The effects of hypoosmotic exposure (0.1 ppt exposure) on the abundance of Cldn3, Cldn5, Cldn23, Cldn28, Cldn7 and Cldn26 in two different epithelial cells: PVCs and MRCs

Figure 3.1: Na\(^+\) influx, efflux and net flux rates in Fundulus heteroclitus MDPP at 0 h, 12 h, 24 h, 3 day and 7 day point during a 7-day acid challenge period

Figure 3.2: Na\(^+\) influx, efflux and net flux rates in Fundulus heteroclitus VAcoast at 0 h, 12 h, 24 h, 3 day and 7 day point during a 7-day acid challenge period
Figure 3.3: Na\textsuperscript{+} influx, efflux and net flux rates in *Fundulus majalis* after HCl injection
Figure 3.4: Claudin 3, -5, -23, -28 mRNA expression level changes in *Fundulus heteroclitus* MDPP gills during a 7-day acid challenge period
Figure 3.5: Claudin 3, -5, -23, -28 mRNA expression level changes in *Fundulus heteroclitus* VAcoast gills during a 7-day acid challenge period
ABSTRACT

The gill, with a large surface area in intimate contact with the environment, is the primary organ of ion and acid-base regulation in fish. These same characteristics make the gill epithelium particularly susceptible to a number of environmental perturbations, including osmotic challenges and metabolic acidosis. For most freshwater fish, the active uptake of the strong ions, sodium and chloride, are intimately linked with the excretion of acid and base equivalents, respectively. However, fish from the genus Fundulus, are unique in their apparent lack of active chloride uptake at the gills. This unique feature makes limiting Cl\(^{-}\) loss through paracellular pathways the only practical strategy for these species in tolerating acute exposure to hypoosmotic conditions especially when dietary chloride is limited. We find that Fundulus grandis can dynamically regulate their paracellular pathway in the gill epithelium at salinities approaching fresh water. We observe the significant up-regulation in the mRNA levels of several claudins in the gill and that some of these claudins exhibit expressional discrepancies between mitochondrion-rich and pavement cells in fish gills, which shows correlations with changes in gill morphology and ion flux rates in fish. Collectively, our data suggest that claudins may play important roles in regulating ion flux across paracellular pathways of Fundulus during osmotic challenges.

The linkage between osmoregulation and acid-base tolerance has long been studied in teleost fish. Failure to identify an active Cl\(^{-}\) uptake system in freshwater Fundulus indicates the uniqueness of these species in osmoregulation and acid-base tolerance. We find that Fundulus heteroclitus
increases Na\(^+\) uptake within the first few hours of metabolic acidosis. We also find that metabolic acidosis induces the changes of mRNA levels of several gill claudin proteins, which may play roles in regulating the permeability of the paracellular pathway to strong ions. Our data show *Fundulus heteroclitus* is capable to cope with great metabolic acidosis within their bodies, which may contribute to their adaptation to internal and external perturbations.
CHAPTER 1: LITERATURE REVIEW

General Principles of Osmoregulation in Teleost Fish

Fish are the largest and most diverse group of vertebrates, with an estimated 20,000 species inhabiting varied aquatic environments worldwide (Vernier, 1989). The majority of fish are stenohaline based on their ability to tolerate only relatively narrow salinity fluctuations. In contrast, a comparably smaller proportion of fish species are referred to as euryhaline, due to their capacity to tolerate large extremes in environmental salinity (Krogh, 1939). Even among euryhaline fishes, there is large diversity amongst fish in their capacity to tolerate salinity extremes (Hwang and Lee, 2007). Some euryhaline species, such as salmonids, are capable of making these salinity transitions only over extended periods of time. In the case of salmonids, they begin their lives in fresh water (FW), move from FW to sea water (SW) after smoltification, and then move back to FW often after a few years (McCormick et al., 1991). Other species, which are typically found in intertidal regions, tolerate more frequent salinity transfers. A classic example includes fish from the genus *Fundulus* from the family *Fundulidae* (Burnett et al., 2007a).

The physiology of euryhaline fish changes dramatically depending on whether the fish is located in FW, which is hypoosmotic to the extracellular fluids of the animal, or in SW, which is hyperosmotic to its internal environment (Evans and Claiborne, 2006; Evans et al., 1999; Hoffmann, 1992). In the former case, fish need to absorb ions actively from the external environment to compensate for the passive loss of ions to FW. In the latter case, animals actively excrete ions to offset the passive loading of ions from SW. In contrast to stenohaline fishes,
many euryhaline species are able to tolerate dramatic shifts in environmental salinity by making physiological adjustments over the course of hours to days to facilitate restoration of ion homeostasis (Evans et al., 2005).

Many studies have focused on euryhaline fishes in order to elucidate the osmoregulatory mechanisms that allow them to cope with fluctuations in environmental salinity (Evans, 2006; Evans, 2008; Evans and Claiborne, 2006; Hwang and Lee, 2007; Wood and Marshall, 1994). It is generally accepted that the gill epithelium is the primary osmoregulatory organ in fish, with the gastrointestinal tract and kidneys also playing important supporting roles, which depend on environmental salinity (Evans et al., 2005). FW-acclimated fish actively absorb ions from the external environment at their gills and minimize ion loss by reducing the paracellular movement of ions at their body surfaces, while actively absorbing ions at their kidney to minimize urinary ion loss. SW-acclimated fish drink SW, from which they actively absorb ions to facilitate water absorption in the intestine. This salt load is subsequently secreted actively at the gills, utilizing a process of transcellular chloride transport and paracellular sodium efflux (Evans, 2006; Evans, 2008; Evans and Claiborne, 2006). Regardless of environmental salinity, the gills are extremely important for osmotic regulation in euryhaline fish (Burnett et al., 2007a; Evans, 2008).

The fish gills are composed of a variety of different cell types, although the mitochondrion-rich (MR) cells and the pavement (PV) cells are the ones most often implicated in osmoregulatory functions. In particular, the MR cells are critical in the active extrusion of ions in SW-acclimated animals, and are
generally associated with active ion uptake in FW fish (Chang et al., 2001; Evans et al., 2005), although their phenotypes vary extensively with salinity. In FW, MR cells have large surface areas with small villi protruding to external environments. In contrast, in SW, MR cells hide underneath the gill epithelial surface in connection to external environments through a small crypt (Wilson and Laurent, 2002). FW- and SW-acclimated fishes possess a wide variety of ion transport proteins, which mediate the active and passive movement of ions. MR cells in both FW- and SW-acclimated fish express many of the same ion transporters, although these proteins differ in their cellular localization (i.e., apical versus basolateral membrane expression), and in their regulation (Hwang and Lee, 2007).

**Ion Transport Models in the Gills of Freshwater (FW)-Acclimated Fish**

Ever since the late 1930’s, there has been general agreement that the unidirectional absorptions of Na\(^+\) and Cl\(^-\) in the freshwater fish gill are independent of one another and linked to the extrusion of proton and bicarbonate, respectively (Krogh, 1938, 1939; Parry et al., 1959). However, there is still considerable debate on the cellular localization and molecular underpinnings of these transport processes (Hwang and Lee, 2007). Although an exhaustive review of these transport models is beyond the scope of my dissertation, I would like to highlight some of the major details of the most widely accepted models (Fig 1.1), and describe the current understanding associated with the role of these transports in the fish gill of FW-acclimated *Fundulus* genus, which is the species of focus for my dissertation.
Ion transport in the gills of most FW teleost exhibit the inextricable link of Na\(^+\) uptake and H\(^+\) excretion either through a Na\(^+\)/H\(^+\) exchanger (NHE) or through a putative apical Na\(^+\) channel, electrochemically linked to a V-type H\(^+\)-ATPase on the fish gill (Edwards and Toop, 2002; Hwang and Lee, 2007; Inokuchi et al., 2008). NHE transports Na\(^+\) from the water into the cell in exchange for proton (or NH\(_4^+\)). Other studies have suggested that un-ionized NH\(_3\) combines with proton to produce NH\(_4^+\), which is trapped after leaving the cell, thus preventing the back flux of H\(^+\) (Hwang and Lee, 2007; Perry et al., 2003a; Perry et al., 2003b; Tresguerres et al., 2005). Either way, this Na\(^+\) and proton exchange (either H\(^+\) directly or H\(^+\) that is chemically combined with NH\(_3\)) is electro-neutral, relying solely on the prevailing electrochemical gradients for both Na\(^+\) entry and proton excretion. At first glance, these mechanisms of exchange
appear problematic. The low concentrations of Na\textsuperscript{+} (e.g., low µM Na\textsuperscript{+} concentrations of some fresh waters) would in fact represent a negative driving force for Na\textsuperscript{+} entry (George et al., 2006b). Furthermore, the intracellular proton concentrations of fish gill cells (pH 7.4) would be insufficient to stimulate Na\textsuperscript{+} uptake, especially under scenarios of environmental acidification (Evans, 2008; Goss and Wood, 1990a).

An alternate model of transepithelial Na\textsuperscript{+} uptake in the FW fish gill was proposed involves the indirect coupling of Na\textsuperscript{+} and H\textsuperscript{+} exchange at the apical membrane (Avella and Bornancin, 1989). This mechanism, which was found to exist in the turtle bladder and skin of FW-acclimated frogs (Duranti et al., 1986), had Na\textsuperscript{+} permeating the apical membrane via an epithelial Na\textsuperscript{+} channel (ENaC) (Evans, 2008) energized by an apical V-type H\textsuperscript{+}-ATPase. The active extrusion of H\textsuperscript{+} via a V-type H\textsuperscript{+}-ATPase would create a localized negative charge along the apical membrane fueling passive Na\textsuperscript{+} entry against a concentration gradient. Intracellular Na\textsuperscript{+} would then exit the cytoplasm into plasma via Na\textsuperscript{+}/K\textsuperscript{+} ATPase (NKA). This mechanism appeared to alleviate concerns raised for the NHE model, which related to the absence of physiologically-relevant electrochemical gradients. Several physiological, radio-isotopic, pharmacological, immunocytochemical, cellular and molecular techniques have provided compelling evidence of the existence of a V-type H\textsuperscript{+}-ATPase in the fish gill (Evans et al., 2005; Hwang and Lee, 2007). For example, bafilomycin, a specific V-type H\textsuperscript{+}-ATPase inhibitor inhibits Na\textsuperscript{+} uptake by up to 90% in tilapia and zebrafish (Biosen et al., 2003; Fenwick et al., 1999). Immunocytochemical
studies using heterologous antibodies raised against various subunits of V-type H\(^+\)-ATPase have localized the protein to the gills of rainbow trout, zebrafish, shark, and tilapia (Biosa et al., 2003; Sullivan et al., 1995; Wilson et al., 2000; Wilson et al., 2002). Vanadate, an inhibitor of the P-type H\(^+\)-ATPase inhibits proton secretion by approximately 50% in rainbow trout, which further indicates the existence of a V-type H\(^+\)-ATPase (Lin and Randall, 1991). Despite all the evidence supporting this model, the existence of ENaC is still in question based on the inability to identify a genomic sequence for ENaC in any of the fully-sequenced genomes of teleost fish. In fact, in *Fundulus heteroclitus*, V-type H\(^+\)-ATPase localizes to the basolateral membrane of the fish gill, where it is thought to play a small role in Cl\(^-\) absorption in FW (Fumi et al., 2002). In this case, carbonic anhydrase breaks down CO\(_2\) into H\(^+\) and HCO\(_3^-\), and H\(^+\) is pumped into plasma facilitating HCO\(_3^-\) exit from cytoplasm (Fumi et al., 2002).

While molecular techniques have put into question the universal adherence of the fish gill to this ENaC/ V-type H\(^+\)-ATPase model, resurgence in the putative role of a NHE in transepithelial Na\(^+\) transport has once again emerged (Hwang and Lee, 2007). One study identified NHE2 mRNA in the gill of longhorn sculpin and killifish, and another study localized NHE3 to MR cells in rainbow trout and blue-throated wrasse (Edwards and Toop, 2002). Amiloride, an inhibitor to NHE, blocks Na\(^+\) accumulation by over 90% in zebrafish embryos (Esaki et al., 2007). Though there is evidence for the existence of NHE, the underlying driving force still remains unclear. Some propose that carbonate anhydrase (CA) plays a key role in this process by converting metabolic CO\(_2\) into HCO\(_3^-\) and H\(^+\). Some
propose that carbonate anhydrase (CA) plays a key role in this process by converting metabolic CO$_2$ into HCO$_3^-$ and H$^+$ which may be secreted to external environments by NHE. A recent study found that the CA inhibitor, ethoxzolamide, reduced Na$^+$ uptake in zebrafish (Biosen et al., 2003). However, one study found that CA is only expressed in non-MR cells in dogfish gill (though more studies confirmed that CA localized to the apical side of both MR cells and pavement cells), which represents a cellular distribution inconsistent with the proposed model (Gilmour et al., 2006).

One of widely accepted models of unidirectional Cl$^-$ uptake for FW fish involves the absorption of Cl$^-$ in exchange for HCO$_3^-$ via an apical anion exchanger (AE) (Evans, 2010; Hwang and Lee, 2007; Shmukler et al., 2005; Tang and Lee, 2007). A Cl$^-$/HCO$_3^-$ exchanger has been immunolocalized to the apical surface of branchial cells in tilapia, although the cellular distribution remains somewhat uncertain. In one study, an oligonucleotide probe (complementary to rat AE cDNA) hybridized to RNA in both the filament and lamellae of the rainbow trout, suggesting that both pavement cells and MR cells contain mRNA transcripts for Cl$^-$/HCO$_3^-$ exchanger (Gary et al., 1996). However, tilapia anion exchanger 1 (AE1) and the basolateral NKA have been co-localized to the gills of pufferfish of FW fish (Wilson et al., 2000; Wilson et al., 2002). As stated previously, carbonic anhydrase (CA) has been identified in fish branchial cells, and is also presumed to be the driving force for Cl$^-$ uptake, in that it provides HCO$_3^-$ from metabolic CO$_2$. One study found that low waterborne Cl$^-$ stimulates Cl$^-$ uptake and enhances CA protein expression in the gills of tilapia,
suggesting a correlation between CA expression and unidirectional Cl\(^-\) uptake (Chang and Hwang, 2004).

Although Cl\(^-/HCO_3^-\) exchanger is considered the main mechanism of Cl\(^-\) uptake in FW-adapted fish, Na\(^+/K^+/2\)Cl\(^-\) co-transport (NKCC) and Na\(^+/Cl^+\) co-transport (NCC) are also considered as candidates for the apical Cl\(^-\) uptake (Evans et al., 2005; Hwang and Lee, 2007). In a study of tilapia embryonic skin and adult gills, a heterologous antibody revealed the presence of apical NKCC in FW-type MR cells, and apical NKCC was suggested to be associated with Na\(^+/Cl^+\) uptake (Hiroi et al., 2005). It is worthy to mention that an active Cl\(^-\) uptake system has not been identified in killifish, which is a departure from general fish model (Burnett et al., 2007b; George et al., 2006b; Patrick et al., 1997; Wood and Marshall, 1994).

NKA is the primary driving force for transepithelial Na\(^+\) transport in fish gill, but is also important in energizing indirectly the active uptake of other ions (Hwang and Lee, 2007; Perry et al., 2003b). Some studies have also suggested that a Na\(^+/HCO_3^-\) cotransporter (NBC) in the basolateral membrane is involved in this step too. One study found expression of a NBC in branchial MR cells in *Osorezan dace* (Hirata et al., 2003). In another study, an isoform of the NBC was identified in the gill tissue of rainbow trout (NCBI GenBank accession ID: AF434166, NBC protein sequence in rainbow trout) (Perry et al., 2003b). NBC could potentially mediate both the Na\(^+\) and Cl\(^-\) transport across the basolateral membrane of the gills, which facilitates Cl\(^-\) enter plasma through basolateral membrane channels. Some studies suggest that specific Cl\(^-\) channels exist (Tang
and Lee, 2007). One study has demonstrated a higher protein expression of CLC-3 Cl⁻ channels in the gills of freshwater puffer fish than in seawater fish (Tang and Lee, 2007). However, though CLC-3 Cl⁻ channels have been cloned and expressed in the gills of tilapia, they have been proposed to function in intracellular Cl⁻ regulation rather than in transepithelial Cl⁻ uptake based on in vitro functional analyses (Hiroaki et al., 2002). Finally, a cystic fibrosis transmembrane regulator (CFTR) has also been proposed to be another candidate for basolateral Cl⁻ transport. Marshall et al localized the CFTR channel in both pavement cells and MR cells in Fundulus heteroclitus, suggesting that CFTR might be involved in Cl⁻ exit into plasma (Marshall et al., 2002). Further studies apparently are needed to verify these competing results.

Early reports have suggested that both Na⁺ and Cl⁻ transports are performed in a single mitochondrion-rich ionocyte, although more recent work proposes that at least 2 or more functionally-distinct MR cell types coordinate these active ion transports (Evans et al., 2005; Galvez et al., 2001; Goss et al., 2001; Hwang and Lee, 2007; Katoh et al., 2001; Scott et al., 2004b). A point needed to mention is that a single model does not apply to all the teleost (Evans, 2008; Marshall and Grosell, 2005). Until now there is still no characterization of a chloride uptake pathway in apical side of gills of Fundulus species (Patrick et al., 1997; Patrick and Wood, 1999). Some studies indicated that Fundulus species maintain plasma chloride by decreasing passive chloride loss from their bodies (Patrick et al., 1997). This is a departure from the general proposed model. One study suggests that Fundulus species maintained Cl⁻
concentration by decreasing the passive loss of the ion by regulating paracellular tight junctions (Scott et al., 2004b). This is a new strategy of maintaining Cl⁻ homeostasis in plasma. This dissertation will address special emphasis on important roles of paracellular pathways regulating ion transport in osmoregulation among Fundulus species.

**Ion Transport Models in the Gills of Seawater (SW)-Acclimated Fish**

![Ion Transport Models in the Gills of Seawater (SW)-Acclimated Fish](image)

Figure 1.2: Traditional model for the mechanism of salt secretion in branchial epithelia of SW-acclimated fish. The distribution of transport proteins in specific cells may vary with fish species. MRC represents mitochondrion-rich cells, CFTR represents cystic fibrosis transmembrane conductance regulators in this figure. (Figure modified from (Evans, 2010)).

SW-acclimated fish actively secrete ions at the gills to counteract the accumulation of ions from ingested SW in the intestine (Evans et al., 2005). The gills of SW-acclimated fish express NKA and Na⁺/K⁺/2Cl⁻ co-transporter (NKCC) on the basolateral membrane, and the cystic fibrosis transmembrane regulator Cl⁻ channel (CFTR) on the apical membrane (Figure 1.2). NKA serves as the driving force for osmoregulation by pumping Na⁺ out of branchial cells and
absorbing K⁺ into cells. This maintains a low intracellular Na⁺ concentration and indirectly leads to the establishment of a negative intracellular membrane potential, which facilitate Na⁺ and Cl⁻ entry via NKCC. Although intracellular Na⁺ is recycled back through NKA, the resulting increase in intracellular Cl⁻ and the highly-negative membrane potential facilitate the passive diffusion of Cl⁻ through the apical CFTR. A positive, blood-side transepithelial potential creates the electromotive force for efflux of Na⁺ into SW via a leaky paracellular pathway between MR cells and accessory cells (AC) (Hwang and Lee, 2007). The permeability of this leaky junction is partly controlled by claudins which will be addressed later (Koval, 2006; Van Itallie and Anderson, 2006). NKA is important for salt transport not only because of its role in Na⁺ transport but also because it is the main driving force for the whole salt secretion process. NKCC is considered as the site of Na⁺ and Cl⁻ entry into cytoplasm facilitated by the electrochemical gradient created by NKA in fish osmoregulation. High expression of NKCC is usually correlated with seawater acclimation in fish. One study showed NKCC mRNA levels were increased in Fundulus heteroclitus gills following transfer from brackish water to sea water (Scott et al., 2004a). Another study found that NKCC expression in bass gills changed in response to salinity change (Tipsmark et al., 2004). An immunofluorescence study showed the expression of NKCC in MR cells in Fundulus heteroclitus too (Marshall et al., 2002).

In terms of Cl⁻ secretion, the Cl⁻ CFTR has been implicated in transcellular Cl⁻ secretion in marine teleost since its initial discovery in the gills of Fundulus
heteroclitus (Singer et al., 1998). Time course studies with Fundulus heteroclitus reveal that expression of apical CFTR mRNA in gills appears at 24h after the transfer from FW to 10% SW while CFTR expression in gills decrease as fish are transferred to FW (Scott et al., 2005; Tipsmark et al., 2002). CFTR mRNA levels in gills of Hawaiian goby also increases during SW acclimation (McCormick et al., 2003). Overall, Cl− secretion from the cytoplasm to sea water is facilitated by the inside negative membrane potential of the gill cells.

**Fundulus as the Premier Model in Environmental Biology and Fish Physiology**

*Fundulus* genus consists of approximately 27 species in North America, with about 11 of them distributed along the coasts of the Atlantic Ocean and the Gulf of Mexico, or in inland regions of North America (Burnett et al., 2007a; Griffith, 1974b; Whitehead et al., 2010).

*Fundulus* species (also called killifish) usually reside in brackish estuaries and salt marshes along coastal regions, however, many of these species are renowned for their abilities to tolerate salinity extremes ranging from FW to approximately 3- to 4-fold times the strength of SW (Griffith, 1974b; Loeb and Wasteneys, 1912; Sumner, 1911). Some *Fundulus* species are often trapped during ebb tide in small ponds, which may become more saline due to evaporative water loss, or more dilute due to large rainstorm events (Marshall, 2003; Marshall et al., 2000; Marshall et al., 1999; Patrick et al., 1997). As such, they have served as an excellent model for studying osmoregulatory physiology and stress biology (Burnett et al., 2007b). Although most *Fundulus* species are thought to have regulatory mechanisms to tolerate these dramatic salinity
gradients, other *Fundulus* exclusively live in FW or SW, but not both, and thus, are less tolerant of fluctuations in environmental salinity (Griffith, 1974b; Whitehead, 2010).

Of these *Fundulus* species, *Fundulus heteroclitus* is most renowned for its environmental plasticity, thus widely used to extend the understanding of strategies to cope with environmental challenges such as changes in salinity, oxygen levels, and temperature (Burnett et al., 2007b; Fangue et al., 2006; George et al., 2006a). *Fundulus heteroclitus* does not migrate during its life cycle, with local sub-populations exhibiting summer home ranges on the order of 30-40 m and restricted winter movements (Burnett et al., 2007a; Chidester, 1920). These characteristics of broad distribution and limited habitat range have made *Fundulus heteroclitus* a popular and powerful field model for studying biological and ecological plasticity to natural environmental challenges. In addition, there is evidence of local adaptation to environmental pollutants of *Fundulus heteroclitus* residing in heavily polluted waters. Consequently, this species, as well as other *Fundulus* species, are used in both laboratory and field studies to elucidate some disease processes and toxicological mechanisms, are used in both laboratory and field studies to elucidate some disease processes and toxicological mechanisms as well (Burnett et al., 2007a).

*Fundulus* species have been studied extensively in the past 60 years, revealing many basic principles that are now accepted as fundamental to our knowledge of euryhaline osmoregulation in teleost fish (Hossler et al., 1985; Karnaky, 1980, 1986; Karnaky et al., 1984; Karnaky et al., 1976). Ion transport
across the gills of SW-acclimated *Fundulus* occurs like it does in other marine teleosts. Cystic fibrosis transmembrane conductance Cl⁻ regulator (CFTR), Na⁺/K⁺/2Cl⁻ co-transporter (NKCC), and NKA coordinate the transcellular secretion of Cl⁻ in MR cells, setting up for the concomitant movement of Na⁺ into water via the leaky-type tight junctions between MR cells and accessory cells. In contrast, the ion transport model for FW-acclimated *Fundulus* is a departure from that of most other fish species (Patrick and Wood, 1999). Generally, the active uptake of Cl⁻ in FW teleost is linked with HCO₃⁻ excretion in MR cells, via a presumptive Cl⁻/HCO₃⁻ exchanger. This process however does not apply to FW-acclimated *Fundulus* species, which does not appear to have active transport of Cl⁻ across the gills (Patrick et al., 1997; Patrick and Wood, 1999; Wood and Marshall, 1994). The limited capacity for active Cl⁻ uptake at the gills of freshwater-acclimated *Fundulus* species places greater emphasis on reducing paracellular Cl⁻ loss or obtaining Cl⁻ from dietary sources in order to maintain Cl⁻ balance. It has been proposed that development of a mechanism to minimize paracellular Cl⁻ loss at the gills was an important evolutionary step allowing certain populations of SW-tolerant *Fundulus* to survive in FW (Laurent et al., 2006; Scott et al., 2004b; Wood and Laurent, 2003; Wood et al., 2010; Wood and Grosell, 2008).

Interestingly, intraspecific differences in FW tolerance appear to exist between populations of *Fundulus heteroclitus*. Based on variations in mitochondrial haplotype, nuclear proteins and microsatellites, *Fundulus heteroclitus* can be divided into at least two clades: a northern and a southern
clade (Bernardi et al., 1993; Gonzalez-villasenor and Powers, 1990). A phylogenetic break in *Fundulus heteroclitus* appears at the Hudson River (approx. 40.5° north latitude), in which northern groups are distinct from southern groups at mitochondrial loci (Gonzalez-villasenor and Powers, 1990; Smith et al., 1998). These two populations exhibit variations in several physiological aspects. For example, northern populations of *Fundulus heteroclitus* exhibit higher fertilization success and survival rates in hypoosmotic exposures than do southern populations (Able and Palmer, 1988b; Fangue et al., 2006; Scott and Schulte, 2005). It is likely that molecular or physiological differences in FW tolerance exist between populations of *Fundulus heteroclitus*. A further study found that northern populations of *Fundulus heteroclitus* were better adapted to FW environments and that minimizing Cl⁻ imbalance through regulation of paracellular pathways appeared to be the key physiological differences accounting for their greater FW tolerance (Scott et al., 2004b; Whitehead et al., 2011). In summary, minimizing chloride loss through regulation of paracellular pathways is an important strategy applied by *Fundulus* species in order to cope with hypoosmotic challenges, which is the issue I will address below.

**Significance of Paracellular Pathway in Osmoregulation**

Ion transport across the paracellular pathways is controlled by tight junction proteins (Tang and Goodenough, 2003)(Fig 1.3). The paracellular pathway is regulated by a series of tight junction proteins, including occludins, claudins, and junctional adhesion molecules, that help demarcate the environmentally-exposed apical membrane from the blood-exposed basolateral membrane of transporting epithelia (Chiba et al., 2008; Van Itallie and Anderson, 2004). Amongst the
members of this protein complex, the claudin superfamily of proteins has received much attention recently due to their importance in regulating the permeability and ion selectivity of the paracellular pathway. Claudins, which are part of the tetraspanin superfamily of transmembrane proteins found in all vertebrate epithelia, contain two extracellular loop domains and constitute the permeability barrier to limit diffusion of solutes between cells (Koval, 2006). The pair of hairpin domains from one cell is able to dimerize with the extracellular domains of an adjacent cell forming a continual seal. Recent studies have demonstrated that claudin proteins are the key components regulating the permeability and the ion selectivity of the paracellular pathway (Koval, 2006; Van Itallie and Anderson, 2004, 2006). It is plausible to infer that claudins play key roles in osmoregulation in *Fundulus* species because Cl⁻ concentration in plasma in *Fundulus* is mainly maintained by decreasing passive loss through paracellular pathways as described above especially when food source is limited. Some studies have shown that some claudin proteins tend to form ‘tighter’ tight junction pores, while others are more likely to form ‘leakier’ tight junction pores (Alexandre et al., 2007; Sas et al., 2008). Claudins typically form homopolymers, but can also form heteropolymers in certain situations (Koval, 2006; Van Itallie and Anderson, 2004).

Until now at least 24 claudins have been found in mammals (Morin, 2009). The claudin family seems to have expanded along chordate lineage due to genome duplication in some fish species with 56 claudins identified in *Fugu rubripes* (Loh et al., 2004). Some studies have suggested that the expansion of
claudin family may due to the unique challenges of fish residing in an aquatic environment which force fish deal with more environmental stressors (Bagherie-Lachidan et al., 2009). In fish, preliminary studies have shown that the mRNA and protein levels of certain claudins in transporting epithelia are responsive to changes in environmental salinity (Bagherie-Lachidan et al., 2008, 2009). Although fish and mammals share many claudin orthologs, roles of claudins may differ between fish and mammals. For example, claudin-7 mRNA expression does not respond to salinity challenges in Fundulus grandis (data not shown), while one study showed that overexpression of claudin-7 decreased paracellular Cl⁻ conductance in LLC-PK1 cells (Alexandre et al., 2007). There is only very limited study indicating claudin distribution among different epithelial

Figure 1.3: A typical model showing the structure of tight junction proteins in paracellular pathways between two adjacent cells (Fromm and Schulzke, 2009)
cell types in fish (Tipsmark et al., 2008a; Tipsmark et al., 2008b; Tipsmark et al., 2008c). Considering two major cell types, MR cells and pavement cells (PV), expressed in fish gills, it is reasonable to infer that claudin proteins may vary among the junctions between MR-MR, MR-PV and PV-PV cells (Phuong et al., 2009).

**General Principles of Acid-base Tolerance in Teleost Fish**

In recent years, there has been significant progress in elucidating the mechanisms of ion and acid-base regulation in FW fish. Numerous studies have described a linkage between the exchange of acid-base equivalents and strong ions in FW-adapted teleost fish. The ‘strong ion difference theory’ was first described by Stewart as a tool to access acid-base status independently of the water titration method (Stewart, 1983). Briefly, both Na\(^+\) and Cl\(^-\) are the major strong cation and anion that have appreciable fluxes across the branchial membrane. A disparity between Na\(^+\) and Cl\(^-\) next fluxes usually indicates a charge imbalance which in FW fish has been equated to the net flux of acidic equivalents (ie., \(J_{Na}^{Na} = J_{Cl}^{Na} - J_{Na}^{Na}\)) (Kirschner, 1997; Patrick et al., 1997; Wood et al., 1984). The correlation has been reported between \(J_{H^+}^{Cl}\) which is measured by titration and the difference between the net Na\(^+\) and Cl\(^-\) fluxes (Patrick et al., 1997). A greater rate of Cl\(^-\) loss over Na\(^+\) loss that is measured following HCl injection represents a net H\(^+\) excretion.

The relationship between acid-base regulation and osmoregulation has been demonstrated by various studies among different teleost fish species. In this model, Na\(^+\) and Cl\(^-\) uptake in FW fish was initially suggested to be through electro-neutral Na\(^+\)/H\(^+\) (NH\(_4^+\)) and Cl\(^-\)/HCO\(_3^-\) exchangers, respectively. This
plausible explanation by assuming the existence of two exchangers went unchallenged for decades. It is not until recently that some studies indicated that this assumption of linkage between acid-base tolerance and osmoregulation is not universal and apparently could not be applied to all fish species. (see below) Some investigated this issue in *Fundulus* species in early 1990’s and found that the mechanisms of ion and acid-base regulation in FW-adapted *Fundulus heteroclitus* departed from the standard model for FW-adapted teleosts (Patrick et al., 1997; Patrick and Wood, 1999). One study pointed out clearly in these studies that anion permeability of paracellular pathways would be selectively elevated in the case of HCl-induced acidosis, which facilitates Cl− excretion through paracellular pathways (Patrick et al., 1997). These studies uncover the importance of paracellular pathways in *Fundulus* species during the recovery from metabolic acidosis and that the regulation of paracellular ion efflux rates play important roles in acid-base regulation, which makes measuring Na+/Cl− flux rates good parameters to understand the acid-base regulatory mechanisms (Evans, 2006; Goss et al., 1992; Patrick and Wood, 1999; Wood and Marshall, 1994).

*Fundulus* species are unique amongst most teleost in their response to disturbances in the acid-base status of their extracellular fluids. These differences include: 1) The non-existence of Cl−/HCO3− exchanger, which puts the necessity of alternative pathways for base secretion; and 2). The relative high flux rates of Na+ across branchial epithelia in FW fish,. *Fundulus* species could modulate Na+ flux rates across paracellular pathways as a way to regulate H+
Briefly, Na\(^+\) and Cl\(^-\) are the major strong ions that have considerable flux rates across the branchial membrane. If a disparity between Na\(^+\) and Cl\(^-\) flux rates exists, a charge imbalance which has been equated to the net acidic equivalents in FW fish may arise. This charge imbalance may the compensated by H\(^+\) transport which is a part of acid-base regulation in fish (Patrick et al., 1997; Wood et al., 1984).

Based on some previous studies in *Fundulus heteroclitus*, acid-base regulatory process involves both transcellular and paracellular transport. In addition, paracellular transport seems to play more important roles compared to other fish species considering that a Cl\(^-\)/HCO\(_3^-\) exchanger may not exist in *Fundulus* species. These discrepancies encourage us to further examine ion and acid-base regulation mechanisms and the nature of their linkage in FW-adapted *Fundulus heteroclitus* as well as the role of the regulation of paracellular permeability which is closely related to ion transport and acid-base regulation. As discussed above, claudin proteins are major components of regulating ion transport and permeability in paracellular pathways, previous studies showed different claudins may exhibit different permeability to ions. A change in permeability to Na\(^+\) versus Cl\(^-\) may attribute to the accomplishment of an osmotic or acid-base regulatory process. We put special emphasis on studying the potential roles of claudins in the mechanisms of acid-base regulation in *Fundulus* species.

**Brief Outline of Main Points of Each Chapter**

Despite these recent advances, our knowledge of mechanisms of osmoregulation and acid-base tolerance in euryhaline fish is still far from
complete. In my dissertation, I will address some of these questions utilizing whole animal, cellular, and molecular approaches in order to reach a better understanding of the underlying mechanisms of osmoregulation and acid-base tolerance in euryhaline *Fundulus* species.

Chapter 2: This chapter aims to uncover the important roles of branchial paracellular pathways in fish osmoregulation. Overall, the collected results demonstrate that *Fundulus grandis* maintains hydromineral balances by rapidly regulating gill permeability to certain ions. More specifically, branchial Cl⁻ permeability decreased significantly as a mechanism to maintain plasma Cl⁻ concentration. This decreased permeability is likely caused by some claudin proteins which regulate the ion transport across paracellular pathways.

Chapter 3: This study aims to initiate investigating the mechanisms of acid tolerance among three populations of *Fundulus* species. In addition, claudin expression is measured to study if some claudins contribute to acid tolerance in two populations of *Fundulus heteroclitus*. The collected data show *Fundulus majalis* does not have the ability to regulate against metabolic acidosis, whereas *Fundulus heteroclitus* are able to compensate quickly metabolic acidosis by regulating the relative transports of Na⁺ and Cl⁻. As an investigation to study if intraspecific differences of acid-base regulation exists among Fundulus heteroclitus as that of osmoregulation does, our data show that there are no differences in responses to acid challenge between two populations of *Fundulus heteroclitus* in this study. There are no differences in responses to acid challenge between two populations of *Fundulus heteroclitus*. Adaptation to acidic
challenge is detected in both populations of *Fundulus heteroclitus*. Differential claudin gene expression is detected and may contribute to this regulatory process.
CHAPTER 2: ALTERATIONS IN CLAUDIN EXPRESSIONS IN THE GILLS OF FUNDULUS GRANDIS FOLLOWING ABRUPT SALINITY TRANSFER

Introduction

Osmoregulation in euryhaline fish involves the coordinated regulation of both transcellular and paracellular pathways in ion-transporting epithelia such as the gills, intestines, and kidney (Evans et al., 2005; Marvao et al., 1994; Shehadeh and Gordon, 1969; Tipsmark et al., 2010). The gill is particularly important in mediating these ion movements due to the extensive surface area of the tissue in direct contact with the external environment, and because of the high water and blood flow rates continually passing across their extracellular surfaces (Evans, 2008; Hwang and Lee, 2007; Wilson and Laurent, 2002). In seawater (SW), the fish gill actively excretes ions to the external environment to balance the passive ion gain occurring at the gills and intestines. In contrast, the freshwater (FW) fish gill actively absorbs ions to compensate for a passive ion loss to the more dilute environment (Chang et al., 2001; George et al., 2006b; Patrick and Wood, 1999). Although the physiology of transcellular ion transport has been studied for nearly a century, comparably little is known regarding the mechanistic basis of paracellular ion movement in fish (Evans, 2008; Evans et al., 2005).

The paracellular pathway is regulated by a series of tight junction proteins, including occludins, claudins, and junctional adhesion molecules, that help demarcate the environmentally-exposed apical membrane from the blood-exposed basolateral membrane of transporting epithelia (Chiba et al., 2008; Van Itallie and Anderson, 2004). Amongst the members of this protein complex, the
The claudin superfamily of proteins has received much attention recently due to their importance in regulating the permeability and ion selectivity of the paracellular pathway (Koval, 2006; Van Itallie and Anderson, 2006). Claudins are tetraspan proteins, which each have a pair of extracellular domains that can form functional dimers with the domains on neighboring cells to form charge and size selective pores (Koval, 2006). There are at least 24 claudin orthologs in mammals (Van Itallie and Anderson, 2006), and at least 56 claudins in Fugu fish, due to genome duplication in fishes (Bagherie-Lachidan et al., 2009; Loh et al., 2004). Each claudin may assume specific functions which are different from those of other claudins (Alexandre et al., 2007; Angelow and Yu, 2007; Coyne et al., 2003b). Expansion of the claudin gene family may be due to the response to the unique aquatic physiological environments in fish (Bagherie-Lachidan et al., 2009). The large numbers of claudin family contribute to completing complex regulations of ion transport across paracellular pathways. Two claudins integrate together as a dimer which functions as a complete unit, which makes elucidation of the functions of claudins complicated (Koval, 2006; Morita et al., 1999). Though there are some claudin orthologs comparable to mammals, the expansion of the claudin family strongly suggest that claudin may behave differently in the function of osmoregulation between mammals and fishes.

Recent studies have shown a correlation between claudin expression changes and salinity acclimation in euryhaline fish. For example, up-regulation of claudin-28a and claudin-30 were found associated to the FW fish gill phenotype. to contribute to in tilapia and a similar observation has been made for claudin-27
in eel (Goggisberg and Hesse, 1983; Tipsmark et al., 2008a; Tipsmark et al., 2008b). Studies have shown that two claudin proteins form a complete functional unit which shows specific permeability to different ions (Alexandre et al., 2007; Alexandre et al., 2005; Angelow and Yu, 2007). This characteristic plays important roles in regulating ion movement across paracellular pathways in epithelia. However most knowledge of claudins obtained until now are from studies in mammals. Little has been investigated on the distributions and functions of claudins in fish (Bagherie-Lachidan et al., 2008, 2009; Clelland et al., 2010; Loh et al., 2004). Considering the changing environments in fish habitats, the study on potential functions of claudins in fish will contribute much to our understanding on mechanisms and regulation of epithelial barriers.

Killifish do not exhibit active Cl⁻ uptake in FW and solely rely on efflux manipulation to maintain ion balance during hypoosmotic exposures when dietary sources of Cl⁻ is limited (Patrick et al., 1997; Patrick and Wood, 1999). This efflux manipulation is achieved by adjusting gill permeability which occurs via paracellular pathways (Patrick et al., 1997; Wood and Marshall, 1994). Northern populations of Fundulus heteroclitus survived better following FW challenge than southern populations during FW exposures because they exhibited lower branchial permeability which lead to less Cl⁻ loss through paracellular pathways (Scott et al., 2004b). Thus, understanding the regulation of branchial permeability of paracellular pathways seems to be the key in uncovering the mechanisms of hypoosmotic tolerances in killifish.
The aim of this study was to investigate the roles of paracellular pathways during hypoosmotic tolerances of the euryhaline *Fundulus grandis*. We examined the plasma Na\(^+\) and Cl\(^-\) concentrations and ion flux rates, as well as gill paracellular permeability in fish before and after the hypoosmotic exposures. *Fundulus heteroclitus* and *Fundulus grandis* are two genetically closely-related species with similar morphologies and ecological niches (Duggins et al., 1989; Hsiao and Meier, 2005). We obtained 6 claudin-like ESTs of *Fundulus heteroclitus* and designed primers that successfully worked in *Fundulus grandis*. We examined mRNA expression of these claudin-like genes in different cell phenotypes in *Fundulus grandis* gills using quantitative PCR. This study is the first study of the roles of paracellular pathways in *Fundulus grandis* and is the first to investigate differential expression of claudins in different cell types of gills in fish during hypoosmotic challenges.

**Materials and Methods**

**Experimental Animals**

Adult *Fundulus grandis* (weight range 3.1-16.8 g) were obtained from a local hatchery (Gulf Coast Minnows, Thibodaux, LA) and acclimated in 330-liter glass aquaria for at least one month prior to experimentation. Fish were housed and handled according to an approved Institutional Animal Care and Use Committee Protocol in facilities managed by the Department of Laboratory Animal Medicine, Louisiana State University and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Fish-holding tanks were part of a recirculating system that received water after filtration through separate particle and biological filters, and sterilization through
an ultraviolet light. Water salinity and temperature, which were monitored daily, were maintained at 5 ppt and 22-24°C, respectively, and nitrogenous waste products, which were measured at least three times a week, were all kept at negligible levels. Fish were fed twice daily at a total ration of 2% body-weight per day with commercial killifish pellets (Cargill Aquaxcel™) and kept on a 12 h light: 12 h dark cycle.

**Tissue Sampling**

One hundred and fifty fish were divided randomly into 5 groups and transferred to 100 liter glass aquaria containing 5 ppt, 2 ppt, 1 ppt, 0.5 ppt and 0.1 ppt water, respectively. These water were prepared by adding sea salt to RO (Reverse Osmosis) water with the calibration of a salinity meter. Water samples of 0.1 and 0.5 ppt were taken daily for determination of water chemistry (Table 2.1), water samples of 5, 2, 1 ppt were calibrated by a salinity meter daily. Fish were sampled at 6 h, 24 h, 3 days, 7 days and 14 days post transfer (n= 6 fish per treatment); fish were not fed for at least 12 h prior to sampling. All the fish were net captured and anesthetized briefly in 0.5g l⁻¹ tricaine methanesulfonate (MS-222). Blood was collected using micro-hematocrit capillary tubes (Fisher Scientific, USA) after severing the spine. Blood was then centrifuged at 3000 rpm for 3 minutes, and blood plasma collected and stored at -20°C awaiting analyses of plasma sodium and chloride concentration. Plasma sodium concentrations were measured by flame atomic absorption spectroscopy (Varian Australia Pty Ltd, Australia) and plasma chloride concentrations were measured using a modified mercuric thiocyanate method (Zall et al., 1956). Gill baskets were removed completely from each fish and washed with deionized water for 10 s.
After the wash, the first left gill arch of each gill basket was processed as described below. The remaining gill basket was cut into small pieces and immersed into RNAlater (Invitrogen, USA) and stored at 4°C for 16 h, before being transferred to -20°C for longer term storage.

Table 2.1 Ion concentration (mM) of the waters used in the experiments.

<table>
<thead>
<tr>
<th>Salinity</th>
<th>[Na⁺]</th>
<th>[Cl⁻]</th>
<th>[Ca²⁺]</th>
<th>[Mg²⁺]</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ppt</td>
<td>0.61±0.04</td>
<td>1.70±0.09</td>
<td>0.40±0.04</td>
<td>0.17±0.02</td>
</tr>
<tr>
<td>0.5 ppt</td>
<td>6.93±0.59</td>
<td>8.21±0.67</td>
<td>2.10±136</td>
<td>0.69±0.08</td>
</tr>
<tr>
<td>1 ppt</td>
<td>14.62</td>
<td>17.03</td>
<td>0.32</td>
<td>1.67</td>
</tr>
<tr>
<td>2 ppt</td>
<td>29.20</td>
<td>34.10</td>
<td>0.64</td>
<td>3.34</td>
</tr>
<tr>
<td>5 ppt</td>
<td>73.12</td>
<td>85.15</td>
<td>1.60</td>
<td>8.35</td>
</tr>
</tbody>
</table>

Values are represented as the mean ± the standard deviation of the mean; n=7, n is the number of water samples measured at each salinity. Ion concentrations of 1, 2, 5 ppt treatments are estimated based on the proportion of the measured ion concentrations of full strength SW (32ppt).

**Sodium and Chloride Flux Experiments**

Sodium and chloride flux experiments were performed using a protocol similar to that described previously (Patrick and Wood, 1999). Thirty *Fundulus grandis* weighing from 4.16 to 7.12 g were acclimated to a 38-liter 5 ppt water tank for two weeks prior to the flux experiments and transferred to 0.5-liter of 0.1 ppt water. On the day before the experiment, six fish were transferred to six individual chambers containing 250 ml of aerated water at 0.1 ppt. The chambers
were covered to decrease fish stress, which appeared to be minimal within 10 min after transfer based on resting status of the fish after initial agitation. Fish were maintained in the dark for 1 h until 0.5 µCi $^{22}$Na$^+$ (PerkinElmer, USA) was added to each chamber. Pre-experiments showed 1 h in dark was enough to eliminate fish stress which is determined by mobile agitation of the fish in the chamber and the deviation of flux rates from control status. Our data showed that there were no significant differences on flux rates before and 1 hour after the transfer (data not shown). The water was well mixed before taking water samples. Two ml water samples were taken at 0, 0.5, 1, 2, 4, and 6 h from the time radioisotopes were added to the water. After the experiments, fish were gently washed, weighed and put back to a 30-liter tank with aerated 0.1 ppt water. The same procedure was repeated at 24 h, 3 days, 7 days and 14 days after the first flux experiments using the same fish. Fish were fed with commercial killifish food (Cargill Aquaxcel™) twice a day, although no food was given the morning prior to the start of flux experiments. The radioactivity of $^{22}$Na$^+$ was counted on a Gamma Radiation Counter (Gamma Counter 5500, Beckman Instruments Inc, USA) and sodium concentration in water samples was measured by flame atomic absorption spectroscopy (Varian Australia Pty Ltd, Australia).

The chloride flux experiments were performed by calculating the rates of disappearance of chloride radioactive isotope from the water and the accumulation rates of isotopes in fish body, which was modified from a protocol previously described (Patrick and Wood, 1999). One ml of freshwater containing 0.25 µCi $^{36}$Cl$^-$ was added to each chamber containing 200 ml of 0.1 ppt water.
Each 1 ml water sample taken during the experiment was added to 5 ml Ultima Gold scintillant (PerkinElmer Life and Analytical Sciences, USA) and the solution was kept in the dark overnight for full interactions. The samples were then counted on a scintillation counter (TRI-CARB 2900TR Liquid Scintillation Analyzer, PerkinElmer Life and Analytical Sciences, USA). All samples had similar quench, so therefore, no quench correction factor was required.

The unidirectional influx rates for Na\(^+\) and Cl\(^-\) were calculated using the change in water isotope radioactivity between successive time points as calculated by:

\[
J_{\text{in}} = \frac{\text{volume}}{\text{weight}} \cdot (\text{cpm}_1 - \text{cpm}_2) \cdot \frac{1}{\text{time}} \cdot \frac{1}{\text{SA}} \quad \text{Eq. 1}
\]

where cpm1 and cpm2 are the radioisotope activities at the start and end of each time period, volume is the exact volume of water in the flux chamber, weight is the fish weight in grams, and SA is the mean specific activity of the water in cpm/µmol.

Net flux rates were calculated by the total ion concentration differences among water samples by the following equation:

\[
J_{\text{net}} = ([\text{ion}_1] - [\text{ion}_2]) \cdot \frac{\text{volume}}{\text{weight}} \cdot \frac{1}{\text{time}} \quad \text{Eq. 2}
\]

where [ion1] and [ion2] are the ion concentrations at the start and end of flux period respectively. All other variables are similar to those described for Equation 1.

Efflux rates were calculated by the differences between influx and net flux rates.
\[ J_e = J_{\text{net}} - J_{\text{in}} \]  

Eq. 3

Only net flux rate were data presented for the 3 day, 7 day, and 14 day time points due to undetectable \( \text{Cl}^- \) influx rates at 3, 7, 14 day time point after fish exposed to 0.1 ppt water.

**SEM and TEM**

Fish gills were fixed in 2% glutaraldehyde-1% formaldehyde in 0.2 M cacodylate buffer for 4 h, then rinsed 5X in 0.1 M cacodylate buffer containing 0.02 M glycine over 12 h period. Samples were post-fixed in 2% osmium tetroxide for 1 h, then rinsed in water, \textit{en bloc} stained in 5% uranyl acetate in the dark for 1 h, rinsed in water 2X, and dehydrated in an ethanol series which is a step-by-step 20-minute dehydration from 30% ethanol to 50%, 70%, 95% and 100% ethanol sequentially. The samples were critical-point dried with liquid \( \text{CO}_2 \) in a Denton CPD, mounted on aluminum SEM stubs, coated with gold: palladium at a ratio of 60: 40 in an Edwards S150 sputter coater, and imaged with a JSM-6610 high vacuum mode SEM.

Emersion of live killifish in lanthanum (La) nitrate emersion was used as a method to differentiate between leaky and tight paracellular tight junction complexes in the fish gill. Briefly, fish were immersed for 15 minutes into a 1.5% solution of \( \text{LaNO}_3 \) dissolved in 5, 2, 1, 0.5, 0.1 ppt water, respectively. Lanthanum went into paracellular pathways if it was leaky while lanthanum could not if it was tight. Lanthanum ion turned into black in TEM images after a series of treatment addressed below. Before fish transfer, the transfer medium was alkalinized to pH 7.8 to avoid precipitation of \( \text{La hydroxide} \). Following exposure, fish were sacrificed, and the gills were dissected from the fish and fixed in 2%
glutaraldehyde-1% formaldehyde in 0.2 M sodium cacodylate buffer. The samples were rinsed in fixative for 10 seconds at least 3 times to remove external remnants of lanthanum nitrate. The samples were then cut into small pieces and treated with osmium for 1 h. After dehydration with ethanol, the gill filaments were treated for transmission electron microscopy (TEM) imaging taken at apical sides of branchial epithelia at magnifications of 26000X and 260000X by the Advanced Microscopy Center in the Department of Biological Sciences at Louisiana State University.

Gill Permeability Experiments: Tritium-labelled polyethylene glycol ($^3$H)PEG-4000; MW=4000, 18.5 MBq/g, American Radiolabeled Chemicals, Inc) is a high molecular weight polymer of ethylene oxide and is a blend of polyers with different degrees of polymerization. It is very soluable to water and widely used as an indicator of epithelial paracellular permeability. Gill permeability was calculated in previous studies using different methods (Kumai et al., 2011; Scott et al., 2004b; Wood et al., 1998). Seventy-two Fundulus grandis were acclimated to 5 ppt water for at least two weeks and then randomly assigned into three groups and transferred to 5, 0.5 and 0.1 ppt water for 0, 1, 3, or 7 days. At each of these time points, six fish per salinity were transferred to individual, darkened, flux chambers containing 1.25 liter of water at the same salinity they had been exposed to post transfer (i.e., 5 ppt, 0.5 ppt, and 0.1 ppt). After a 1 h acclimation period, 25 µCi $^3$H]PEG-4000 was added to each container and two water samples (1 ml each) were taken at time 0 and 6 h after addition of $^3$H]PEG-
Fish were weighted and sacrificed at the end of each 6-hour exposure period.

Fish intestine was gently removed carefully to ensure no purging of intestinal contents. Each intestine and its associated gut contents were digested in 1M HNO$_3$ overnight at 60$^\circ$C and the digested content was centrifuged at 4000 rpm for 5 min with an aliquot of the supernant diluted to a v:v ratio of 1:2 with liquid scintillant (Ultima Gold scintillant liquid, Perkin Elmer Life and Analytical Sciences, USA). The 1 mL water samples were diluted with 4 mL deionized water, and diluted to a v:v ratio of 1:2 with liquid scintillant. The radioactivity of these samples was counted using a scintillation counter (TRI-CARB 2900TR Liquid Scintillation Analyzer, PerkinElmer Life and Analytical Sciences, USA). The parameter of gill paracellular permeability is the clearance of external radioactive isotopes by calculating the appearance of radioactivity within fish body minus uptake of that in intestine versus time. This method excludes drinking effect which represents uptake of radioactivity in intestine and thus is more accurate than traditional methods (Scott et al., 2004b).

\[
\text{Gillp} = \frac{[\text{isotopes in fish body}]_a - [\text{isotopes in intestine}]_a}{\text{weight}} / \text{time}
\]

Eq. 4

where [isotopes in fish body]$a$ represents amount of isotopes in fish body at the end of experiment, [isotopes in intestine]$a$ represents the amount of isotopes in intestine at the end of the experiment. Time represents duration of the experiment. Weight represents fish weight at the end of the experiment.
**Total RNA Isolation**

Total RNA from gill tissue was isolated using TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions and total RNA concentrations measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). Single-stranded cDNA synthesis (using 2µg total RNA per 20 µL reaction) was primed at poly(A) tail by reverse transcription using reverse transcription kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Carlsbad, California, USA).

**Claudin mRNA Levels in Isolated Gill Cells**

Forty-eight *Fundulus grandis* were acclimated to 5 ppt water for at least two weeks, then divided into two groups and transferred to 5 or 0.1 ppt water. Fish (n=6 per salinity) were sampled after 6 h, 24 h, 3 days, and 7 days post transfer. Gill epithelial cells were isolated from each fish using a protocol previously described (Galvez et al., 2002). Briefly, epithelial cells from each fish were separated into different cell types by using a three-step Percoll gradient consisting of 1.03, 1.06, 1.09 g.ml⁻¹ Percoll in a similar protocol. After the isolation, 300 nM fluorescent Mito-tracker Red was applied to cells obtained from different layers to check percentages of MR cells in each fraction to ensure proper cell type separation. Mitochondrion-rich cells (MRCs) displayed extensive, strong fluorescent staining within cell boundaries while non-mitochondrion-rich cells only showed sparse and weak fluorescent staining inside the cells. After the separation of the cells from each layer, I counted MRC number in each field of view under fluorescent microscopes. MRCs consist of 87-95% (91.6%±0.02) of total cells in 1.06-1.09 layer while that is only less than 5% (4%±0.017) in 1.03-
1.06 layer. Total RNA was isolated from epithelial cells collected from 1.03-1.06 and 1.06-1.09 g.ml\(^{-1}\) layers using Trizol reagents. RNA was converted to single-stranded cDNA (using 2µg total RNA per 20 µL reaction) by reverse transcription (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Carlsbad, California, USA).

**Quantitative PCR**

*Fundulus heteroclitus* claudin-like EST sequences (DR047164, DN951669, CV819612, DR441634, DR441341, DR441481) were obtained using the National Center for Biotechnology Information (NCBI). These ESTs were submitted to a BLAST search against the non-redundant nucleotide database and assigned the nomenclature, claudin-3, claudin-5, claudin-7, claudin-23, claudin-26, and claudin-28, respectively, based on their highest sequence similarity to *Takifugu rubripes* claudin nucleotide sequences. These EST sequences were used to develop primers for qPCR using Primer 3 software (Primer3) ensuring that a GC base content between 40-60%, a theoretical annealing temperature of 60 °C, and a primer length of approximately 21 nucleotides were maintained. All primers amplified single products as demonstrated by agarose gel electrophoresis and denaturation analysis following individual real-time PCR (qPCR). Primer sequences are listed in Table 2.2.

The qPCR analysis was performed using a SYBR Green detection system (SYBR Green core Reagent, Applied Biosystems, USA) according to the manufacturer’s instructions. Reactions were carried out with 5 µL cDNA, 10 nM forward and reverse primers, and 2 µL SYBR Green reagent in a total volume of 20 µL. A three-step cycling protocol was used with 40 cycles of 50 °C for 2 min,
95°C for 10 min, and 95 °C for 15 s. Critical threshold (Ct) values were calculated using the adaptive baseline function in the ABI Prism 7000 SDS software (ABI Prism 7000, Applied BioSystems). The amplification efficiencies for each primer set was calculated by serially-diluting cDNA derived from the gills of control fish, and using these dilutions to obtain a linear regression relationship of Ct. For all primer sets, amplification efficiency varied between 92.1% and 105.5%, with 100% efficiency representing a theoretical doubling in the amount of cDNA after each cycle.

Relative changes in mRNA levels were calculated by the $2^{-\Delta\Delta\text{Ct}}$ method using the formula, $\Delta\text{Ct} \text{ target (treatment-control) - } \Delta\text{Ct ref (treatment-control)}$ (Galvez et al., 2007), where target refers to the claudin genes, and ref refers to the reference gene, 18s ribosomal RNA (18s rRNA). Controls represent the 5.0 ppt-acclimated fish at each time point, and treatments represent either the 0.1 ppt or 0.5 ppt fish under hypoosmotic challenges. The Ct values for 18s rRNA varied only moderately among the different treatments with a standard error of 0.057 within each plate of samples plate. The mean $\Delta\Delta\text{Ct}$ value of each 5.0 ppt control was expressed as 1, and relative change in mRNA levels of hypoosmotic treatments were expressed relative to this value. A terminal dissociation step (95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s) was added to verify the presence of only one amplicon in the PCR reaction.

**Statistical Analysis**

All data collected were expressed as mean ± SE (n) where n equals the number of fish in an experimental group. Plasma chemistry data were analyzed by one-way ANOVA with Student-Newman-Keuls test. After the test of
homogeneity of variance, gill permeability data were analyzed by one-way
ANOVA. Equal variance was checked on the data of claudin expression levels in
different cell types before a two-way ANOVA statistical method was applied to
test the significance of claudin expressional levels among 5, 0.5, and 0.1 ppt
samples. A significance level of $P<0.05$ was chosen. All statistics were done by
SPSS version 17.

Table 2.2 Sequences of forward and reverse primers used to measure *Fundulus
grandis* mRNA claudin levels using quantitative PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Direction</th>
<th>Sequence 5'→3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cldn3-qPCR</td>
<td>Forward</td>
<td>AAGCTTCTTCTTGACCCAGCA</td>
</tr>
<tr>
<td>Cln3-qPCR</td>
<td>Reverse</td>
<td>CCCACCACGTGAGACTAAT</td>
</tr>
<tr>
<td>Cldn5-qPCR</td>
<td>Forward</td>
<td>TGGGACTTGCTATGTGCAGTA</td>
</tr>
<tr>
<td>Cldn5-qPCR</td>
<td>Reverse</td>
<td>ACGGAGGAGATGGTGAGTGAG</td>
</tr>
<tr>
<td>Cldn7-qPCR</td>
<td>Forward</td>
<td>CATCATCCTGATCCGGAGCTT</td>
</tr>
<tr>
<td>Cldn7-qPCR</td>
<td>Reverse</td>
<td>TGGCACCCTCAATTATAGCC</td>
</tr>
<tr>
<td>Cldn23-qPCR</td>
<td>Forward</td>
<td>CGAACAAACAACAAACCAGC</td>
</tr>
<tr>
<td>Cldn23-qPCR</td>
<td>Reverse</td>
<td>GAAATCCCTTGCTCGAGTGTA</td>
</tr>
<tr>
<td>Cldn26-qPCR</td>
<td>Forward</td>
<td>GGACTGACATCGTGATTTT</td>
</tr>
<tr>
<td>Cldn26-qPCR</td>
<td>Reverse</td>
<td>TGGCCACCATCAGTCTCTAG</td>
</tr>
<tr>
<td>Cldn28-qPCR</td>
<td>Forward</td>
<td>TCTGGAGTTGCCTCAAGGAC</td>
</tr>
<tr>
<td>Cldn28-qPCR</td>
<td>Reverse</td>
<td>ATGATTGTGTGGGCAGTCCA</td>
</tr>
<tr>
<td>18s-qPCR</td>
<td>Forward</td>
<td>TTCCGATAACGAGGAGAC</td>
</tr>
<tr>
<td>18s-qPCR</td>
<td>Reverse</td>
<td>GACATCTAAGGAGCTACAG</td>
</tr>
</tbody>
</table>
Results

Plasma Chemistry

One hundred and fifty-six Fundulus grandis were acclimated to 5ppt water for at least 2 weeks before they were divided randomly and transferred to 5, 2, 1, 0.5, 0.1 ppt water for hypoosmotic challenges respectively. Plasma from each sacrificed fish was collected, then [Na⁺] and [Cl⁻] of these samples were measured as the direct parameters of fish responses to hypoosmotic challenges. Plasma [Na⁺] significantly decreased 6 h after the transfer to 0.1 ppt water, reached its lowest point at 24 h and tended to recover thereafter, but could not recover completely even 14 days after the transfer. There was only a significant difference at 6 h after the transfer in 0.5 ppt group. No significant differences were found at 24 h, 3, 7 and 14 days (Fig 2.1A).

Plasma [Cl⁻] data showed significant decreases at all time-points after the transfer at 0.1 ppt with the lowest point at 6 h compared to control water (5 ppt). No significant differences found in 0.5 ppt group (Fig 2.1B). Plasma [Na⁺] and [Cl⁻] did not show disturbances within 1, 2 and 5 ppt groups.

Unidirectional Sodium and Chloride Fluxes

Changes in sodium and chloride flux rates give direct exhibition of regulations in sodium and chloride ion movement across fish epithelia during hypoosmotic challenges.

During the first 6 h after Fundulus grandis were transferred from 5 ppt to 0.1 ppt, the average sodium influx rate was 260 μmol⁻¹·kg⁻¹·h⁻¹ and the efflux rate was approximately at -770 μmol⁻¹·kg⁻¹·h⁻¹. There was a significant net efflux rate
during the first 6 h. The influx rates remained relatively constant 1 day and 3
days after the transfer while the efflux rates decreased significantly at 1 day and

![Graph A](image)

**A**
Plasma $[\text{Na}^+]$ (mM)

![Graph B](image)

**B**
Plasma $[\text{Cl}^-]$ (mM)

Figure 2.1 Plasma sodium (A) ($N=6$) and chloride (B) ($N=6$) levels in *Fundulus grandis* before and after transfer from 5 ppt water to 2 ppt, 1 ppt, 0.5 ppt and 0.1 ppt water respectively. Data are expressed as means ± S.E.M. * indicates significance from pre-transfer 5 ppt control ($P<0.05$).

3 days and no significant net flux rates were found. Both influx and efflux rates
increased significantly 7 days after the transfer but the net flux rate was not
significantly different from zero. There was no difference in efflux rates between
first 6 h and 14 days. After 14 days post transfer, the influx rate increased to
approximately 730 µmol·kg⁻¹·l⁻¹ with the efflux rate at 760 µmol·kg⁻¹·l⁻¹. No net flux was detected 14 days after the transfer (Fig 2.2A).

The average chloride influx rate in the first 6 h after the transfer to 0.1 ppt

![Graph A](image)

![Graph B](image)

Figure 2.2: Total (net) flux and unidirectional fluxes of Na⁺ (A) and Cl⁻ (B) in *Fundulus grandis* after transfer from 5 ppt water to 0.1 ppt water (*N*=6) versus time. Positive values represent influx (*J*₁), negative values represent efflux (*J*₂). a, b were used to indicate the significant differences in influx rates; a’, b’ were used to indicate the significant differences in efflux rates; a”, b” were used to indicate the significant differences in net flux rates.
water was 250 \( \mu \text{mol}^{-1} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) with the average efflux rate at -790 \( \mu \text{mol}^{-1} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \). A significant net efflux rate of -540 \( \mu \text{mol}^{-1} \cdot \text{kg}^{-1} \cdot \text{h}^{-1} \) was found during the first 6 h. By 1 day, no difference of influx rate was found while there was a significant decrease in efflux rate. From 3 days post-transfer, influx and efflux was so minimal that it was difficult to detect them. As discussed earlier, *Fundulus* species do not have active chloride uptake systems in FW. Minimizing chloride loss is the strategy to maintain plasma chloride levels. This strategy seemed to happen 3 days after FW exposure. Only net flux rate was measured during this period but no significant difference was detected (Fig 2.2B).

**Scanning Electron Microscopy (SEM) Study**

The branchial epithelial morphology exhibits distinct differences between a FW fish and a SW one. SEM study was applied in this experiment to give direct evidence showing this transition of gill morphology in fish under hypoosmotic challenges. Our data found that gills in *Fundulus grandis* at 5 ppt water showed typical ‘SW’ type morphology. Pavement cells (PVCs) occupied more than 90% of surface area and mitochondrion-rich cells (MRCs) represented a small crypt at apical sides (Fig 2.3). After 6 h post-transfer to 0.1 and 0.5 ppt, there was a transient morphological change in MRCs which showed that cell membrane surfaces were more exposed to the external environment with the appearance of micro villi. These cells looked closer to typical ‘FW-type’ MRCs. The apical surfaces were square, triangular or rounded. The population of this type of cells was about 20% that of PVCs. PVCs showed more foldings on the cell surface compared to 5ppt group. After 1 day, FW-type MRCs in 0.1 ppt water fish
continued showing this morphological change but the population of these types of cells decreased. However morphology of gills in 0.5 ppt water fish tended to go back to ‘SW-type’ morphology 1 day after the transfer. No apparent differences including surface areas, shape of edges in PVC epithelia were found on the morphology of PVCs throughout the experiments at all salinity and time points (Fig 2.3).

![Figure 2.3 Gill SEM images of *Fundulus grandis* transferred from 5 ppt to 0.1 ppt and 0.5 ppt group at 6 h, 24 h, 3 days and 7 days. PV represents pavement cell. MR represents mitochondrion-rich cell which are exhibited as apical black crypts. Each bar represents 5 µm.](image)

**Gill Permeability Study**

Gill permeability is the characteristic showing the difficulty of an ion passing through epithelia. This study used these data as an indicator explaining changes in sodium and chloride flux rates. The rate of disappearance of tritium-labeled polyethylene glycol ([H]PEG-4000; MW=4000) across the gills did not show significant change in the *Fundulus grandis* 5 ppt control group basing on a statistical analysis among control groups. This rate did not show significant difference at 6 h in the 0.5 ppt group compared to the 5 ppt control group,
however it did decrease significantly at 1, 3 and 7 days post-transfer. The rate of $^3$[H]PEG-4000 disappearance showed significant decrease at all time-points in 0.1 ppt group compared to 5 ppt control (Fig 2.4) These data showed that fish exhibited prompt responses of regulating paracellular permeability to hypoosmotic challenges and that the lower the salinity was, the faster the responses were.

![Graph showing total gill permeability rate](image)

Figure 2.4 Total gill permeability rate (ml kg$^{-1}$ h$^{-1}$, previously described in equation 4 in Materials and Methods part) of PEG-4000 in *Fundulus grandis* after transfer from 5 ppt water to 5 ppt, 0.5 ppt and 0.1 ppt water respectively. Gill paracellular permeability represents the rate of external solution entering fish body by calculating the appearance of radioactivity within the fish body minus uptake of the radioactivity in intestine versus time. Data are expressed as means ± S.E.M. * indicates significant difference from 5 ppt control ($P<0.05$).

**Transmission Electron Microscopy Study**

The use of lanthanum nitrate provides useful information on differentiating the “open” and “close” of paracellular pathways. Lanthanum went into paracellular pathways if it was leaky while lanthanum could not if it was tight. Lanthanum ion turned into black in TEM images after a series of treatment. Our data showed lanthanum could penetrate into paracellular pathways between MRCs and adjacent cells in 5, 2, 1 ppt group. However an apparent penetration was not observed in paracellular pathways in 0.5 and 0.1 ppt groups (Fig 2.5).
Figure 2.5 Junctions between MRCs and adjacent cells in *Fundulus grandis* 3 days after the transfer from 5 ppt water to 5 (A, a), 2 (B,b), 1 (C,c), 0.5 (D,d), 0.1 (E,e) ppt water. A (a), B (b), C (c): lanthanum penetrated paracellular pathways between mitochondrion-rich cells and adjacent cells. D (d), E (e): lanthanum could not penetrate paracellular pathways between mitochondrion-rich cells and adjacent cells. Each black box in A, B, C, D, E contains a paracellular pathway with a magnification of 26000X. The picture (a,b,c,d,e) on the right showed the paracellular pathways at a magnification of 260000X. Scale bars represent 1 µm.

**Gene Identification and Claudin Expression Profiles**

We first examined expressional patterns of these 6 claudins in gills of *Fundulus grandis* following hypoosmotic exposures. Transfer of 5 ppt-acclimated
*Fundulus grandis* to lower salinity induced different claudin expression patterns in fish gills.

Figure 2.6 Changes of claudin expressional levels in response to hypoosmotic challenges versus time. The effects of hypoosmotic exposure (0.1 ppt and 0.5 ppt exposure) on the abundance of Cldn3 (A), Cldn5 (B), Cldn23 (C), Cldn28 (D), Cldn7 (E) and Cldn26 (F) in gills of *Fundulus grandis* for 6 h, 1 day, 3 days and 7 days. Relative claudin mRNA expression was determined relative to controls with no hyposomotic challenge. All expressional changes were normalized to expression of EF1a (we used both, we first used 18s a little, then we used EF1a) A T-test was used to assess the expressional changes of claudins in gills of *Fundulus grandis* between 5ppt control and 0.5 ppt, 0.1ppt experimental groups (P<0.05).
Claudin-3 expression showed no difference at 6 and 24 h after 0.1 ppt transfer. A robust 24-fold expression increase was detected by 3 days and then decreased by 7 days. Claudin-3 expression in 0.5 ppt group was more progressive with an 8-times increase found by 7 days. However claudin-3 expression levels showed discrepancies between MRCs and PVCs. Claudin-3 expression showed a sharp increase 24 h in MRCs after the transfer and remained at high expression levels thereafter while only a slow increase was detected in PVCs 3 days after the transfer (Fig 2.6A, 2.7A).

Claudin-5 increased 3.6 folds 24 h after the exposure to 0.1 ppt water and remained significantly different from control group at 3 and 7 day time point. No significant differences detected in 0.5 ppt group until 7 days after the transfer. Increases of claudin-5 expression occurred exclusively in MRC 24 h after the exposures, whereas no expressional change was detected in PVCs throughout the experiment. These findings confirmed that claudin-5 was mainly expressed in MRCs instead of PVCs. The data also indicated that claudin-5 may be a functional part contributing to osmoregulatory mechanisms in fish during hypoosmotic exposures. (Fig 2.6B, 2.7B).

Claudin-23 showed an increase in both 0.1 and 0.5 ppt group 3 days after the exposure, however this increase persisted only in 0.1 ppt group 7 days after the exposure. Expression of claudin-23 increased 6 h after the exposure and this up-regulation persisted across the experiments in MRCs. Only a transient increase 24 h after the exposure was detected in PVCs. These finding indicated hypoosmotic changes of the environments. (Fig 2.6C, 2.7C).
Figure 2.7 The effects of hypoosmotic exposure (0.1 ppt exposure) on the abundance of Cldn3 (A), Cldn5 (B), Cldn23 (C), Cldn28 (D), Cldn7 (E) and Cldn26 (F) in two different epithelial cells: PVCs and MRCs for 6 h, 1 day, 3 days and 7 days. Relative claudin mRNA expression was determined relative to controls with no hypoosmotic challenge. A T-test was used to assess the expressional changes of claudins in the same type of cells between 5ppt control and 0.1ppt experimental group (P<0.05).

that claudin-23 was mainly expressed in MRCs and was responsive to

Claudin-28 expression showed an abrupt decrease 3 days after transfer to 0.1 ppt which is correlated to the expressional decrease in PVCs. These data suggested that claudin-28 was mainly expressed in PVCs. No expressional
change was detected in MRCs (Fig 2.6D, 2.7D). Claudin-7 and claudin-26 did not show expressional changes in both experiments, which suggested both were unresponsive to hypoosmotic challenges and may assume a function of structure in paracellular pathways. (Fig 2.6E, 2.7E, 2.6F, 2.7F).

**Discussion**

The gulf killifish, *Fundulus grandis*, and its sister taxa the Atlantic killifish, *Fundulus heteroclitus*, reside in coastal marshes that are subject to frequent and episodic oscillations in salinity, dissolved oxygen, and temperature (Marshall, 2003; Wood and LeMoigne, 2001). Their ability to tolerate these dynamic habitats has made them a popular model to study the physiological basis of environmental stress tolerance (Burnett et al., 2007a). Although tending to inhabit brackish to marine environments (Kaneko and Katoh, 2004; Wood and Grosell, 2008, 2009), euryhaline killifish are exposed to reduced environmental salinity while feeding, or during natural or anthropogenic freshwater inputs such as rainstorm flooding events (Marshall, 2003). Killifish appear to cope with short bouts of fresh water by making rapid physiological adjustments (Marshall, 2003; Wood and Grosell, 2008, 2009); it is only with prolonged exposure to fresh water that fish are then required to make more substantive compensatory adjustments to their ion-transporting epithelia (Wood and Grosell, 2008).

Few studies have attempted to delineate the salinity at which the gill epithelium transitions from a SW type to a FW phenotype. Interestingly, most *Fundulus* species are ostensibly marine teleost with the ability to retain their SW physiology at salinities approaching FW (Feldmeth and Waggoner, 1972; Griffith, 1974a; Haag and Warren, 2000). Copeland and Philpott (Copeland, 1950;
Philpott and Copeland, 1963) suggested killifish make this physiological transition at a salinity between 2.0 and 0.5 ppt; however no concrete data were provided in these studies to support this claim. Recently, we have shown that *Fundulus heteroclitus* acclimated to full-strength SW can tolerate abrupt transfer to 0.4 ppt with no significant effects to plasma osmolality over 14 days post-transfer (Whitehead et al., 2010). In the present study, killifish were acclimated to 5.0 ppt since we had previously shown that the gill surface morphology retains its SW phenotype at this salinity, and since we wanted to test this SW to FW phenotypic transition over a relatively small salinity range. Interestingly, we found that plasma Na\(^+\) and Cl\(^-\) concentrations were only significantly decreased at salinities of 0.5 ppt and below, and that only exposure to 0.1 ppt elicited a sustained reduction in plasma Cl\(^-\) concentration from pre-transfer controls (Fig 2.1). This is in accordance with our scanning electron microscopy data (Fig 2.3) showing that the gills of *Fundulus grandis* transition to a FW phenotype occurs at a salinity of 0.1 ppt. Although the gills of fish exposed to 0.5 ppt expressed features consistent with a FW phenotype after only 6 h post exposure, the apical crypts typical of a SW-acclimated fish gill reemerged by 1 d. The surface morphology of mitochondrion-rich gill cells in 0.1 ppt-exposed killifish is consistent with the surface characteristics of the cuboidal cell first described by Laurent et al (Laurent et al., 2006); cells analogous to the mitochondrion-rich PV cells that are presumed to have high active Na\(^+\) transport capacity but only limited ability to actively transport Cl\(^-\) (Laurent et al., 2006). These data also support the presence of fully-developed MR cell types beneath the gill surface, which can be quickly
expressed on the surface upon osmotic challenge (Katoh et al., 2001), and a gill that transitions from a SW to a FW phenotype at approximately 0.5 ppt.

Maintenance of osmotic balance in euryhaline fish during hypoosmotic exposure requires both a reduction of paracellular loss and a longer term stimulation of solute absorption in existing or newly-formed cells (Lin et al., 2004; Marshall et al., 2005). These physiological processes can be monitored indirectly using unidirectional solute movement in whole animals, which acts as a proxy of gill function. Unidirectional influx and efflux rates of Na⁺ in Fundulus heteroclitus ranged from 15000-18000 µmol·kg⁻¹·h⁻¹ in 100% SW and 3000 µmol·kg⁻¹·h⁻¹ in 10% SW, but decreased to below 1000 µmol·kg⁻¹·h⁻¹ during FW exposure (Wood and Marshall, 1994). In the present study, Na⁺ influx rate was approximately 250 µmol·kg⁻¹·h⁻¹ and Na⁺ efflux rate was approximately 750 µmol·kg⁻¹·h⁻¹ in Fundulus grandis after 6 hours exposure to 0.1 ppt water (i.e., FW) (Fig 2.2). This net Na⁺ imbalance recovered to normal (i.e., net Na⁺ flux approaching 0) after 1 day exposure as a result of reduction in Na⁺ efflux over this acute time frame. Na⁺ influx remained relatively constant until day 3 post transfer to 0.1 ppt, but then gradually increased thereafter, indicative of an increase in active Na⁺ uptake via a transcellular pathway. This stimulation of active Na⁺ influx paralleled a secondary, concomitant increase in Na⁺ efflux such that net Na⁺ balance remained negligible. One study found that Fundulus heteroclitus could not maintain sodium balance in their bodies when the concentration of sodium in external environments was below 0.98mM which was called balance point (Wood and Marshall, 1994). The sodium concentration of 0.1 ppt water in this study was
less than 0.7mM. Two reasons may explain this contradiction: 1. *Fundulus grandis* has a lower balance point compared to *Fundulus heteroclitus*. 2. *Fundulus grandis* was fed during the experiments in this study so dietary compensation of sodium may help animal maintain sodium levels in fish plasma.

Cl⁻ influx and efflux rates were both below 1000 µmol·kg⁻¹·h⁻¹ in *Fundulus grandis* 6 hours after exposure to 0.1 ppt water, but decreased to a negligible rate by day 3 post exposure (Fig 2.2). One study reported that Cl⁻ influx and efflux rates were above 15000 µmol·kg⁻¹·h⁻¹ in SW-adapted *Fundulus heteroclitus* (Wood and Marshall, 1994), suggesting a rapid and dramatic decrease in both unidirectional rates upon transfer to FW. Previous studies have demonstrated that *Fundulus heteroclitus* have no appreciable active Cl⁻ uptake in FW, and suggest that regulation of paracellular Cl⁻ loss is a key strategy of hypoosmotic tolerance in FW-tolerant *Fundulus* populations (Scott et al., 2004b). Some studies have reported that euryhaline teleost transferred from SW to FW were able to regulate plasma osmolality within days, but often did so at an osmolality lower than that pre-transfer (Kalujnaia, 2007). This is consistent with the plasma Cl⁻ concentrations of the 0.1 ppt fish observed here, which stabilized by day 3 post-transfer, but at concentrations significantly below those of the 5.0 ppt control fish. These finding indicated that animal may lower chloride concentration in plasma as another strategy to cope with hypoosmotic challenges besides the strategy to minimize passive chloride loss.

Wood and Grosell (2008; 2009) reported that transepithelial potential, which is an important determinant of the electrochemical driving force of gill ion
transport, was relatively refractory to the short-term transfer of SW-acclimated *Fundulus heteroclitus* to FW, and that only with longer exposure to FW did the gills of *Fundulus heteroclitus* functionally switch into that of a FW animal (Wood and Grosell, 2008, 2009). The gills of brackish water-acclimated *Fundulus* exhibited high non-selective cation permeability largely via the paracellular shunt pathways (Wood and Grosell, 2008, 2009). Brackish water-acclimated *Fundulus heteroclitus* exhibited a positive diffusional transepithelial potential (TEP), which facilitates Na\(^+\) extrusion via paracellular shunt pathways (Wood and Grosell, 2009). The TEP of the *Fundulus* in natural environments is interpreted as a diffusional potential dictated by the relative permeability of the branchial epithelia to the two dominant ions (Na\(^+\) and Cl\(^-\)) in extracellular fluid and the external environments (Wood and Grosell, 2008). Upon acute exposures to FW, the TEP will become negative and limits Na\(^+\) loss immediately. However this negative TEP in plasma side does not hamper the uptake of Cl\(^-\) due to the fact there is no active Cl\(^-\) uptake in the gills of *Fundulus* species (Patrick and Wood, 1999; Wood and Marshall, 1994). Regardless, keeping Cl\(^-\) loss to negligible amounts but maintaining extensive paracellular permeability to Na\(^+\) is a unique feature of the gills of *Fundulus* species in FW.

Numerous studies have shown that claudins, a key constituent of the tight junction complex, are the principal proteins forming barrier properties regulating permeability of paracellular pathways (Koval, 2006; Van Itallie and Anderson, 2004, 2006). Although not well studied, there is growing evidence that claudins are likely important regulators of paracellular ion movement in the fish gill, and
are under dynamic regulation during osmotic challenges (Bagherie-Lachidan et al., 2009; Clelland et al., 2010; Tipsmark et al., 2008a). A transcriptional change in the abundance of specific claudins or claudin isoform switching could contribute to the transformation of the fish gill from a ‘leaky’ epithelium to a ‘tight’ one in euryhaline fish during hypoosmotic challenges.

Although the ability of gills to remodel with varying environmental salinity is well reported (Katoh et al., 2001; Laurent and Dunel, 1980; Wilson and Laurent, 2002), few investigations have described the cellular mechanisms regulating their transient and long-term responses to osmotic stress. The current study demonstrated significant increases in the mRNA levels of claudin 3, 5, and 23 in the gills of Fundulus grandis during hypoosmotic exposures, while that of claudin-28 decreased following FW exposure (Fig 2.6). Claudin-3 protein levels in the gills of tilapia have been shown to increase following transfer from SW to FW (Tipsmark et al., 2008a), which is in accordance with the increase in mRNA levels in this study. It is possible that claudin-3 is involved in forming ‘tighter’ paracellular pathways which favor FW tolerance. We also found that claudin-3 mRNA levels increased in both PVCs and MRCs (Fig 2.7), suggesting this isoform may be associated with PVC-PVC and PVC-MRC tight junctional complexes in FW-acclimated killifish, and that it might associated with the increased “tightening” of the gill epithelium in FW. Acclimation of Fundulus heteroclitus to FW is known to elicit the proliferation of a cuboidal-type MR cell, this study also found that the mitosis rate of these ‘cuboidal cells’ reached the peak at 12 h after the exposure (Laurent et al., 2006). This increased mitosis rate
may correlate with increased expression of some claudins which are mainly expressed in these cuboidal cells. Our data found the increased expression of claudin-3 occurred 24 h after the transfer. It is plausible to infer that this sharp up-regulation may be probably correlated with these newly expressed 'cuboidal cells'. These 'cuboidal cells', which show different morphology from typical FW-type MRCs which exhibit active Cl' uptake as previously described in teleost (Evans et al., 2005), are possibly responsible for the lack of Cl' uptake in Fundulus species (Laurent et al., 2006).

Claudin-5 mRNA expression levels increased significantly in Fundulus grandis gills following hypoosmotic exposure, however, unlike the situation for claudin-3 mRNA expression, transcript abundance was highest in the MR cell fraction (Fig 2.6, Fig 2.7). A recent study indicated that claudin-5 contributed to forming a ‘tight’ brain barrier in zebrafish (Abdelilah-Seyfried, 2010), which contrasts the reported stimulatory effect on paracellular permeability of this claudin in mammals (Wen et al., 2004). Interestingly, one study showed that a significant portion of SW-type MRCs were replaced by FW-type MRCs following 3 days transfer from SW to FW (Katoh and Kaneko, 2003). In the current study, claudin-5 mRNA levels in the 0.1 ppt gills were significantly increased above that of the 5 ppt controls at 24 h post transfer, and reached levels of 10-fold above controls by 3 d and 7 d post transfer. It is possible that this increased claudin-5 expression may contribute to forming a “tighter” paracellular pathway and be related to long-term adaptation of hypoosmotic challenges.
Claudin-23 mRNA levels were significantly increased after hypoosmotic exposure and this increase was also predominately localized to the MR cell fraction (Fig 2.6, Fig 2.7), which indicated its role in FW tolerance. These are the first set of data reported so far outlining a potential role of this claudin isoform in fish osmoregulation. Interestingly, this increase in claudin-23 expression occurs as early as 6 h after the transfer. Taken that morphological transformation occurs at 6 h in the SEM study (Fig 2.3) into consideration, these data suggest that claudin-23 expression increase may relate to short-term transformation in MRCs in Fundulus grandis which was described in another study earlier (Katoh and Kaneko, 2003). There is also an increase of claudin-23 in PV cell fraction 3 days after the transfer, which may possibly be due to contamination during cell separation.

In contrast, claudin-28 mRNA levels decreased significantly in Fundulus grandis following FW exposure (Fig 2.6, Fig 2.7). However, this decrease in transcript abundance was observed in the PV cell fraction, suggesting a possible role of PVCs, and not only MR cells, in hypoosmotic tolerance. Interestingly, another study did not show any effect of salinity transfer on claudin-28 mRNA levels in Atlantic salmon (Tipsmark et al., 2008b), suggesting species-specific differences of this claudin in response to osmotic response. The absence of claudin 28 in mammals suggests that this isoform may confer unique attributes to the paracellular pathway in fish. Our data did not detect claudin-7, -26 expression changes in Fundulus grandis with hypoosmotic challenge. One study found that overexpression of claudin-7 decreased paracellular Cl⁻ conductance and
increased paracellular Na\(^+\) conductance in LLC-PK1 cells (Alexandre et al., 2005). These controversial findings suggest that further investigation is needed in order to reach a better understanding.

Despite these expressional changes of claudins described above, it is important to point out that the nomenclature of the claudins in this study may not be very accurate. Due to extensive gene duplication in fish compared to mammals, existence of subunits of claudins is prevalent (Loh et al., 2004). This study however does give valuable information on the relation of claudins and FW tolerance in *Fundulus* species, which is the first one investigating on this question.

We used radiolabeled-PEG 4000 as a marker of paracellular permeability to provide insights in the possible function of claudins during acclimation to hypoosmotic conditions. Previous studies have administered radiolabeled PEG-4000 into fish by intraperitoneal injection and then monitored the appearance of the marker in water. This method is based on the assumption that the transcellular movement of PEG-4000 was negligible (Kumai et al., 2011; Scott et al., 2004b). However, despite of this assumption, PEG-4000 is able to permeate kidney glomeruli and enter into water via urination (Scott et al., 2004b), which makes estimation of extrarenal routes of PEG-4000 permeation problematic. As such, we decided to add PEG-4000 to the water and measure its appearance into the animal, after removing any contribution associated with drinking. Our data found that amount of PEG taken into gastrointestinal tract accounted for 2-21% of total amount of PEG into the body in *Fundulus grandis* (data not shown), which indicated effect of drinking is substantial in FW *Fundulus grandis*. Thus
by applying PEG into external medium and excluding the effect of drinking, the method we used in this study gave more accurate measurement on evaluating paracellular permeability. Our data showed an acute reduction of paracellular permeability occurs promptly after the hypo-osmotic exposure. In addition, we recorded abrupt decrease in both Na\(^+\) and Cl\(^-\) efflux rates. It is likely that the sharp decrease of Na\(^+\) and Cl\(^-\) efflux rates were caused by the decrease of paracellular permeability, which was significant in minimizing ion loss in the early stage of post-transfer period and maintaining plasma ion balance. It is surprising to discover that branchial paracellular permeability did not show differences between 0.5 ppt and 0.1 ppt group 24 h after the transfer in this study (Fig 2.4). In order to reach a better understanding of these data, we applied lanthanum staining in TEM study depicting the paracellular pathways. We found there was no lanthanum penetration into the paracellular pathways in 0.5 and 0.1 ppt group while significant penetration into paracellular pathways in 5, 2 and 1 ppt group (Fig 2.5). This finding gave strong evidence that Fundulus grandis shut down paracellular pathways to minimize passive ion loss during hypoosmotic exposures. However this robust decrease in paracellular permeability is unlikely to be the result of structural transformation or protein expression changes which usually takes hours or days to happen. Some studies indicated that acute regulation of claudins by phosphorylation may play a role in regulation of the paracellular permeability (Angelow and Yu, 2007). This is not surprising since claudins are the major components of tight junctions regulating paracellular permeability (Koval, 2006). Phosphorylation has been linked to both increases
and decreases in tight junction assembly and function. There are some studies indicating that a group of kinases like protein kinase A and myosin light chain kinase are involved in the regulation of claudins or other membrane proteins (Koval, 2006), however this regulatory process is still unclear and further investigation is needed.

In summary, three strategies were adopted by Fundulus species during acclimation to hypoosmotic exposure basing on this and previous studies: 1. Decreasing Na\(^+\) loss by switching the TEP from positive to negative (Wood and Grosell, 2009); 2 Minimizing Na\(^+\) and Cl\(^-\) loss by decreasing permeability of paracellular pathways; 3 Adjusting plasma [Na\(^+\)] and [Cl\(^-\)] at a lower level to lessen the stress of ion maintenance. In addition, this study is the first to give experimental evidence on the transition point from ‘SW morphology’ to ‘FW morphology’ in fish osmoregulation. The information of claudin expressions in this experiment will contribute to a better understanding on their roles in fish osmoregulation. In addition, this study gave valuable information on the roles of claudins during hypoosmotic exposures in Fundulus grandis. Further work needs be done on exploring the function of the whole family of claudins in terms of fish osmoregulation from protein levels to reach a better understanding of the underlying mechanisms.
CHAPTER 3: ACID-BASE TOLERANCES AMONG *FUNDULUS* SPECIES

**Introduction**
Under freshwater conditions, teleost fish are hyperosmotic to their environment, and must actively absorb osmolytes such as Na\(^+\) and Cl\(^-\) to compensate for the passive loss of ions through paracellular pathways (Evans and Claiborne, 2006). One interesting feature is that the transport of strong ions in plasma (Patrick et al., 1997), such as Na\(^+\) and Cl\(^-\) in freshwater teleost relies on the counter movement (i.e., excretion) of acid and base equivalents into the surrounding environment as a requirement for the maintenance of electro-neutrality (Marshall and Grosell, 2005). Compared to animals living on land, fish have a limited capacity for respiratory compensation of non-respiratory, acid-base disturbances, largely due to physical constraints of living in an aquatic environment (Evans and Claiborne, 2006). Although changes in renal excretion and intercellular buffering may contribute to their regulation, ion and acid-base homeostasis primarily occurs at the gills (Evans and Claiborne, 2006; Evans et al., 2005).

The gill epithelium is a dynamic ion-transporting epithelium that manipulates the rates of sodium and chloride transport to achieve both ion and acid-base homeostasis (Evans, 2008; Hwang and Lee, 2007; Wilson and Laurent, 2002). Numerous studies have described a linkage between acid-base tolerance and transepithelial ion exchange in the gills of FW-acclimated fish (Evans et al., 1999; Hyde and Perry, 1987; Marshall and Grosell, 2005; Patrick and Wood, 1999; Salama et al., 1999; Wilson et al., 1995; Wood and Marshall, 1994). In most species, Cl\(^-\) is removed from the water in exchange for a basic equivalent, HCO\(_3^-\),
whereas Na\(^+\) is removed from the water in exchange for an acidic equivalent, H\(^+\) (Goss et al., 1992; Heisler, 1984). Although considerable controversy exists regarding the mechanism of Na\(^+\) and H\(^+\) exchange in the gills, most fish species have a direct coupling of Cl\(^-\) and HCO\(_3\)\(^-\) exchange in the gill, through a putative anion exchanger on the apical membrane of the tissue. Recent studies, however, suggest that *Fundulus* species are amongst a few teleost known to have little to no active transport of Cl\(^-\) at the gills (Patrick and Wood, 1999). As a result, several studies have implicated the restriction of paracellular Cl\(^-\) loss as a requirement for hypoosmotic tolerance; a mechanism that likely delineates species and/or population tolerance of fresh water in fish from the genus *Fundulus*. Furthermore, lack of this transport system likely imparts differences in the regulation against acid-base disturbances.

One study found that metabolic acidosis in *Fundulus heteroclitus* initiated by the intraperitoneal injection of HCl had no effect on Na\(^+\) or Cl\(^-\) influx rates, but rather stimulated an increase of Cl\(^-\) loss and attenuation of unidirectional Na\(^+\) efflux (Patrick et al., 1997). This study drew on the possible role of regulation of paracellular loss of ions as a mechanism of acid-base regulation (Evans et al., 2005; Goss and Wood, 1990a; Goss and Wood, 1990b; Patrick et al., 1997; Patrick and Wood, 1999; Wood and Marshall, 1994). These studies highlight the importance of the paracellular pathway and the associated tight junction proteins in acid-base regulation.

The claudins are a group of proteins which span bilayers 4 times. Some studies show claudins are the key proteins in regulating paracellular permeability
and solute transport (Koval, 2006). Claudins of a given cell interact with other claudins in the adjacent cells and two claudin proteins form a functional dimer which is charge-selective or size-selective (Angelow and Yu, 2007; Coyne et al., 2003a; Coyne et al., 2003b; Van Itallie and Anderson, 2006; Wen et al., 2004). Until now at least 24 claudins have been found in mammals (Angelow and Yu, 2007; Koval, 2006). The claudin family seems to have expanded along chordate lineage due to genome duplication in fishes, with a total of 56 claudins identified in Fugu fish (Loh et al., 2004). Some conjectured that the expansion of claudin family may due to the response to the unique aquatic physiological environments in fish (Bagherie-Lachidan et al., 2009). Though there are some claudin orthologs comparable to mammals, the expansion of the claudin family strongly suggest that claudin may behave differently in the function of osmoregulation between mammals and fishes.

*Fundulus heteroclitus* populations exhibit both physiological and adaptative responses to cope with the changing environments they inhabit (Burnett et al., 2007b). This study aims to uncover underlying mechanism of acid-base tolerance by comparing responses to acid challenge of two *Fundulus* species: *Fundulus heteroclitus* and *Fundulus majalis*. A special effort will be put on two populations of *Fundulus heteroclitus* to study if intraspecific variation of acid tolerance exists. In addition, some claudin expressions will be investigated to study if there is any potential claudin involved in acid-base tolerances between two populations of *Fundulus heteroclitus* since previous studies suggested that claudins play key roles in regulating paracellular ion transport (Koval, 2006).
Materials and Methods

Experimental Animals

*Fundulus heteroclitus* (weight range of 3.8-9.6 g) were collected from Piscataway Park, Maryland (termed *Fundulus heteroclitus* MDPP) and Chincoteague National Wildlife Refuge, Chincoteague Island, Virginia (termed *Fundulus heteroclitus* VAcoast) during June 2011, and transferred to Louisiana State University, where they were held for at least 1 month. *Fundulus heteroclitus* MDPP were held at 0.1 ppt, which was approximately the salinity that they were collected from in the field. *Fundulus heteroclitus* VAcoast were collected from water at 32 ppt, but maintained in the laboratory at 18 ppt, until approximately one month prior to experimentation, when they were gradually transferred to 0.1 ppt over the course of a week, then acclimated at this salinity for the duration of experimentation. *Fundulus majalis* VAcoast, a typical marine killifish, were collected from Chincoteague National Wildlife Refuge, Chincoteague Island, Virginia during June 2011, and transferred to Louisiana State University, where they were held in seawater originally and gradually brought down to 0.1 ppt freshwater over a course of two weeks.

All populations were acclimated for at least one month at 0.1 ppt in 250-liter glass aquaria. Fish were housed according to an approved Institutional Animal Care and Use Committee Protocol in facilities managed by the Department of Laboratory Animal Medicine, Louisiana State University and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Fish-holding tanks were part of a recirculating system that received water after filtration through separate particle and biological filters, and
sterilization through an ultraviolet light. Prior to experimentation, water salinity
and temperature, which were monitored daily, were maintained at 0.1 ppt and 22-
24°C, respectively, and nitrogenous waste products, which were measured at
least three times a week, were all kept at negligible levels. Fish were fed twice
daily at a total ration of 2% body-weight per day with commercial killifish pellets
(Cargill Aquaxcel™) and kept on a 12 h light: 12 h dark cycle.

**Tissue Sampling and Flux Experiment**

Thirty fish per population (*Fundulus heteroclitus* MDPP and *Fundulus*
heteroclitus VAcoast; weighing from 3.8 to 9.6 g) which were already acclimated
to 0.1 ppt water for at least one month were injected by intraperitoneal injection
with 1000 nEq.g⁻¹ HCl (using a stock of 140 mM HCl) and immediately
transferred back to 0.1 ppt. Twenty-four of these fish were transferred to glass
aquaria containing 0.1 ppt, whereas the remaining 6 fish were used immediately
in radioisotopic flux experiments as described below. At 12h post transfer to 0.1
ppt, an additional fish (n=6) were transferred from the glass aquaria to individual
flux chambers as described below. The remaining fish (n=18) were injected from
days 1 to 7 post transfer with 1000 nEq.g⁻¹ HCl, and utilized at 1d, 3d, and 7 days
for radioisotopic flux experiments. Six fish of *Fundulus majalis* VAcoast were
injected by intraperitoneal injection with 1000 nEq.g⁻¹ HCl (using a stock of 140
mM HCl) and immediately transferred to 0.1 ppt. Six *Fundulus majalis* VAcoast
were injected with 1000 nEq.g⁻¹ HCl (using a stock of 140 mM HCl) and and
immediately transferred back to 0.1 ppt water. Since this species could not
survive after the HCl injection, no further flux experiments were done during 12h,
24h, 3d, 7d time points.
Sodium flux experiments were performed in opaque, plexiglass containers containing 250 mL 0.1 ppt water, according to a protocol previously described (Patrick and Wood, 1999). Briefly, each fish was transferred to an individual flux chamber, and allowed to acclimate to the conditions for exactly 1 hour. Pre-experiments showed 1 h in dark was enough to eliminate fish stress which is determined by mobile agitation of the fish in the chamber and the deviation of flux rates from control status. Our data showed that there were no significant differences on flux rates before and 1 hour after the transfer. After 1 h, 0.5 uCi $^{22}$Na$^+$ (as NaCl; PerkinElmer, USA) was added to each chamber and mixed well by gentle aeration. Fish were held under static conditions for the entire 6 h flux period. Water samples (2 mL per time point) were taken at 0, 1, 2, 3, 4, 5, 6 h from the start of the flux period. After flux experiments (described below), all the fish were net captured and anesthetized briefly in 0.5 g.l$^{-1}$ tricaine methanesulfonate (MS-222). Gill baskets were removed from each fish and washed with deionized water for 10 seconds. After the wash, two gill arches of each fish was cut into small pieces and immersed into 1 ml RNAlater (Invitrogen, USA) solution and stored at 4$^\circ$C for 16 h, then transferred to -20$^\circ$C awaiting RNA isolation as described below. Similar fluxes were repeated on fish at 12 h, 24 h, 3 days and 7 days. Fish were fed with commercial killifish food twice a day. No food was given to fish the morning prior to the flux experiments. $^{22}$Na$^+$ radioactivity was counted on a Gamma Radiation Counter (Gamma Counter 5500, Beckman Instruments Inc, USA). Sodium concentration of water samples
was measured by flame atomic absorption spectroscopy (AA240FS, Varian Australia Pty Ltd, Australia).

The unidirectional influx rates for Na\(^+\) were calculated using the change in water \(^{22}\)Na\(^+\) radioactivity between successive time points as calculated by:

\[
J_{in} = \frac{volume}{weight} \cdot (cpm1 - cpm2) \cdot \frac{1}{time} \cdot \frac{1}{SA}
\]

where cpm1 and cpm2 are the radioisotope activities at the start and end of each time period (i.e., 0-1h, 1-2h, 2-3h, 3-4h, 4-5h, and 5-6h), volume is the exact volume of water in the flux chamber, weight is the fish weight in grams, and SA is the mean specific activity of the water in cpm/µmol.

Net flux rates, which represent the difference between unidirectional influx and efflux rates, were calculated using the change in total Na\(^+\) concentration in water between successive time points according to the following equation:

\[
J_{net} = ([ion1] - [ion2]) \cdot \frac{volume}{weight} \cdot \frac{1}{time}
\]

where [ion1] and [ion2] are the total Na\(^+\) concentrations at the start and end of each time period (i.e., 0-1h, 1-2h, 2-3h, 3-4h, 4-5h, and 5-6h).

Unidirectional efflux rate was calculated for each fish by the difference between unidirectional Na\(^+\) influx rate and net Na\(^+\) flux rate.

**Total RNA Isolation**

Gill tissue total RNA was isolated by TRI reagent (Invitrogen, Carlsbad, California, USA) with RNA quality confirmed by gel electrophoresis (1% agarose). RNA concentration and purity were determined by spectrophotometry using a Nanodrop spectrophotometer before and after DNase
treatment (NanoDrop Technologies, Wilmington, Delaware, USA). Absorbance values (Abs 260/280) in all samples were 1.8-2.1. Two microgram DNase-treated total RNA was used in a cDNA synthesis by reverse transcription using random primers with a reverse transcription kit (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Carlsbad, California, USA).

**Quantitative PCR**

Four claudin-like EST sequences of *Fundulus heteroclitus* (DR047164, DN951669, DR441634, DR441481) were acquired from the National Center for Biotechnology Information (NCBI). A BLAST search against the non-redundant nucleotide database was processed based on their highest sequence similarity to *Takifugu rubripes* claudin nucleotide sequences and these ESTs were assigned the nomenclature, claudin-3, claudin-5, claudin-23, claudin-38, respectively. Primer 3 software was used to develop primers based on these ESTs for qPCR analysis later. All designed primers were ensured a GC base content between 40-60%, a theoretical annealing temperature of 60 °C, and a length of approximately 21 nucleotides maintained. A single product was obtained by agarose gel electrophoresis after each primer amplification. These primer sequences are listed in chapter 2 (Table 2.2).

Quantitative real-time PCR (qPCR) analysis was performed using a SYBR Green detection system (SYBR Green core Reagent, Applied Biosystems) according to the manufacturer's instructions. Reactions were carried out with 5 µL cDNA, 10 nM forward and reverse primer, and 2 µL SYBR Green reagent in a total volume of 20 µL. A three-step cycling protocol was used with 40 cycles of 50 °C for 2 min, 95 °C for 10 min, and 95 °C for 15 s (ABI Prism 7000). Critical
threshold (Ct) values were calculated using the adaptive baseline function in the ABI Prism 7000 SDS software. The amplification efficiencies for each primer set were calculated by serially-diluting cDNA derived from the gills of control fish, and using these dilutions to obtain a linear regression relationship of Ct versus the natural logarithm of relative cDNA concentration. For all primer sets, amplification efficiency varied between 92.1% and 105.5%, with 100% efficiency representing a theoretical doubling in the amount of cDNA after each cycle.

Relative changes in mRNA levels were calculated using the $2^{-\Delta\Delta Ct}$ method (Galvez et al., 2007) using the formula, $\Delta Ct$ target (treatment-control) - $\Delta Ct$ ref (treatment-control), where target refers to the claudin genes, ref refers to the reference gene, (18 S ribosomal RNA (18S rRNA), controls represent the 0.1 ppt-acclimated fish at each time point, and treatments represent either the 0.1 ppt HCl-injected fish. The Ct values for 18s rRNA varied only moderately among the different treatments with a standard error of 0.057. The mean $\Delta\Delta Ct$ value of each 0.1 ppt control was expressed as 1, and relative change in mRNA levels of hypoosmotic treatments expressed relative to this value. A terminal dissociation step (95 °C for 15 s, 60 °C for 20 s, and 95 °C for 15 s) was added to verify the presence of only one amplicon in the PCR reaction.

**Statistical Analysis**

All data collected are expressed as mean ± SE (n) where n equals the number of fish in an experimental group. Statistics on flux data and gill claudin expression was done using one-way ANOVA with Student-Newman-Keuls test. A significance level of $P<0.05$ was chosen. All statistics were done by SPSS version 17.
Results

Unidirectional Sodium Fluxes

The unidirectional Na\(^+\) flux rates for both *Fundulus heteroclitus* populations and *Fundulus majalis* were measured in order to detect potential responding differences among fish during acidic challenges. These data showed that the unidirectional Na\(^+\) and Cl\(^-\) flux rates of fish were significantly affected by metabolic acidosis induced by intraperitoneal injection of HCl, and the magnitude and time-course of this response was dependent on the amount of time fish were kept under acidic conditions (Fig 3.1, 3.2, 3.3).

Acidosis resulted in a significant positive net Na\(^+\) flux in *Fundulus heteroclitus* MDPP between 3h to 6h following acidosis (Fig. 3.1A). Similar effects were seen in the *Fundulus heteroclitus* VAcoast population, except that a significant increase in net Na\(^+\) flux was not elicited until 4 hours following acidosis (Fig 3.2A). In both cases, the initial increase in net Na\(^+\) flux was associated with an increase in unidirectional Na\(^+\) influx, but with no significant effect on Na\(^+\) efflux rate (Fig 3.1A; 3.2A). These data showed both populations of *Fundulus heteroclitus* exhibited similar responses to acidic challenges.

The effect of Na\(^+\) and Cl\(^-\) flux rates in response to one shot of HCl infusion ceased within 24 hours. In order to study the long-term effect of acidic challenges, fish received continuous infusion of HCl at 12h, 24h, 3d, 7d, respectively. During subsequent acid infusions (i.e., 1-7d of acidosis), both net Na\(^+\) flux and unidirectional Na\(^+\) influx rates were also increased for both *Fundulus heteroclitus* populations. The major difference seen following repeated acid infusions was that the acidosis-induced increase in unidirectional Na\(^+\) influx was initiated
sooner after acidosis (Fig. 3.1B), but tended to recover back to control levels by the end of the 6h recovery period. For example, the unidirectional Na\(^+\) influx rate in *Fundulus heteroclitus* MDPP that received a second HCl injection at 24h transfer to 0.1 ppt was elevated by 3h post acidification, although it recovered to

![Graph showing Na\(^+\) flux rates](image)

Fig 3.1: Na\(^+\) influx, efflux and net flux rates in *Fundulus heteroclitus* MDPP at 0 h (A), 24 h (B), 3 day (C) and 7 day (D) point during a 7-day acid challenge period. Positive bars represent influx rate. Negative bars represent efflux rate. Solid bars represent net flux rate. * represents significant difference in influx rates compared to control (0 h); *' represents significant difference in efflux rates compared to control (0 h); *" represents significant differences in net flux rates compared to control (0 h) (p<0.05).

control levels by 6h (Fig 3.1B). The lack of effect of metabolic acidosis on unidirectional Na\(^+\) efflux meant that changes in net Na\(^+\) flux were largely influenced by changes in influx (Fig 3.1B). In contrast, the unidirectional Na\(^+\)
influx rate in the *Fundulus heteroclitus* VAcoast populations was only increased at 5 h post acid infusions, although the net Na\(^+\) flux was significantly increased by 3 h, as seen in *Fundulus heteroclitus* MDPP. Both populations did not show significant differences in Na\(^+\) efflux rates during this period (Fig 3.2B).
only 2 h, but remained elevated despite there only being an increase in unidirectional Na⁺ influx at 3h post acidosis. No significant differences in Na⁺ efflux rates were detected (Fig 3.1C). There were some discrepancies compared to Fundulus heteroclitus VAcoast which showed significant differences in Na⁺ influx rates as early as 2 h. Fundulus heteroclitus VAcoast showed significant net Na⁺ gain at 3 h, 4 h, 5 h, 6 h point (Fig 3.2C).

After 7 d of daily acid infusions, both populations were responding quickly by stimulating Na⁺ influx and net flux by 2h, and recovering to control levels by 3h or 4h post acidification for the Fundulus heteroclitus MDPP and Fundulus heteroclitus VAcoast, respectively (Fig 3.1D, 3.2D). Once again, acidification did not affect unidirectional Na⁺ efflux rates in either population at any time point following administration of a metabolic acidosis treatment (Fig 3.1D, 3.2D).

In comparison with the effects seen in Fundulus heteroclitus, metabolic acidosis elicited severe impairment of Na⁺ homeostasis and led to mortality in Fundulus majalis. Unidirectional Na⁺ influx was not stimulated by acidification by intraperitoneal injection with 1000 nEq.g⁻¹ HCl (Fig 3.3). Both Na⁺ efflux and net flux rates were highly negative after only 1h; an effect that persisted until their death. These data exhibited significant differences of responses to acidic challenges between two species of Fundulus: Fundulus heteroclitus and Fundulus majalis. An increase of Na⁺ influx rates was stimulated in both populations of Fundulus heteroclitus while this increase was not elicited in Fundulus majalis. In addition, both populations of Fundulus heteroclitus survived after HCl infusion while Fundulus majalis VAcoast failed to survive.
**Influence of Metabolic Acidosis on Claudin mRNA Levels**

Four claudin ESTs were selectively studied because previous investigation (see chapter 2) showed these four claudins expressional levels changed in response to hypoosmotic challenges. Claudin 3-like protein mRNA levels in the gills of *Fundulus heteroclitus* MDPP were significantly increased at 24 h post HCl injection, and this significant difference persisted through 7d acidification. A similar trend was observed in the gills of *Fundulus heteroclitus* VAcoast following acidification (Fig 3.4A, Fig 3.5A).

![Graph showing Na+ influx, efflux and net flux rates in Fundulus majalis after HCl injection. Positive bars represent influx rate. Negative bars represent efflux rate. Solid bars represent net flux rate. * represents significant difference in influx rates compared to control (0 h); *' represents significant difference in efflux rates compared to control (0 h); *" represents significant differences in net flux rates compared to control (0 h) (p<0.05).](image)

Claudin 23-like protein mRNA expressions in *Fundulus heteroclitus* MDPP increased significantly after 12 h, 24 h, 3 d, and 7 d acidification. In contrast,
*Fundulus heteroclitus* VAcoast only shows these significant increases at 24 h, 3 d and 7 d time point (Fig 3.4C, Fig 3.5C).

No significant differences are detected on claudin 5-like and claudin 28-like protein mRNA levels throughout the experiments compared to control group (Fig 3.4B, Fig 3.4D, Fig 3.5B, Fig 3.5D).

Fig 3.4: Claudin 3 (A), -5 (B), -23 (C), -28 (D) mRNA expression level changes in *Fundulus heteroclitus* MDPP gills during a 7-day acid challenge period. * represents significant difference in mRNA expression levels compared to control (p<0.05)

**Discussion**

The linkage between osmoregulation and acid-base tolerance in FW-adapted fish has been established for decades. One study suggested that Na⁺
was exchanged for endogenous H⁺ or NH₄⁺ and Cl⁻ was exchanged for endogenous HCO₃⁻ (Krogh, 1938). This process was determined in the gills of freshwater fish via independent electroneutral exchanges (Evans et al., 2005; Marshall and Grosell, 2005). Independent manipulation of these Na⁺/acid and Cl⁻/acid base exchangers were thus applied in various studies and a significant advance in understanding of acid-base regulation was achieved in the past several decades (Cameron, 1978; Cameron, 1984; Claiborne et al., 1997; Goss et al., 1998; Goss et al., 1992; Heisler, 1984; Maetz, 1976; Marshall and Grosell, 2005).

Fig 3.5: Claudin 3 (A), -5 (B), -23 (C), -28 (D) mRNA expression level changes in Fundulus heteroclitus VAcoast gills during a 7-day acid challenge period. * represents significant difference in mRNA expression levels compared to control (p<0.05).
This model of Na\textsuperscript{+}/acid and Cl\textsuperscript{-}/base exchangers does not apply for all fish species. The linkage between ion regulation and acid-base transport in *Fundulus heteroclitus* appears to be very different from that in other fish species (Patrick and Wood, 1999). The main difference between *Fundulus* species and other fish species involves an active Cl\textsuperscript{-} uptake system in *Fundulus* species which requires further study, that is, *Fundulus* species does not show an active Cl\textsuperscript{-} uptake while most other teleost do. (Patrick et al., 1997; Wood and Marshall, 1994). Lack of active Cl\textsuperscript{-} uptake in *Fundulus* allows a unique model species which departs from the traditional model. Intraspecific variation exists between two populations of *Fundulus heteroclitus*: northern and southern populations (Scott et al., 2004b). Based on variations in mitochondrial haplotype, nuclear proteins and microsatellites, *Fundulus heteroclitus* can be divided into at least two populations: a northern and a southern population (Adams et al., 2006; Bernardi et al., 1993; Duvernell et al., 2008; Smith et al., 1998; Whitehead, 2009; Whitehead, 2010; Whitehead and Crawford, 2006; Whitehead et al., 2010; Whitehead A, 2010). Northern populations more readily tolerate hypoosmotic challenges than southern populations due to their better ability to minimize passive Cl\textsuperscript{-} loss through branchial paracellular pathways (Scott et al., 2004b). Based on previous studies, *Fundulus heteroclitus* MDPP belongs to northern population while *Fundulus heteroclitus* VAcoast belongs to southern population (Adams et al., 2006; Smith et al., 1998; Whitehead, 2009; Whitehead, 2010; Whitehead and Crawford, 2006; Whitehead et al., 2010; Whitehead A, 2010). Furthermore, net Na\textsuperscript{+} and Cl\textsuperscript{-} flux differential is a very practical and useful means for estimating
acid-base balance *in vivo* in that net amount of acid secreted equals to net amount of Na\(^+\) uptake (Patrick and Wood, 1999). The above information construct the background of this study to investigate if intraspecific variation exists between two populations of *Fundulus heteroclitus* during acidic challenge via characterizing Na\(^+\) flux rates across fish gills.

Generally both populations of *Fundulus heteroclitus* showed active Na\(^+\) uptake after HCl injection throughout the experimental period (Fig 3.1, Fig 3.2). This is consistent with some previous studies which demonstrated increased Na\(^+\) uptake after acid load (Patrick et al., 1997). Patrick et al showed active Na\(^+\) uptake occurs 3 h post injection (Patrick et al., 1997). The findings of the current study are consistent with those findings for both populations of *Fundulus heteroclitus*. This net Na\(^+\) gain was possibly caused by increased activity of Na\(^+\)/acid exchangers in the apical membrane of branchial cells. As stated above, net Na\(^+\) and Cl\(^-\) flux difference is a very practical and useful means for estimating acid-base balance *in vivo* (Patrick and Wood, 1999). The amount of net Na\(^+\) uptake corresponds to that of net acid secretion. The acid secretion in *Fundulus heteroclitus* is likely to be complete within 6 h post HCl injection (Fig 3.1, 3.2). Interestingly, this active Na\(^+\) uptake occurred much earlier as the experiments progressed. At 7 d time point, this active Na\(^+\) uptake occurred 2 h after HCl injection on both populations of *Fundulus heteroclitus*. This is possibly due to the increased expression of this Na\(^+\)/acid exchangers caused by previous HCl injections. All previous studies were conducted over shorter durations (Evans et
al., 2005; Patrick et al., 1997; Wood and Marshall, 1994), thus this study is the first to demonstrate this adaptive process of acid tolerance in fish.

However significant differences were not detected in this adaptive process between the two populations of *Fundulus heteroclitus* (Fig 3.1, 3.2). Other studies have shown that intraspecific differences do exist among *Fundulus heteroclitus* populations (Able and Palmer, 1988a; Scott et al., 2004b), but it is not likely the case for acid excretion or Na\(^+\) uptake.

*Fundulus majalis* is a marine fish and can tolerate FW when salinity is gradually decreased. After the acid load, no increase of Na\(^+\) uptake was detected even 6 hours after the injection (Fig 3.3). There was a significant Na\(^+\) loss, which was possibly due to increased permeability to Na\(^+\) at paracellular pathways after acid load. These data indicated that *Fundulus majalis* failed to survive during acidic challenge because of its inability to take up Na\(^+\) through a Na\(^+\)/H\(^+\) exchange system. Their failure to regulate permeability to Na\(^+\) at paracellular pathways during acidic challenge may also contribute to their death. There is no published data on acid tolerance of *Fundulus majalis* until now. These data are the first to illustrate the physiological processes of Na\(^+\) transport in *Fundulus majalis* during acidic challenge.

Previous work has demonstrated the importance of claudin proteins in epithelial paracellular pathway regulations (Koval, 2006). This study selectively investigated mRNA expressional levels between two populations of *Fundulus heteroclitus* post HCl injection. Our data show that claudin 3-like protein mRNA levels decreased post HCl injection (Fig 3.4A, Fig 3.5A). Tipsmark et al found
claudin 3 protein levels were higher in tilapia in FW than that in SW, which indicates that claudin 3 may contribute to a ‘tighter’ epithelia (Tipsmark et al., 2008a). For Fundulus heteroclitus undergoing HCl exposure, an increased paracellular permeability to Cl− would facilitate the excretion of Cl− and thus reach a better ability to maintain acid-base balance (Wood and Marshall, 1994). In this experiment, a ‘tighter’ epithelia was not desired because a ‘leaky’ paracellular pathway was needed in order to facilitate Cl− excretion. It is plausible to infer that the decreased claudin 3-like protein in Fundulus heteroclitus during acidic challenge may be related to increased permeability to Cl− in paracellular pathways, which facilitates acid tolerance. These findings together indicate the roles of claudin 3-like proteins in regulating Cl− transport across paracellular pathways. Further study is needed for a better understanding of the mechanisms of this process.

Claudin 5-like protein mRNA expressional levels showed no significant differences throughout the experiment in both populations of Fundulus heteroclitus post HCl injection (Fig 3.4B, Fig 3.5B). There is no direct study on roles of claudin 5 protein in acid-base tolerance in fish. However a recent study showed claudin-5 contributed to forming a ‘tight’ brain barrier in zebrafish (Abdelilah-Seyfried, 2010). Some studies have indicated that claudin-5 decreased paracellular permeability in vivo (Wen et al., 2004). These findings in zebrafish and mammal are not in accordance with this study, which indicated a controversial role of claudin-5 protein in regulating permeability and that different
Claudin isoforms may cause distinct functions. The specific roles of claudin 5-like proteins in *Fundulus heteroclitus* gills are unclear and need further investigation. Claudin 23-like protein levels increased on both populations of *Fundulus heteroclitus* after HCl injection (Fig 3.4C, Fig 3.5C). To the author’s knowledge, there are no known reports regarding claudin-23 function in fish osmoregulation or paracellular permeability in mammals. The up-regulation of claudin-23 in osmoregulation and acid-base tolerance in *Fundulus* species indicated an important role of this protein though specific contribution of this protein is still not clear now.

Claudin 28-like protein expression remain unchanged which may indicate it is not actively involved in acid-base regulation in *Fundulus* species (Fig 3.4D, Fig 3.5D). However Claudin-28 expression levels decreased in *Fundulus grandis* following FW exposure based on the study in Chapter 2. Another study did not find claudin-28 expression change in Atlantic salmon following salinity transfers (Tipsmark et al., 2008b). No further information could be obtained from mammals because it does not exist in mammal. The actual role of this claudin 28-like protein in acid tolerance in *Fundulus heteroclitus* needs further investigation.
CHAPTER 4: PROSPECTIVES

Previous studies show that *Fundulus* species exhibit unexpected complexity in gene expressions. This unexpected complexity includes divergence in population genetics, physiological adaptation variation, transcriptional difference, etc. Comparative analyses among *Fundulus* species provide a powerful approach to understand physiological and evolutionary variation. This powerful tool can be used to elucidate the genetic bases of target gene expression changes. Similar comparative experiments within *Fundulus* can be applied to seek better understandings on underlying transcriptional processes and regulatory mechanisms responsible for population adaptation to environmental stressors such as salinity, temperature, pH, pollutant etc.

One of the key characteristics during adaptation to salinity challenges in euryhaline fish is the morphological changes in branchial epithelia. A series of factors may contribute to this morphological transformation such as hormones, phosphorylation on some transmembrane proteins, osmotic sensing receptors on the cell membrane etc. However a definite description to this question is far from complete. In addition, signal transductions for cell differentiation and apoptosis need further investigation. A combination of various approaches such as morphological, molecular, and gene transcriptional study will contribute to reach a better understanding of this big picture.

Despite the improved understandings in fish osmoregulation and acid-base tolerance in the past several decades, most of territories are still not fully charted. For example, How does osmotic sensing work? What are the specific signal
transduction processes involved in protein expression changes responsible for solute transport across epithelia? What contributes to interspecific and intraspecific variation in fish osmoregulation? Experiments of whole animal, in vitro, molecular biology, morphology, comparative analysis could help us reach a better understanding in this issue.

*Fundulus* species which osmoregulatory mechanism is a departure from the traditional model serve as excellent candidates studying fish osmoregulation. Failure to locate an active Cl⁻ uptake system in these species makes branchial paracellular ways assume more important roles in maintaining ion homeostasis in fish during hypoosmotic challenges. Claudins, the key components regulating permeability of paracellular pathways, require extensive studies in order to elucidate the actual functions of specific claudins. Immunohistochemistry staining to specific claudin proteins, gene knockdown (siRNA, morpholinos, etc), protein structure analysis will all provide direct valuable information to address this question.

Participation of claudin proteins is crucial for determining the appropriate permeability characteristics of FW gill epithelia. Unfortunately there is not much information available on regulatory mechanisms of these proteins. Some studies find cortisol, prolactin and growth hormone are the upstream regulators of claudin proteins. However it is hard to explain some controversial findings. For example, cortisol exhibits increased expression during both hypo- and hyperosmotic challenges. The general nature of cortisol effect turning into a specific development in either direction remains unknown. Interactions between cortisol
and other endocrine or paracrine factors or with the osmotic strain itself need further investigation.

The linkage between osmoregulation and acid-base tolerance in fish is far from complete too. Previous studies show extensive interspecific and intraspecific variation in osmoregulation and acid-base tolerance among fish species. Do these variations come from differential gene expression? Or from the availability of some specific genes? How much effect do ecological environments exert on these variations? Obviously comparative analyses study will help us elucidate these questions.
REFERENCES CITED


Avella, M., Bornancin, M., 1989. A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (Salmo gairdneri. 142, 155-175.


Clelland, E.S., Bui, P., Bagherie-Lachidan, M., Kelly, S.P., 2010. Spatial and salinity-induced alterations in claudin-3 isoform mRNA along the


Gilmour, K.M., Bayaa, M., Kenney, L., McNeill, B., Perry, S.F., 2006. Type IV carbonic anhydrase is present in the gills of spiny dogfish (Squalus acanthias). Comparative and Evolutionary Physiology 292, 556-567.


Goss, G.G., Wood, C.M., 1990b. Na\textsuperscript{+} and Cl\textsuperscript{−} uptake kinetics, diffusive effluxes and acidic equivalent fluxes across the gills of rainbow trout. 2. Responses to bicarbonate infusion. J. Exp. Biol. 152, 549-571.


Krogh, A., 1938. The active transport of ions in some freshwater animals. 25, 335-350.


Loeb, J., Wasteneys, H., 1912. On the adaptation of fish (Fundulus) to higher temperatures. J. Exp. Zool. 12, 543-557.


Sumner, F.B., 1911. Fundulus and fresh water. Sci. 34, 928-931.


Tipsmark, C.K., Madsen, S.S., Seidelin, M., Christensen, A.S., Cutler, C.P., Cramb, G., 2002. Dynamics of Na\(^+\),K\(^+\),2Cl\(^-\) cotransporter and Na\(^+\),K\(^+\)-ATPase expression in the branchial epithelium of brown trout (Salmo
trutta) and Atlantic salmon (Salmo salar). Journal of Experimental Zoology 293, 106-118.


Whitehead, A., 2010. The evolutionary radiation of diverse osmotolerant physiologies in killifish (Fundulus sp.). Evolution 64, 2070-2085.


VITA

Shujun Zhang is the oldest son among the three of Weidong Zhang and Xiaofeng Wang. He graduated with a Bachelor of Medicine degree from Tongji Medical College in Wuhan, China, in July of 2003. Then he started doing his residency in Liver Cancer in Tongji Hospital and obtained his Master degree from Huazhong University of Science & Technology in 2005. He entered an orthopedic residency program in Wuhan Orthopedics Hospital in August of 2005. He started his doctoral work in Louisiana State University in Baton Rouge, Louisiana from fall of 2006. Mr. Zhang will graduate with the degree of Doctor of Philosophy in Biological Sciences in the Summer of 2012.