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Expression of Claudins in Gill and Opercular Membranes in Relation to Osmoregulation in Euryhaline Atlantic Killifish, Fundulus heteroclitus

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Expression of Claudins in Gill and Opercular Membranes in Relation to Osmoregulation in Euryhaline Atlantic Killifish, *Fundulus heteroclitus*

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by

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This thesis is approved for recommendation to the graduate council.

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Abstract

Atlantic killifish (*Fundulus heteroclitus*) are euryhaline teleosts, which means they can thrive in environments with varying salinities, ranging from concentrated seawater (SW) to dilute fresh water (FW) and thus survive in estuaries. To be able to maintain ionic and osmotic homeostasis in these diverse salinities, osmoregulatory organs like gill and opercular membrane must be able to adjust the permeability and transport of water and ions. The surface epithelia in these organs need to prevent the passive flux of ions and water in a drastically changing environment, while being able to switch between active ion secretion or uptake dependent upon environmental salinity. The tight junctions between the epithelial cells are critical to this phenotypic plasticity. The claudin (Cldn) proteins define tight junction permeability and molecular variants predicted to be important in gill and opercular membranes (*cldn30c, cldn32a, cldn10c, cldn10d, cldn10e, cldn10f)* are the focus of this thesis. The studies tested the organs distribution of these *cldn*s and examined their regulation in gill and opercular membranes by salinity, prolactin, and cortisol. Analysis of the organ distribution largely confirmed that they are highly expressed in gill and opercular membranes when compared with muscle, liver, intestine, kidney and brain. While salinity acclimation generally did not affect mRNA levels, Cldn30c protein expression was elevated in FW compared to SW. Hormone *ex vivo* gill explant experiments showed that the FW hormone prolactin specifically stimulated *cldn30c* and *cldn32a* , both hypothesized to be critical to salt retention in freshwater. In contrast, cortisol affected *cldn10c* and *cldn10f* with a putative role in salt secretion in seawater. The thesis demonstrated the specific hormonal control of *cldns* and the significance of post-translational mechanisms was emphasized by the difference in salinity induced Cldn30c mRNA and protein levels.

Acknowledgments

To my husband, Sam, words can't express how grateful I am for your unwavering support of my dreams. I couldn't have done this without you.

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Project Objectives

The objective of this project was to advance our knowledge of function and regulation of intercellular tight junction proteins of the claudin (Cldn) family. These proteins control passive permeability in gill and skin and thus safeguard regulation of blood volume and osmotic consistency in aquatic organisms. The objective was pursued through a combination of *in vivo* and *ex vivo* culture experiments.

When fish are surrounded by concentrated seawater (SW), the gill needs to limit osmotic water loss and influx of ions while complexes of ion transporting cells (ionocytes) actively secrete monovalent ions. According to current models, sodium efflux is electrically coupled to trans-cellular chloride secretion and involves a paracellular cation-selective pathway thought to be controlled by specific Cldn10 paralogs.

In contrast, freshwater (FW) fish must limit osmotic water influx and ion loss to the dilute environment while ionocytes actively take up salts from the environment. Ion uptake does not involve paracellular transport; accordingly, freshwater acclimation is thought to be associated with the enhancement of tight junction seals that support salt retention. The change is believed to be associated with the expression of barrier Cldns like Cldn30c and Cldn32a.

From the above-described models in SW and FW fish, it is clear that estuarine species experiencing fluctuations in their environments must have a high degree of functional plasticity and that such species can be beneficial when studying the function and regulation of gill and integument.

The objective of the present master thesis was to use the estuarine Atlantic killifish (*Fundulus heteroclitus*) to study the significance, function, and regulation of expression of the Cldn paralogs mentioned above in gill and opercular skin. To this end, the projects aimed to examine

organ distribution, regulation by salinity, and regulation by hormones of the selected Cldns. It was planned to use transport proteins particular to respectively, the FW and SW gill phenotype as reference points. The final objective was to study the effect of Cldn30c knockdown on paracellular permeability.

Introduction

Fish are the most diverse group of vertebrates on Earth, with over 25,000 species. Fish species have evolved to be radically different from one another. The evolution resulted in many fish adapting to different environments that can vastly vary in salinity. Some fish are stenohaline, meaning that they can only tolerate a narrow range of salinities and typically live in only FW (<0.5 ppt or <15 mOsm/L) or SW (35 ppt or 1050 mOsm/L). Other kinds of fish are euryhaline, meaning they can live in varying salinities and successfully switch between hyposmoregulation when in concentrated SW and hyperosmoregulation when in dilute FW. Euryhaline teleosts maintain a relatively steady plasma osmotic concentration between 300 and 400 mOsm/L in all environments (Evans et al., 2005). Salmon, for example, are fish that live most of their lives in seawater with high salt content but migrate to freshwater rivers to spawn. Atlantic killifish (*Fundulus heteroclitus*) are found in estuaries where the salinity of their environment can vary based on weather or ocean current conditions. Due to their ability to rather quickly switch their methods of osmoregulation based on phenotypic plasticity of the osmoregulatory organs killifish are an excellent model to understand the regulation and basal function of these organs since functional changes can be induced by changing their environmental salinity. The gill is the organ most predominately used for osmoregulation (Evans et al., 2005) and because the opercular membrane represents a good gill model applicable for electrophysiology (Marshall and Bellamy, 2010), these two organs are the focus of this thesis.

Atlantic Killifish

Atlantic killifish (*F. heteroclitus*) are one of the most common fish found on the East coast of North America. These fish are small, typically between 50 and 105 mm in length, and usually

weigh less than 8 grams (Abraham, 1985). Because they are small, Atlantic killifish make for great research as housing large numbers of them is relatively easy. They were once the most common wild fish utilized in research (Rosen, 1973 – Abraham, 1985). The male killifish is more vibrant in color than their female counterparts. After the first year of life, female killifish are typically larger than the males. While these are not large enough to be considered game or useful for human consumption, Atlantic killifish play a major role in the food chain in the ecosystem of the Atlantic estuaries. In the wild, killifish eat mosquito larvae, making them invaluable for maintaining the population of mosquitos in the swamps of Florida (Abraham, 1985).

Life Cycle

The vibrant yellow color found on the underbelly of the male killifish appears more saturated and shinier during the mating season. Female killifish produce ova with the amount determined by the size of the fish but can range between 200-800 over a 3-5 day span in the early spawning season. These fish can spawn up to 8 times in a single season, and most of the fertilized eggs are able to survive until hatching (Abraham, 1985).

The killifish eggs mature in air and take 7 to 8 days to reach maturity in the wild. After hatching, the fish go through a larval stage where they grow scales and fin rays. Once the larva takes on the characteristics of the killifish, they are considered juvenile. Atlantic killifish are considered adult and sexually mature once females reach a length of 38 mm and males reach a length of 32 mm, and this maturation usually goes into the second year of life. This young adult stage has a 99.5% mortality rate, but if they survive, the Atlantic killifish have an expected life span of about 2-3 years (Abraham, 1985).

Environment

Because killifish have an upturned mouth, they are well equipped for surface feeding. However, the Atlantic killifish will eat from the surface or mid-water and occasionally feed on the river bottom should the need arise. These fish are omnivores with a diet that consists of algae, plankton, small shellfish, insect eggs, and other sources that are found in the estuaries. The fish swallow their food whole, so the size of the gape of their mouths limits the size of prey. The predators of the Atlantic killifish are birds, larger fish, and eels that call the Atlantic coast estuaries home (Abraham, 1985).

F. heteroclitus are eurythermal as well as euryhaline. In the northern United States specifically, killifish are exposed to rapid temperature changes of around 15 ^{o}C in either direction. Because the estuaries commonly have rapid salinity changes, the killifish are able to abruptly change the method of osmoregulation being utilized to maintain homeostasis. The ability to compensate for temperature and imbalance allows Atlantic killifish to thrive in a multitude of environments (Abraham, 1985).

Osmoregulation

Atlantic killifish are a part of less than 10% of teleosts that are euryhaline and able to maintain ionic and osmotic homeostasis when placed in environments with significantly varying salinities. These fish can tolerate a range of salinity from FW to over full-strength SW (Shultz & McCormick, 2013). Found in estuaries where the salinity is constantly varied, Atlantic killifish must be, and are, able to change the mode of osmoregulation quickly and the gill itself invert the direction of salt transport from active uptake in dilute environment to ion secretion is concentrated seawater.

No matter the salinity of the environment, fish need to osmoregulate. Osmoregulation occurs in fish gills, gut, and renal system. There are many different ions that organisms need to balance, including sodium, chloride, calcium, and bicarbonate. Euryhaline fish, like the Atlantic killifish, use specialized cells with specific transport proteins, and fish permeability can also be modulated by changing tight junctions and aquaporins in surface cells; these changes adjust passive fluxes of solute and water as well as active ion transport in or out to maintain a healthy balance of ions in the blood.

Intercellular Junctions

Vertebrate epithelial cells have four different kinds of intercellular junctions; The tight junction (*zonula occludens*), adherens junction (*zonula adhaerens*), and desmosomes (*macula adhaerens*) were observed and described using electron microscopy in 1963 (Farquar & Palade, 1963). Gap junctions were observed and characterized using electron microscopy in 1989 (Swenson et al., 1989). Each kind of junction has specific types of integral proteins embedded into them. The structures of the intercellular junctions are illustrated in figure 1.1 below.

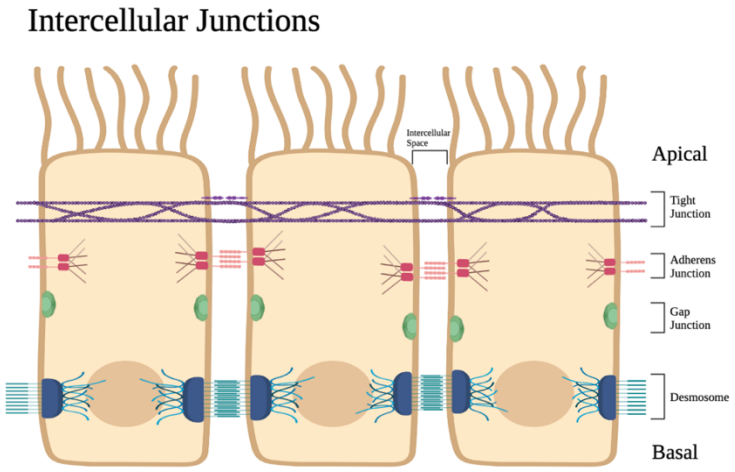

Fig. 1.1– Diagram illustrating the 4 types of intercellular junctions and methods of communication, illustrated on ciliated epithelial cells. Illustration created on BioRender.com, influenced by Eftekhari et al., 2020 and Tsukita et al., 2001.

Tight Junctions

Tight junctions are the most apical of epithelial cell-cell junctions and are a point between cells that create separation between apical and basolateral membranes, limit solute movement between adjacent cells, and act as a sealed connection point between adjacent cells (Shin et al., 2006). Tight junctions serve two purposes in the cells; they maintain polarity for the cell and regulate ion, water, and other molecular passage through the intercellular space (Campbell et al., 2017). Tight junctions consist of a multitude of different proteins with different functions. In vertebrates, tight junctions have scaffolding proteins (i.e., ZO proteins) and transmembrane proteins (i.e., occludins, cldns, and junctional adhesion molecules (JAMs)) (Günzel & Fromm, 2012). The structures found within the tight junction are illustrated in figure 1.2.

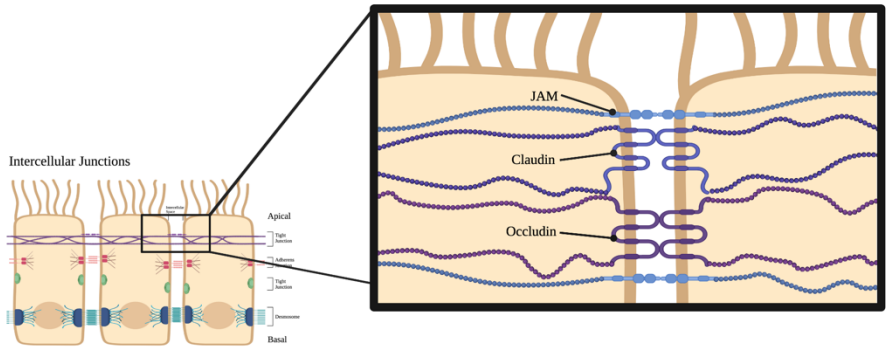

Fig. 1.2 – Diagram representing the types of proteins found within tight junctions. Illustration created on BioRender.com, influenced by Eftekhari et al., 2020.

Claudins

Cldn proteins are found within tight junctions of cells and regulate ion permeability. These proteins belong to a large gene family that contains many paralogs. Cldns have 4 transmembrane helices, 2 extracellular domains, and internal C- and N-termini (Van Itallie & Anderson, 2006), illustrated in figure 1.3.

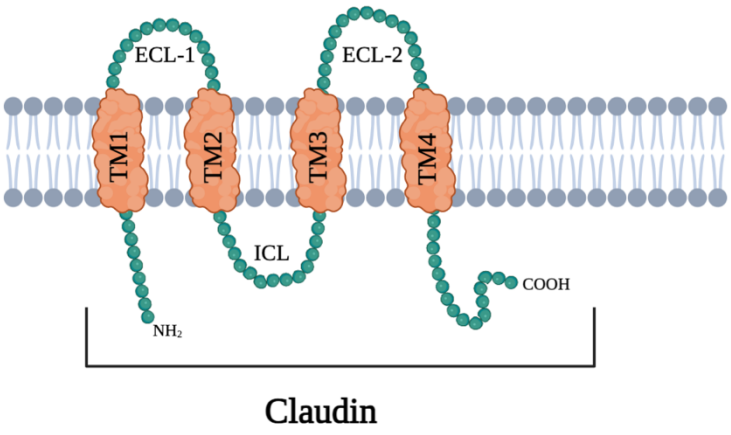

Fig. 1.3: Diagram of the common structure of a claudin protein, inspired by description in Van Itallie & Anderson, 2006. Diagram created on BioRender.com. Transmembrane regions = TM, extracellular loops = ECL, intercellular loop = ICL. Claudin is shown woven through the lipid bilayer of a cell membrane.

Within the tight junction, claudins play a heavy role in limiting paracellular water and solute permeability and may do this in a general manner or do it selectively (Chasiotis et al., 2012). Some claudins are considered to be part of 'leaky junctions' and to be non-selective, whereas others allow permeation for only specific types of ions through the formation of pores. These pores are often selective to the charge of the ions (Shen et al., 2011) and may in some cases even conduct water transport as claudin-2 and claudin-15 in the mammalian kidney (Rosenthal et al., 2019).

Seawater Osmoregulation

Atlantic killifish are found in the estuaries on the east coast of the United States, and the water in those estuaries can reach salinities up to 35 parts per thousand (ppt), or 1050 mOsm, which is the salinity of the Atlantic Ocean. The fish are in a hyperosmotic environment in SW, as their blood osmolarity is around 380 mOsm/kg, or 12 ppt. Under these conditions the fish get constantly lose water osmotically and gain ions from the environment especially across the gill and the fish must therefore drink SW to volume regulate and retain as much water as possible. The subsequent intestinal water uptake by osmosis is secondary to active NaCl uptake by the enterocytes, and the kidney excretes a sparce isosmotic urine to limit water loss and gets rid of divalent ions specifically (Evans et al., 2005). Due to the high salt load from the ingested water the gill has to

actively excrete NaCl to safeguard blood osmolality. The mentioned passive (dotted arrows) and active (arrows) mechanisms in seawater is illustrated in Figure 1.4.

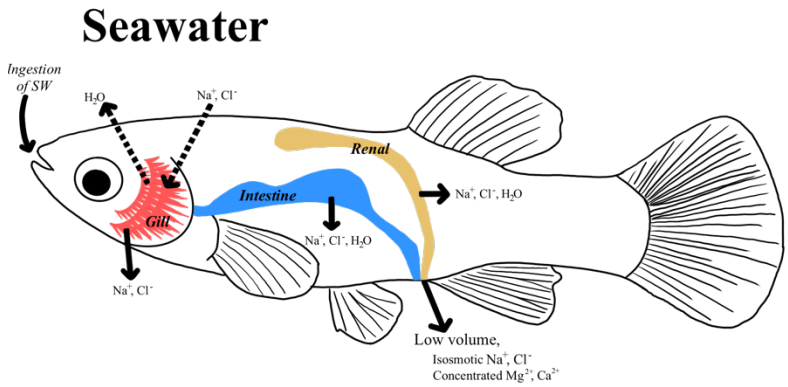

Fig. 1.4 – Mechanism of osmoregulation in killifish living in seawater or a hyperosmotic environment. Dotted arrows indicate passive flow, solid arrows indicate active processes. Figure based on/modified from Randall, Burggren, French, *Animal Physiology*, 1997

Hormonal Control of Osmoregulation in the Seawater Environment

Hormones play a significant role in the osmoregulation of Atlantic killifish in seawater and freshwater environments.

Cortisol is a corticosteroid hormone that the interrenal gland produces in fish (Greenwood et al., 2003). For a while, cortisol was considered a hormone utilized primarily in seawater acclimation due to the ability of cortisol injections to reverse the reduction of ion excretion caused by hypophysectomy in Atlantic killifish in SW (Pickford et al., 1970). However, more recent studies have shown that cortisol levels increased in freshwater acclimation of many species of teleost fish (McCormick, 2001). Cortisol is often referred to as a stress hormone. Large amounts of stress occur when fish are transferred from one environment of one salinity to another environment with significantly different salinity. This is demonstrated on a hormonal level when

cortisol levels rise, whether transferred from SW to FW or vice-versa, as environmental transfers are stressful, and cortisol rises because of stress (Takei & McCormick, 2013, Mommsen, 1999).

Mitochondrion-Rich Cells and Transport Proteins of the Seawater Fish

Ionocytes are specialized cells that regulate ion exchange. They are often located in the interlamellar regions of the gill filament, and many of these epithelial ionocytes are also rich in mitochondria (Evans et al., 2005). Mitochondrion-rich cells (MRCs) are a relatively abundant type of ionocyte in the osmoregulatory organs of the Atlantic killifish. We see them in the highest concentrations in the killifish gill and opercular membranes, with the opercular membrane having up to 60% of cells as MRCs (Evans et al., 2005). These cells were first discovered and described by Keys and Wilmer in 1932 and were described to have distinct basolateral folding sheets, significant Na^+/K^+-ATPase (Nka) activity, and numerous mitochondria (Keys and Willmer, 1932). When euryhaline fish are suddenly exposed to a hyperosmotic environment, cortisol levels increase, and the increase in cortisol is correlated with an increase in the size and number of MRCs. Cortisol facilitates the upregulation and insertion of NKA and Na^+, K^+, $2Cl^-$ cotransporter (Nkcc into the basolateral membrane and the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel into the apical membrane of the MRCs. This is all instrumental to excretion of salt from MRCs (Hiroi et al., 2005, McCormick, 2001), which is required for acclimation to and osmoregulation in SW. The apical membrane of MRCs is morphologically simple with a complex subapical region with a tubular and vesicular network (Laurent, 1984). There are tight junctions between pavement cells (PVCs) and MRCs, which were illustrated in figure 1.2, and the MRCs also interact with accessory cells (ACs). Currently, the functions of seawater ACs is unknown.

The teleost MRCs can mediate the transport of chloride ions from the cell and into the environment against a strong gradient. The mechanism in which this occurs in the teleost gill is as described: Na^+, K^+, and Cl^- enter the cell of the gill epithelia through the basolateral Nkcc. This secondary active transport is driven by sodium's chemical gradient that is maintained by Nka that also helps create a negative membrane potential when 3 Na^+ ions are pumped out of the cell in exchange for 2 K^+ ions. This negative membrane potential facilitates chloride excretion through the apical channel, commonly known as the Cftr chloride channel. The electrical potential across the gill epithelium induces the Na^+ across leaky tight junctions found between the ACs and the MRCs. This process is illustrated in figure 1.5.

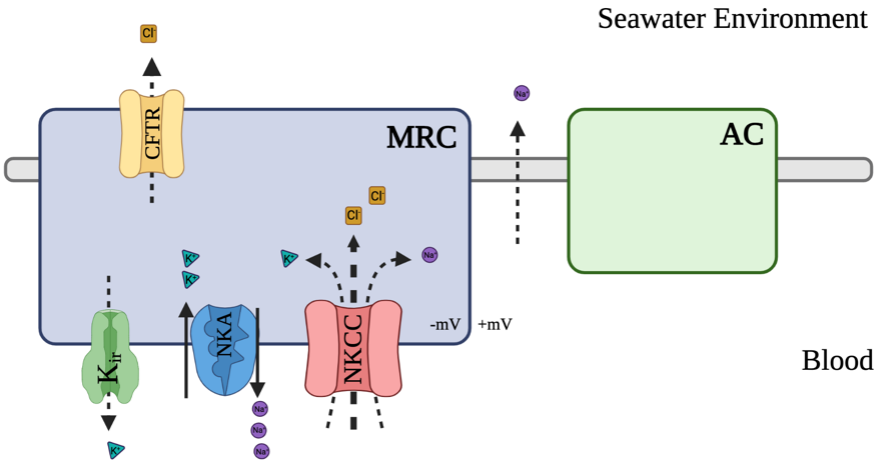

Fig. 1.5 – Mechanism of Na^+, K^+, and Cl^- transport by seawater (SW) teleost gill mitochondrion-rich cells (MRCs). AC = accessory cell K_{ir} = K^+ channel, NKA = Na^+/K^+ ATPase, NKCC = Na^+ ,K^+,$2Cl^-$ cotransporter, CFTR = cystic fibrosis transmembrane conductance regulator. Dotted arrows indicate passive flow, solid arrows indicate active processes. Diagram created on BioRender.com, diagram modified from Evans et al., 2005.

Putative Cation-Selective Pore Forming Claudin-10 Paralogs

As discussed earlier, claudins are proteins found within the tight junctions of epithelial cells and play an important role in the regulation of ion permeability. There is some evidence that select

Cldn10 paralogs are involved in the formation of cation-selective pores in the tight junction protein of gill and opercular membranes of fish placed in a hyperosmotic environment. Cldn10e was found to be increased in Atlantic salmon when transferred from FW to SW (Tipsmark et. al, 2008). Claudin-10d and claudin-10e were found to have nearly no expression in pavement cells and high expression in MRCs, leading to the conclusion that they are most likely utilized in seawater acclimation and osmoregulation (Bui & Kelly, 2014). The paralogs Cldn-10d and Cldn10e both had a marked increase in mRNA in killifish transferred from FW to SW after at least 3 days, and Cldn-10c and Cldn-10d showed a 4-fold increase in mRNA abundance when transferred from SW to 2SW (double salinity of SW), and during these transfers, they only experienced mild interruptions in osmotic and ionic homeostasis (Marshall et al., 2018). The mechanism by which the fish were able to maintain this balance was studied by Marshall and coworkers (2018) who found that based on *in silico* analysis suggested that ions were dehydrated upon movement through the junction, implying that the Cldn10 paralogs create a junction that is not permeable to water. Anti-parallel alignment study of the Cldn10 paralog sequences suggested that pore formation through ionic attractions was a possible function of those proteins.

Freshwater Osmoregulation

The Atlantic killifish live in estuaries where the salinity can vary greatly, as the river water is FW, and it will sometimes mix with SW. That means that the killifish are occasionally exposed to a hypoosmotic environment to their plasma ion concentration. With an osmolality of approximately 2 mOsm/L in the FW environment, the killifish cannot drink any water, which would dilute the ion concentration in their systems. The fish gill actively take up what salt they can from the environment to compensate for passively loss of ions across the gill and with the

urine. Due to the influx of water and the low concentration of NaCl, the killifish must excrete high volume, low concentration urine. Despite the diluted urine, there is still a significant loss of salt and minerals, and to make up for that loss, the fish must also eat food rich in salt and minerals. This mechanism with passive (dotted arrows) and active (arrow) processes is illustrated in figure 1.6 below.

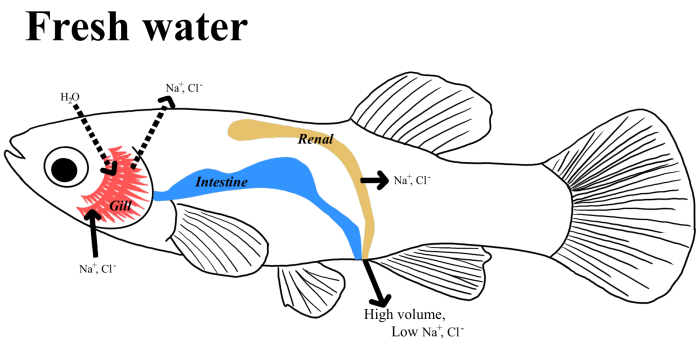

Fig. 1.6 – Mechanism of osmoregulation in killifish living in freshwater or a hypoosmotic environment. Dotted arrows indicate passive flow, solid arrows indicate active processes. Figure based on/modified from Randall, Burggren, French, *Animal Physiology*, 1997

Hormone Control of Osmoregulation in a Freshwater Environment

Similar to when in SW, fish living in FW use hormones to regulate plasma osmolality. Living in an environment with a salinity unlike the plasma of the fish causes stress, and stress leads to an increase in cortisol secretion. As referenced earlier (McCormick, 2001), cortisol is often considered a SW regulating hormone, but the stress response seen in FW leads to a similar increase in cortisol compared to SW.

Another hormone that is used by fish to osmoregulate is prolactin. Prolactin is a pituitary hormone that has a clear effect on the gill function of fish in FW environments. In 1959, Pickford and Phillips published a paper that demonstrated that prolactin was necessary for survival for FW fish. Pickford and Phillips performed hypophysectomies on killifish, resulting in

the fish being unable to produce prolactin on their own. Unless treated with prolactin, the killifish could not survive in FW. (Pickford & Phillips, 1959). Prolactin has also been shown to reduce gill NKA activity in certain euryhaline teleosts, but not in others. Killifish were among those in which prolactin decreased gill NKA but stimulated kidney NKA (Pickford et at., 1970). There is some recent research that suggests that prolactin influences certain types of Cldn proteins insertion into the tight junctions of MRCs, specifically in hypoosmotic environments (Tipsmark et al., 2016).

Hypoosmotic-Specific Osmoregulatory Cells and Proteins

Ionocytes are vital for the osmoregulation of fish, and depending on the environment, there are specific cells that are in use. Due to the osmotic pressure of the environmental water on the cells of the fish, the epithelial cells must be able to uptake what few ions are present from the fresh water. There are 3 types of ionocytes that are present and active in the gill of FW fish: the H^+-ATPase (HR) cells, the NKA-rich (NaR) cells, and the Na^+-Cl^- cotransporter (NCC) cells. FW fish must actively take up Na^+ to counteract the passive efflux caused by the low salinity of the environment, and the NCC cells use NCC protein channels to do so. NaR cells contain an epithelium calcium channel (ECaC) that allows calcium to enter the cell apically from the environment, after which it moves from the cytosol to the blood side by secondarily active transport driven by NKA (Hwang, 2011). The uptake of chloride by the freshwater fish is usually considered to be performed by the Cl^-/HCO_3^- exchange (Evans et al., 2005). The NCC cells are named like the cotransporter protein found within them and in the Atlantic killifish, ncc2 is

abundant and vital for osmoregulation in FW (Breves, et al., 2020). An illustration of the FW is shown in figure 1.7, modified from Hwang, 2011.

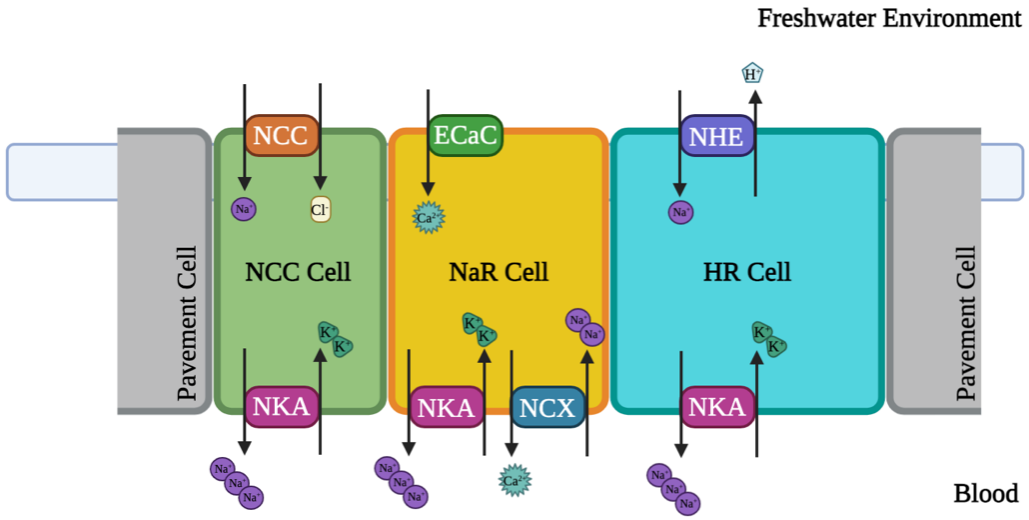

Fig. 1.7–Modified from Hwang, 2011. Designed using BioRender.com. Illustration depicting the three types of ionocytes found in the gill and epithelium of freshwater fish. Freshwater ionocytes included are Na^+/K^+-ATPase rich (NaR) H^+-ATPase rich (HR), and Na^+,Cl^- cotransporter (NCC). The protein channels included are: Na^+/K^+-ATPase (NKA), Na^+,Cl^- cotransporter (NCC), epithelium-Ca^{2+} channel (ECaC), Na^+/Ca^{2+} ion exchanger (NCX), and Na^+/H^+ exchanger (NHE).

Aquaporins are water channel proteins that also play a large role in the maintenance of osmotic homeostasis. Aquaporins (AQPs) form pores in the plasma membrane of cells and regulates water movement across membranes (Madsen et al., 2015). Of particular interest for this study is aquaporin-3 (Aqp3) because it is thought to be a primary water pore in the gill and opercular membranes. When exposed to SW, salmon gill showed a decreased expression of *aqp3* mRNA as compared to the levels found in the FW acclimated salmon (Tipsmark et al., 2010), suggesting Aqp3 is quantitatively more important in the FW gill.

Cldns are another vital protein used in the osmoregulation of fish in dilute environments. Because there is low environmental osmotic pressure and ionic concentrations that can lead to

disastrous passive water influx and ion loss, the junctions between the surface cells in FW fish epithelia must be very tight. Tight junctions between cells contain Cldns (as mentioned earlier), but the Cldns utilized by fish in FW differ in function than those in SW fish. For example, *cldn30c* and *cldn32a* (or claudin-4 and claudin-3 respectively in Whitehead et al., 2011) are seen to be upregulated significantly at a transfer to a dilute environment (Whitehead et al., 2011). Cldn30 is thought to tighten up the space between cells to prevent ion loss in sudden a salinity shift to a dilute environment but prevent influx of ions in sudden salinity shifts to a concentrated environment in Japanese medaka (Bossus et al., 2017) and *cldn30* is shown to increase in salmon and tilapia acclimated to fresh water (Tipsmark et al., 2008a; Tipsmark et al., 2016), and decline in tilapia acclimated to seawater for 4 days (Tipsmark et al., 2008b).

Materials and Methods

Experimental Animals

The Atlantic killifish, *F. heteroclitus,* that we have in our care for research are purchased from Aquatic Research Organisms, Inc. (Hampton, NH, USA). The fish were maintained indoor at Department of Biological Sciences (University of Arkansas, Fayetteville, USA). The room that housed the tanks was climate controlled to be at constant room temperature (20 °C) and fish were kept under a 14/10 h light/dark photoperiod. In between experiments the fish were kept in a stock tank (2000 L) with recirculating mechanically and biologically filtered de-chlorinated Fayetteville tap water adjusted to a salinity around 12 ppt using Instant Ocean Sea salt (Spectrum Brands, Blacksburg, VA, USA). Salinity was checked and adjusted weekly. Fish were fed with Purina trout pellets (Delaware, OH, USA) an Tetramin tropical flakes (Tetra, United Pet Group, Blacksburg, VA, USA) 3 times a week unless fasting was required for an experiment. Ammonia nitrogen was tested periodically with API Ammonia, NH_3/NH_4^+ Test Kit to ensure a safe living environment. Handling and experimental procedures were approved by the IACUC of the University of Arkansas (AUP # 18134 and 22003).

Sampling

Killifish were randomly selected from the tanks and anesthetized (100-200 mg MS-222/l water), blood was be drawn from blood was drawn from the caudal vessels via heparinized microcapillary tubes and the fish then euthanized by cervical transection and pithing of the brain prior to tissue collection. Length, mass, sex, and any other observations (like eggs, milt, or physical abnormalities or damage) of each fish were recorded.

Muscle Water Content

Muscle was collected from the tail musculature and the water content was measured gravimetrically. This measure of body hydration levels was used as a proxy for the osmoregulatory ability of the fish in different environments. Samples were weighed and left to dry at 70 °C for 48 hours and then re-weighed and the muscle water content of each sample calculated.

RNA Extraction

Samples to be used in mRNA analysis were placed in TRI Reagent® (Sigma Aldrich, St. Louis, MO, USA) and were flash-frozen using either dry ice or liquid nitrogen to be stored at -80 °C until further processing. Total RNA was extracted following the manufacturer's protocol. RNA concentration and purity were checked in triplicate using Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). The purified RNA was then kept frozen at -80 °C until further use. Protocol modified from Tipsmark et al., 2008b.

cDNA Synthesis

After RNA concentration was checked, the RNA was used to synthesize complementary DNA (cDNA) using High Capacity cDNA Reverse Transcriptase kit following manufacturer protocol (Applied Biosystems, Foster City, CA). The volume of total RNA needed to be used in a 20 uL synthesis was typically 500 - 1000 ng of total RNA calculated. Samples were placed in thermocycler (Eppendorf Mastercycler gradient), using the program below.

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Quantitative polymerase chain reaction (qPCR)

Relative levels of mRNA of target genes were determined using PowerUp™ SYBR™ Green Master Mix (2x) (Applied Biosystems, Life Technologies, Carlsbad, CA, Thermo Fisher Scientific). Using a 96-well plate, 1 µL of cDNA was added to a well, followed by 14 µL of a master mix consisting of 3 µL of 1 µM primer mixture, 3.5 µL of nuclease free H2O, and 7.5 µL 2x PowerUp™ SYBR™ Green Master Mix. The qPCR analyses were performed using BioRad CFX96 platform thermocycler (BioRad, Hercules, CA, USA) using the following settings:

qPCR Settings	Temperature (°C)	Time (s)	Repeats
UDG Activation	50	120	-
Dual-Lock DNA Polymerase	95	120	-
Denature	95	15	40
Anneal/Extend	60	60	40
Melting Curve Analysis	Ramp Rate (°C /s)	Temperature (°C)	Time (s)
Step 1	1.6	95	15
Step 2	1.6	60	60
(Dissociation Step) Step 3	0.15	95	15

Data analyzed and collected using Bio-Rad CFX Manager software, Microsoft Excel, and GraphPad Prism.

Protein Extraction

Gill and opercular membrane tissues were placed in chilled SEI buffer and immediately frozen using dry ice or liquid nitrogen and were stored at -80 °C until use. Samples were thawed and removed from SEI buffer (300 mM sucrose, 20mM Na2-EDTA, 50 mM imidazole, pH 7.3) to be homogenized in SEID buffer (300 mM sucrose, 20mM Na2-EDTA, 50 mM imidazole, 0.1 % sodium deoxycholate w/v, pH 7.3) with protease inhibitor cocktail (P8340 100x Sigma Aldrich, St. Louis, MO). To separate the target protein from the cellular debris and intact cells, the homogenate was centrifuged at 4 °C for 10 minutes with 5,000 x g. The supernatant was removed and placed into new tubes, then centrifuged again for 60 minutes with 20,000 x g to form a crude membrane fraction. After centrifugation, the supernatant was removed from the tube and the protein pellet was reconstituted in SEID with protease inhibitors. Protein concentration and quality were checked in triplicate using Nanodrop 2000 Spectrophotometer. Protocol modified from Tipsmark et al., 2008b.

Preparation of Protein

Using the protein extracted from the crude membrane fraction proteins were prepared and separated in denaturing SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) to be used in a Western blot. Samples for loading were prepared with NuPage LDS sample buffer (Life Technologies) and 50 mM dithiothreitol and denatured on a heat block (10 min, 70 °C). The samples were allowed to cool at room temperature and were briefly centrifuged to collect any evaporated water. A similar amount of proteins were loaded in all wells on the same gel (5-15 µg mL^{-1}) and in some cases, the samples were frozen until use in SDS-PAGE at -20 °C short-term or -80 °C for long-term storage.

SDS-PAGE

Proteins were separated in a 4–12% Bis-Tris gel with 2-(N-morpholino)ethanesulfonic acid sodium dodecyl sulfate (MES SDS; Novex Life Technologies, Thermo Fisher Scientific) running buffer at 200 V for 30 min (Mini Gel Tank, A259, Novex Life Technologies) following the user guide provided by the manufacturer and included Precision Plus Protein™ Standards All Blue (Bio-Rad).

Western Blotting

The Mini Blot Module (Novex Life Technologies, Thermo Fisher Scientific) was used for the Western blotting to a Nitrocellulose membrane according to the instructions provided by the manufacturer using a NuPAGE™ Transfer Buffer (Novex Life Technologies)) with 10% methanol. The module was built following manufacturer instructions, and all necessary layers were soaked in the transfer buffer. The sponges, filter paper, gel, and membrane were layered in the module appropriately for transfer. Transfer buffer was added to the module and the tank. The blot ran at 10V for 60 minutes, with the value of the current being closely monitored for error. Once the run ended, the module was taken apart and the nitrocellulose membrane was placed in 20 mL of LI-COR blocking buffer for 1 hour at room temperature. Primary antibodies were prepared in 10 mL of blocking buffer and following incubation, the used blocking buffer was removed from the plate. The primary antibody mixture was added to the plate with the membrane, and the plate was incubated overnight at 4 ºC. The following morning, the membrane was removed from the antibody mixture and washed with Tris-buffered saline with Tween-20 (TBS-T). The secondary antibody mixture was prepared in LI-COR blocking buffer and was added to the plate following the washes and removal of TBS-T. The plate was incubated at room temperature for 1 hour shielded from light. After the incubation with the secondary antibodies,

the membrane was washed again shielded from light. Once the final wash was finished, the membrane was removed from the plate and was allowed to dry for at least 1 hour in the dark before viewing using the LI-COR Odyssey.

Organ Distribution

The distribution of mRNA of numerous claudin proteins in multiple organs was checked through qPCR. Killifish organs were sampled and stored as described above for RNA extraction. Killifish gill, opercular membrane, kidney, anterior and posterior intestine, liver, muscle, and brain were removed from 8 fish (4 male, 4 female; 4 FW-acclimated, 4 SW-acclimated) and RNA was extracted as described above. The RNA was transformed into cDNA, then used to perform a qPCR. The targeted claudin proteins are listed in table X.X with the primer sequences.

RPL7 and ef1a were used as normalization genes. Standard curves were obtained.

Claudin-30c Environmental Effects

3 fish tanks were prepared with the salinities of 0 ppt, 10 ppt, and 35 ppt to represent fresh water (FW), brackish water (BW), and seawater (SW) respectively. Each tank had 8 killifish rehomed into them for at least 3 weeks to allow for long-term acclimation by the killifish. The killifish were then sacrificed and the gills, opercular membranes, and muscle were sampled from each fish. Half of the gill arches from each fish were saved for mRNA analysis and the other half were saved for Western blotting. The opercular membranes were only used for mRNA analysis. The muscle was used for muscle water content analysis.

The antibodies used in the Western blot are shown in the table below, with each mixture dissolved in 10 mL of LI-COR blocking buffer.

Primary Ab	Tested Reactivity	Host Species	Concentration	Absorbs	Company
Beta-actin	Dictyostelium, Drosophila, Gecko, Lizard	Mouse	1:2000	-	Developmental Studies Hybridoma Bank
NKA-alpha 5 subunit	Avian, Drosophila, Fish, Frog, Honeybee, Human, Insect, Mackerel, Mammal, Mosquito, Zebrafish	Mouse	1:1000	-	Developmental Studies Hybridoma Bank
Claudin 30c		Rabbit	1:1000	-	
Secondary Ab					
Anti-Mouse	Mouse	Goat	1:20000	680 nm	
Anti-Rabbit	Rabbit	Goat	1:10000	800 nm	

Hormone Control of Membrane Protein Expression

A total of 3 experiments were run in order to determine how cortisol and prolactin affect the mRNA expression of select membrane proteins involved in salinity homeostasis. 6 killifish, 3 of each sex, were selected from the main brackish water tank for dissection for each experiment. Fish were sacrificed as described above. Organ sections were placed in Dulbeccos Modified Eagles Medium (DMEM) treated with 50 U mL^{-1} of penicillin and 50 µg mL^{-1} of streptomycin (Invitrogen, Carlsbad, CA, USA) on a 6-well plate until use in the experiments. 2 pieces of each organ from each fish were placed wells on a 24-well plate in media containing different hormone concentrations.

For the prolactin experiment, there was a pre-incubation control where the samples were homogenized in TRI reagent immediately after sampling concluded. The other treatment groups consisted of a no hormone control (0 µg mL^{-1}), 0.1 µg mL^{-1}, 1 µg mL^{-1}, and µg mL^{-1}. For the

cortisol experiment, there was a pre-incubation control, no hormone control (0 μg mL^{-1}), 0.1 μg mL^{-1}, 1 μg mL^{-1}, and 10 μg mL^{-1}. For the combination experiment, there was no hormone control (0 μg mL^{-1}), cortisol at 10 μg mL^{-1}, prolactin at 1 μg mL^{-1}, and a combination of both at 10 μg mL^{-1} of cortisol and 1 μg mL^{-1} of prolactin.

RNA was extracted from each sample and transformed into cDNA for use in qPCR. Primers used are in table below.

Target	**Primer Name**	**Sequence**
EF1(a)	Fhef1a-FP-Schulte	Gggaaagggctccttcaagt
	Fhef1a-RP-Schulte	Acgctcggccttcagctt
RPL7	Rpl7-Fhe-FP1	GTCCGTAAGGTGACCAGGAA
	Rpl7-Fhe-RP1	TCTCACGCCTGTACATCTGC
Cldn30c	FP1-Cldn30c-killi	GCGCCATCTTCATCATTTCT
	RP1-Cldn30c-killi	GGAGCTCCAACATTGGGTTGT
Cldn32a	Fhe-Cldn32a-FP1	GATATTGGCTGCCCAAAAGA
	Fhe-cldn32a-FP1	GTCTACCCCAGTCCCTCACA
Cldn10c	Fu-Cldn10c-fp2	CGCACGGAGATCACACATAC
	Fu-Cldn10c-rp2	AGTCTTCCTGGTGGTGTTGG
Cldn10d	Fu-Cldn10d-fp4	CGGTGATCATGTACGTGGAG
	Fu-Cldn10d-rp4	TACTCTGTGGGAAGGGTGGA
Cldn10e	Fu-Cldn10e-FP2	CTCTGCGGAGAAGGAGAAGA
	Fu-Cldn10e-RP2	GAGAAGCTGTGGTGGGCTTA
Cldn10f	Fu-Cldn10f-FP4	ACTTATATCGGCGGAGCAGA
	Fu-Cldn10f-RP4	ATAAGCAGTAGGCGGCAAGA
ECaC	FP1_ECaCkillifish	ATCGTGCTCATCGGTTTTTC
	RP1_ECaCkillifish	GGAAGGAGGTGTATGCAGGA
NCC2b	Ncc2b-fp1-fundulus	GTGGTGACCAATGTGACTGC
	Ncc2b-rp1-fundulus	CGATTTCAGGACCCAAGGTA
Aqp3	Aqp3-fp1-fundulus	TCAACTTTGCCTTTGGCTTT
	Aqp3-rp1-fundulus	GGTCACTGCAGGGTTCAGAT
NKCC1	Fhe_nkcc1a_FP	CCCGCAGCCACTGGTATT
	Fhe_nkcc1a_RP	GCCATCTGTGGGTCAGCAA
CFTR	Fhe_CFTR_FP_Scott2004	AAT CGA GCA GTT CCC AGA CAA G
	Fhe_CFTR_RP_Scott2004	AGC TGT TTG TGC CCA TTG C

Results

Organ Distribution of Claudins in FW and SW Acclimated Killifish

Select organs from Atlantic killifish were sampled by another graduate student (Julie Starling) from long-term acclimated (>3 weeks) FW and SW killifish. The RNA extracted from this sampling was used in an organ distribution study of select claudin proteins with expected functionality in the gill. Expression of each claudin was detected in all organs and generally had the highest expression in gill and opercular membranes by 1-4 orders of magnitude when compared to skeletal muscle, liver, anterior intestine, posterior intestine, kidney, and brain (Fig. 3.1). There were a few exceptions to this as, for instance, *cldn30c* was expressed at similar levels in gill and muscle and *cldn10e* was highly expressed in the posterior intestine. Expression in the gill and opercular membrane was comparable for all claudins except *cldn32a*. Because no effect of salinity was observed, organ data from FW and SW are combined (Fig. 3.1).

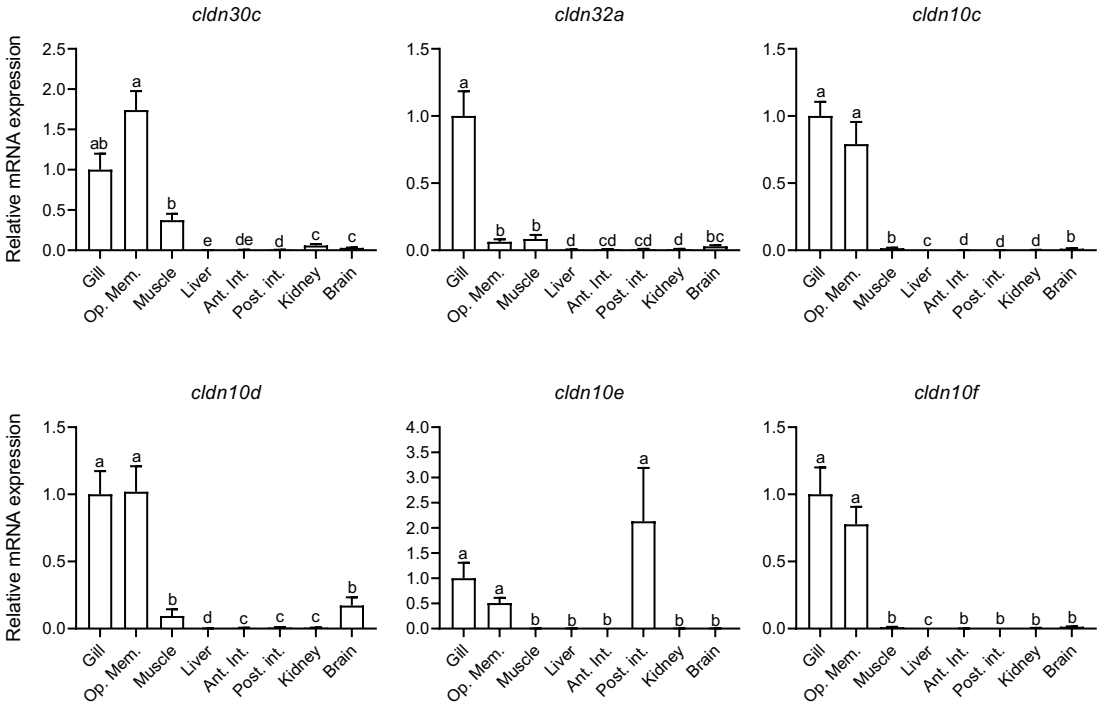

Fig. 3.1: mRNA expression in gill, opercular membranes (Op. Mem.), skeletal muscle (muscle), liver, anterior intestine (Ant. Int.), posterior intestine (Pos. Int.), kidney, and brain from Atlantic killifish. Values are mean + s.e.m. of four fresh water and four seawater fish (four males and four females) that were long-term acclimated (> 3 weeks) to their respective salinity. Transcript levels are shown in arbitrary units as calculated relative to the geometric mean of two normalization genes (*ef1a* and *rpl7*) and subsequently normalized relative to the expression in gill (=1). The data were analyzed with two-way ANOVA and there were no significant effects of salinity or interaction. Shared letters above bars indicate no significant difference between organs as determined using Tukey's multiple comparisons test ($p<0.05$).

In addition to the mRNA expression experiment, an organ distribution was completed at the protein expression level of Cldn30c (Fig. 3.2). While no statistics could be performed due to the low number of replicates, the data confirms consistent protein expression in both the gill and opercular membranes with the apparent highest levels in the latter. Cldn30c bands were detected on at least one Western blot in all organs excluding the kidney and liver.

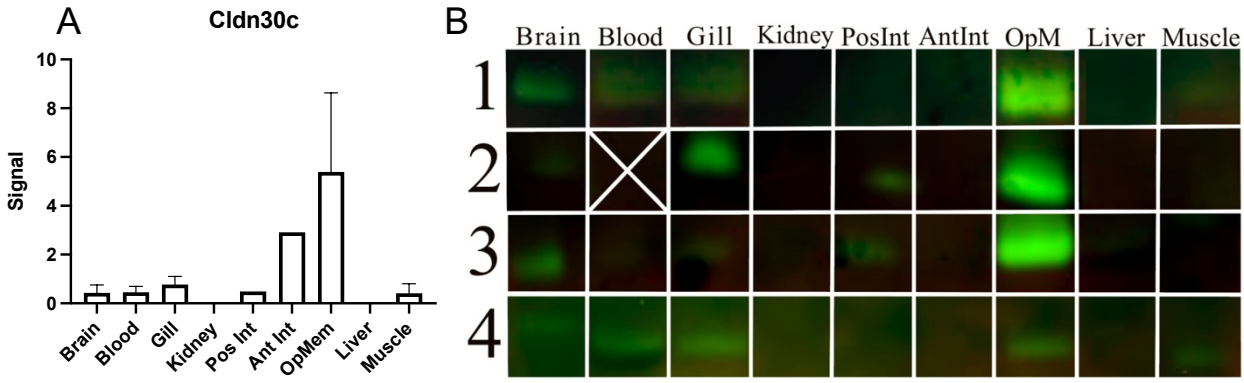

Fig. 3.2: A: Graphical representation of Cldn30c expression in brain, blood, gill, kidney, posterior intestine (Pos Int), anterior intestine (Ant Int), opercular membranes (OpMem), liver, and muscle from Atlantic killifish. The four fish (mixed sex) sampled were long-term acclimated to brackish water (12 ppt). Values are mean + s.e.m. and Cldn30c signals graphed were normalized ß-actin. B: The four separate Western blots shown in the grid were probed with Cldn30c antibody. For some organs, no bands were detected (kidney, liver) on any of the 4 Western blots.

Effect of Salinity on Claudin Expression in Opercular Membrane and Gill

As discussed above, there was no significant difference in claudin mRNA expression between long-term acclimated FW and SW fish. However, to add an important comparison group an additional experiment sampled opercular membrane and gill from fish acclimated for 3 weeks to isosmotic BW (12 ppt), dilute FW, and concentrated SW.

The relative opercular membrane mRNA expression of the target claudin proteins are as shown in figure 3.3. At the opercular membrane mRNA level, *cldn10e* levels were significantly higher in FW samples than in SW and BW samples, but the other claudin level comparisons were nonsignificant.

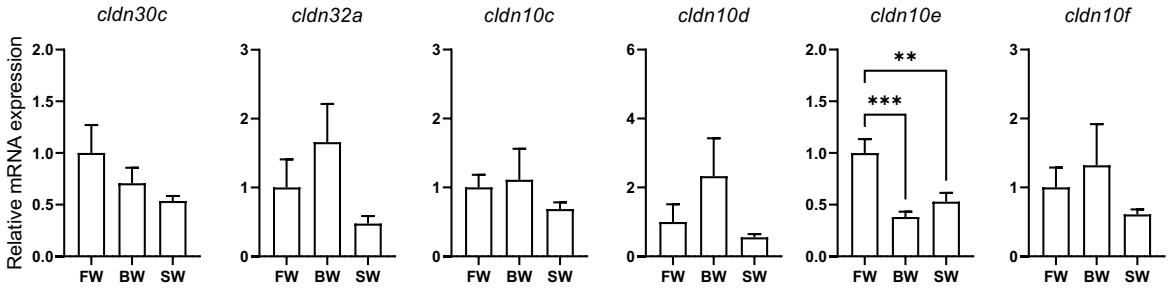

Fig. 3.3: Opercular membrane claudin mRNA expression in Atlantic killifish long-term acclimated to FW, BW, and SW. Values are mean + s.e.m. of 8 fish per salinity (four males and four females). Target genes were normalized to *rpl7* and are shown relative FW mean (=1). The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Asterisks with brackets indicate significant differences between data points (**p<0.01, ***p<0.001).

The relative gill mRNA expression of the target claudin proteins are as shown in figure 3.4. In the gill, *cldn10d* levels were significantly higher in SW than FW fish, but no significant effect was observed in the other claudins, although there seems to be a trend of increased expression as the salinity of the environment increased.

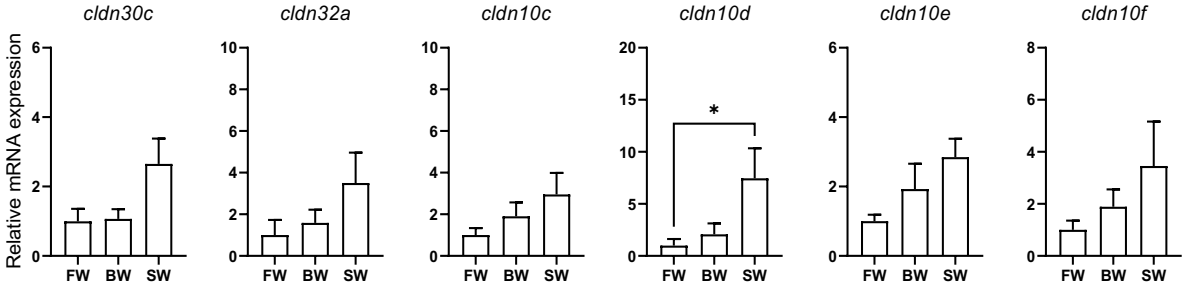

Fig. 3.4: Gill claudin mRNA expression in Atlantic killifish long-term acclimated to FW, BW, and SW. Values are mean + s.e.m. of 8 fish per salinity (four males and four females). Target genes were normalized to *rpl7* and are shown relative FW mean (=1). The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Asterisk with bracket indicates a significant difference between data points (*p<0.05).

Protein analysis was performed on gill samples from the same sampling, with the addition of 8 fish from each environment from a later sampling. Samples from FW, BW, and SW long-term acclimated fish were compared using Western blotting (Fig. 3.5). There was no effect on salinity on the loading control.

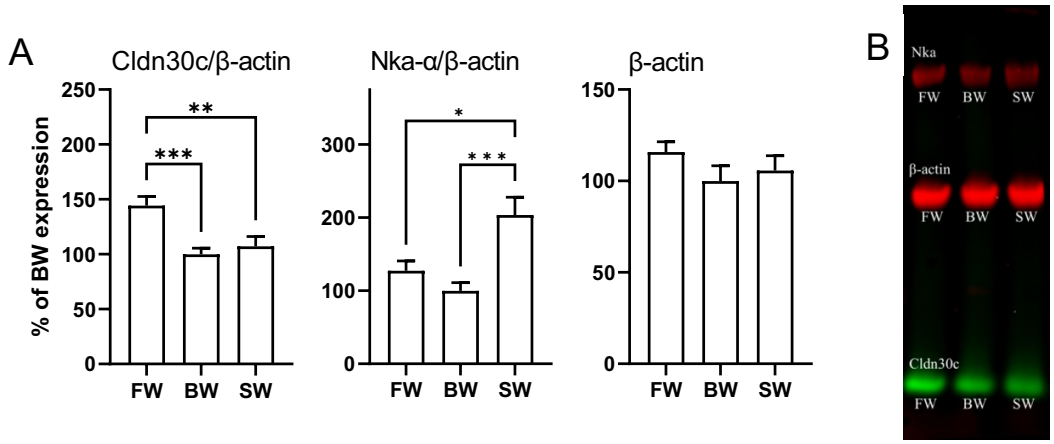

Fig. 3.5: A: Gill Cldn30c, Nka α subunit (Nka-α) and ß-actin protein expression in gill of Atlantic killifish acclimated long-term to FW, BW, and SW. Values are mean + s.e.m. of 15 fish per salinity (mixed sex). Cldn30c and Nka-α fluorescence intensity were normalized to ß-actin and are shown as percent of the mean for the BW group. The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Asterisks with brackets indicate significant differences between data points (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
B: Representative section of Western blot performed. Nka-α and ß-actin detected at 680 nm, Cldn30c detected at 800 nm.

At the protein level, Cldn30c expression was ~40-50% higher in fish acclimated to FW than BW and SW. Nka protein expression was higher in fish acclimated to SW by ~75% when compared to FW fish and by ~100% when compared to BW fish.

Time Course of Claudin-30c mRNA and Protein Expression

Given the difference in mRNA and protein expression in long-term acclimated fish and our initial hypothesis, this experiment took a closer look at the expressions during time-course

acclimation to a dilute environment (FW). Following transfer from BW to FW, 8 killifish from 4 different time points (0 hours, 24 hours, 72 hours, and 168 hours) were used to sample muscle tissue, gill, and opercular membranes. The muscle water content for each time point over the period of 1 week was observed. The opercular membrane and gill *cldn30c* and *ncc2b* mRNA expression and gill Cldn30c protein expression were analyzed.

The muscle water content of the fish showed an increase as the fish spent more time in FW which was significant after 168 hours (Fig. 3.6).

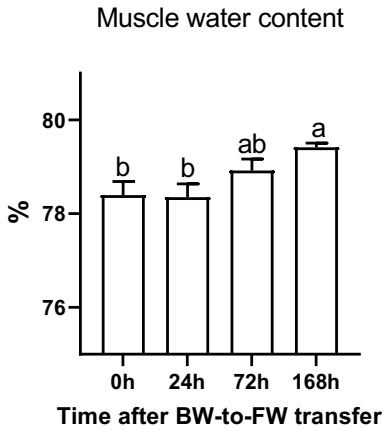

Fig. 3.6: Muscle water content of Atlantic killifish short-term acclimated from BW to FW. Values are mean + s.e.m. with 8 fish per time point (mixed sex). Results are shown in percent muscle water. The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Shared letters above bars indicate no significant difference between time points ($p<0.05$).

The gill mRNA expression of *ncc2b* and *cldn30c* was analyzed after transfer to a dilute environment (Fig. 3.7). After BW to FW transfer, there was a transient increase in mRNA expression of *ncc2b* 24 h, after which the level returned to BW levels. No significant increase in

the amount of *cldn30c* transcript abundance after FW transfer.

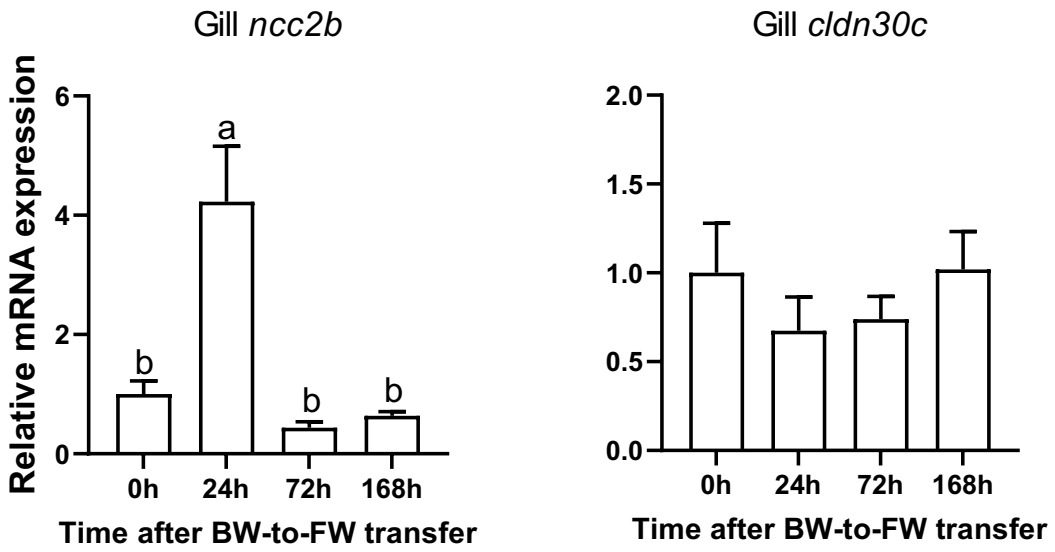

Fig. 3.7: Time-course of *ncc2b* and *cldn30c* mRNA expression in gill of Atlantic killifish during acclimation from BW to FW. Values are mean + s.e.m. with 8 fish per time point (mixed sex). Results target genes normalized to the geometric mean *ef1a* and *rpl7* and shown relative to the mean at time 0 (=1). The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Shared letters above bars indicate no significant difference between time points ($p<0.05$).

The expression of Cldn30c protein showed an increase as the time in FW increased. There was a significant increase in the abundance of Cldn30c at the 72- and 168-hour time points as compared to any other time point (Fig. 3.8).

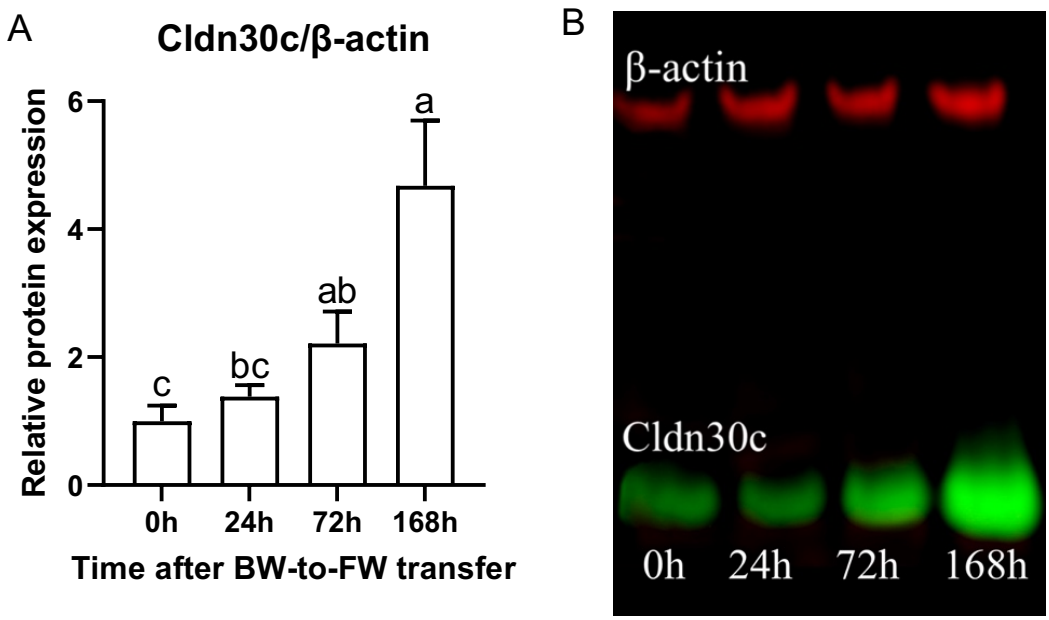

Fig. 3.8: A: Time-course of gill protein expression when killifish acclimate from BW to FW. Values are mean + s.e.m. of 8 fish (mixed sex) per time point. Cldn30c fluorescence intensity was normalized to ß-actin and is shown relative to the mean for the BW group (0 h). The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Shared letters above bars indicate no significant difference between time points ($p<0.05$). B: Representative section of Western blot performed. Cldn30c detected at 800 nm and ß-actin detected at 680 nm.

The mRNA expression of *ncc2b* and *cldn30c* in the opercular membrane was analyzed after transfer to a dilute environment (Fig. 3.9). The *ncc*2b transcript abundance increased 24 hours and 168 hours after transfer to FW. There was found to be no statistically significant change in

the amount of *cldn30c* expression over time in the FW.

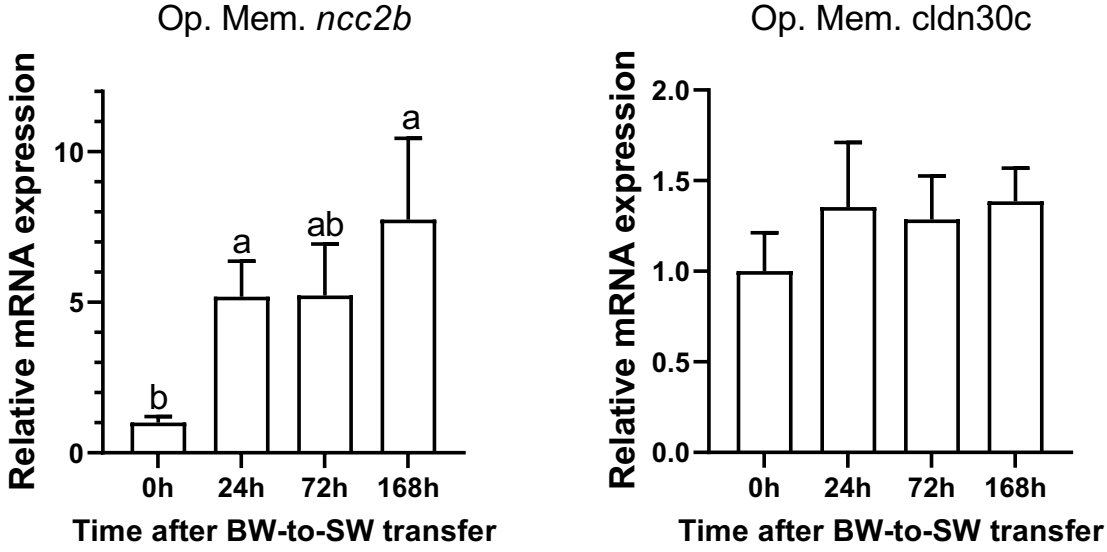

Fig. 3.9: Time-course of *ncc2b* and *cldn30c* mRNA expression in opercular membrane (Op. Mem.) of Atlantic killifish during acclimation from brackish water to fresh water. Values are mean + s.e.m. with 8 fish per time point (mixed sex). Results show target genes normalized to the geometric mean *ef1a* and *rpl7* and shown relative to the mean at time 0 (=1). The data were analyzed with one-way ANOVA followed by Tukey's multiple comparisons test. Shared letters above bars indicate no significant difference between time points ($p<0.05$).

Prolactin and Cortisol Control of Gill Claudins and Transport Proteins Examined In Gill Explant Cultures

There is not much known about hormonal control of tight junction proteins and salinity-specific osmoregulatory proteins in estuarine fish like Atlantic killifish. To explore how those mechanisms may work, killifish gill explants were incubated overnight (16 hours) with prolactin or/and cortisol after which mRNA expression of key gill claudins (*cldn30c*, *cldn32a*, *cldn10c*, *cldn10d*, *cldn10e*, *cldn10f*) in the gill explants were analyzed. Important transport proteins (*aqp3*, *ecac*, *ncc2b*, *nkcc1a*, *cftr*) were also analyzed as reference points.

The expression levels for the targets from the control groups were pooled from the cortisol and prolactin experiments (n= 5-6+5-6=11-12) and are as shown in figure 3.10. There was found to be a significant decrease in the expression of the mRNA of all targets after 16-hour incubation with the exception of *cldn30c*, *cldn10e* and *nkcc1a* (Fig. 3.10).

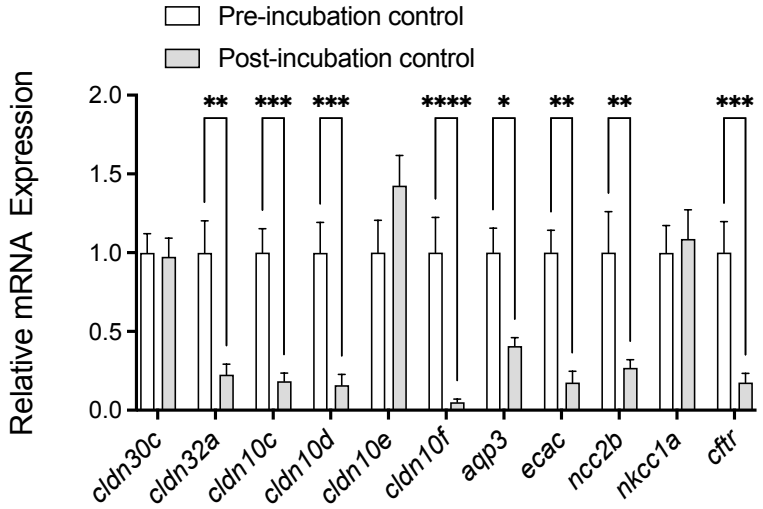

Fig. 3.10: mRNA expression of *cldn30c*, *cldn32a*, *cldn10c*, *cldn10d*, *cldn10e*, *cldn10f, aqp3*, *ecac*, *ncc2b*, *nkcc1a*, *cftr* in gill explants from Atlantic killifish either processed immediately after sampling (pre-incubation control) or incubated in DMEM overnight (16 hours, post-incubation control). Values are mean + s.e.m. of gill explants from 11-12 fish (mixed sex). Results show target genes normalized to the geometric mean *ef1a* and *rpl7* and shown relative to the mean initial sampling time (pre-incubation control). The data were analyzed with mixed-effects analysis followed by Bonferroni's multiple comparisons test. Asterisks with bracket indicate significant difference between data points (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001).

To test main factor effects in a two-way design and analyze for possible interactions, a cortisol-prolactin combination experiment was performed using the gill explant model using hormone concentrations that should be relevant based on the literature. The gill explants were incubated for 16 hours in DMEM or DMEM containing either 1 μg mL^{-1} of prolactin, 10 μg mL^{-1} of cortisol, or 1 μg mL^{-1} of prolactin and 10 μg mL^{-1} of cortisol. The combination explant experiment yielded some interesting results and in figure 3.11 we see the expression of transporter proteins. Prolactin treatment increased the expression of *aqp3* by more than 3 times while cortisol significantly inhibited this transcript. The two hormones had a synergistic effect with the combined treatment causing a 20-fold increase in the expression of *aqp3*. Expression of *ecac* was doubled by cortisol but unaffected by prolactin. For *ncc2b*, a synergistic effect of the two hormones elevated the expression 5-fold in the combination group. For *cftr* and *nkcc1a*, no significant effects were observed even if cortisol tended to increase their levels.

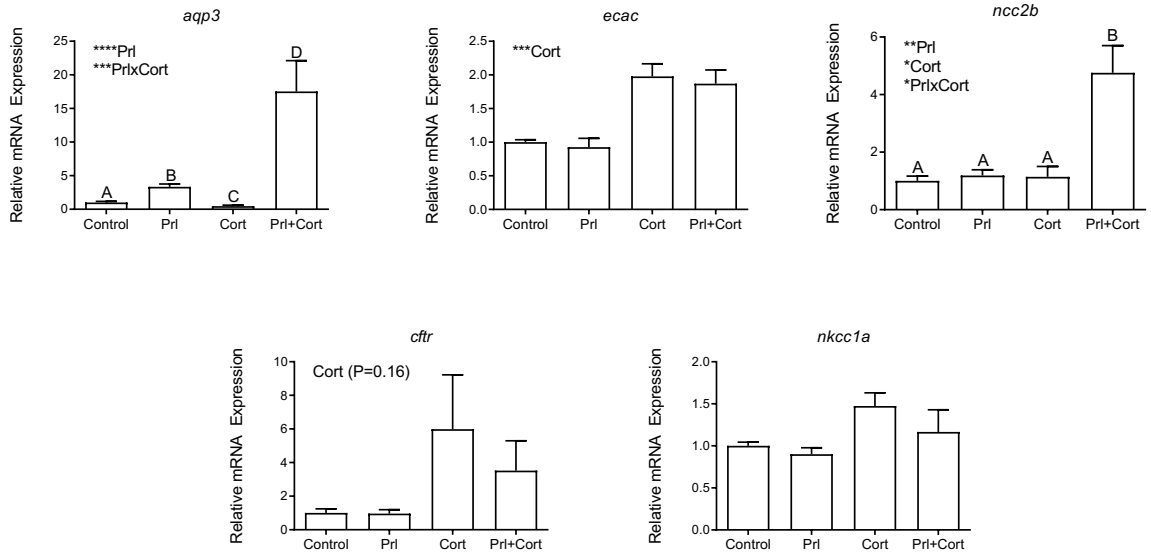

Fig. 3.11: mRNA expression of transporter proteins (*aqp3*, *ecac*, *ncc2b*, *nkcc1a*, *cftr*) in gill explants from Atlantic killifish incubated that had been incubated in DMEM overnight (control), in 1 µg mL^{-1} of prolactin (Prl), 10 µg mL^{-1} of cortisol (Cort), and a combination of both prolactin and cortisol at those concentrations. Results show target genes normalized to the geometric mean *ef1a* and *rpl7* and shown relative to the mean value without hormone. The data was analyzed with a mixed-effects model two-way analysis. Asterisks next to Prl or Cort indicate a significant main factor effect of the hormone while asterisks next to PrlxCort indicate a significant interaction between hormones (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). When there was a significant interaction, main factor analysis was followed by Tukey's multiple comparisons test to identify the kind of interaction. Shared letters above bars indicate no significant difference between time points ($p<0.05$). Values are mean + s.e.m. of gill explants from 5-6 fish (mixed sex).

For the claudin mRNA expression in the combination experiment, we observed main factor effects but no significant interaction between factors (Fig. 3.12). There was an overall stimulatory effect of prolactin on the mRNA expression of *cldn30c* and *cldn32a*, but no effect of cortisol on these two genes. While there were no effects of prolactin on any of the claudin-10 paralogs, cortisol significantly stimulated *cldn10c* and *cldn10f*, but had no effect on *cldn10d* and *cldn10e*.

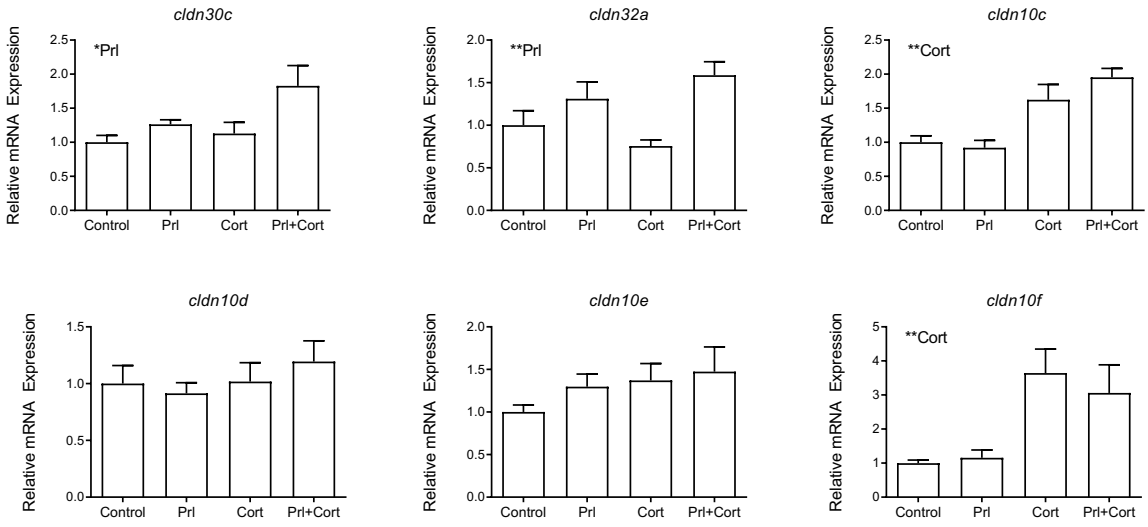

Fig. 3.12: mRNA expression of transporter proteins (*cldn30c*, *cldn32a*, *cldn10c*, *cldn10d*, *cldn10e*, *cldn10f*) in gill explants from Atlantic killifish incubated that had been incubated in DMEM overnight (control), in 1 µg mL^{-1} of prolactin (Prl), 10 µg mL^{-1} of cortisol (Cort), and a combination of both prolactin and cortisol at those concentrations. Results show target genes normalized to the geometric mean *ef1a* and *rpl7* and shown relative to the mean value without hormone. The data was analyzed with a mixed-effects model two-way analysis. Asterisks next to Prl or Cort indicate a significant main factor effect of the hormone while asterisks next to PrlxCort indicate significant interaction between hormones (*P<0.05, **P<0.01). There was a significant interaction between main factors. Values are mean + s.e.m. of gill explants from 5-6 fish (mixed sex).

Discussion

Euryhaline fish like the Atlantic killifish can maintain ionic and osmotic homeostasis while moving between areas with a wide range of salinities, utilizing specialized proteins like claudins found in the gill, opercular membrane, and other osmoregulatory organs. The studies in this master thesis use the estuarine species to identify the significance of claudin paralogs in the gill and opercular membrane in osmoregulation in different environments. The project contains an examination of organ distribution, salinity-regulated, and hormone-regulated expression of claudin and other transport proteins vital for osmoregulation. It was found that most claudins found in abundance in the gill are also seen with high expression in the opercular membranes. The salinity-regulation study showed that mRNA and protein regulation of claudins do not follow the same pattern, suggesting post-translational control. The hormone-regulation study generally illustrated an increase in the expression of FW-acclimating proteins when exposed to prolactin in high concentrations and an increase in the expression of SW-acclimating proteins when exposed to high cortisol concentrations.

Organ Distribution

In teleost fish, the main organ that determines the ability to maintain hydromineral balance is the gill. This is because this respiratory surface is both the main site for passive water and ion fluxes that needs correction, but also that gill ionocytes are responsible for active compensatory ion transport (Evans et al., 2005). A number of specific claudin paralogs are found in gill tight junctions and their regulation by salinity and osmoregulatory hormones in euryhaline fish suggest that they play a large part in homeostasis (reviewed in Chasiotis et al., 2012). The current organ distribution study in Atlantic killifish confirmed that *cldn30c*, *cldn32a*, *cldn10c*, *cldn10d*, *cldn10e,* and *cldn10f* transcripts are most prominently expressed in the gill, and except

for *cldn32a,* also the opercular membrane (Fig. 3.1 and 3.2). This is similar to findings for *cldn30c* and *cldn10e* in Atlantic salmon and Mozambique tilapia (Tipsmark et al., 2008b; Tipsmark et al., 2016) and for *cldn30c*, *cldn10c*, *cldn10d*, *cldn10e* and *cldn10f* in Japanese medaka (Bossus et al., 2015) and suggests an evolutionarily conserved role in the gill. In the case of claudin-10e, the posterior section of the intestine had a similar high expression to the gill and opercular membrane which is dissimilar to findings in salmon and medaka (Tipsmark et al., 2008; Bossus et al., 2015). This would suggest a role of this claudin in controlling paracellular water and solute handling in the intestine, specific to the species Atlantic killifish. A transcriptomic study in several Atlantic killifish strains found *cldn30c* and *cldn32*a to be the most abundant gill *cldn* transcripts (Whitehead et al., 2011), and even though no other organs were tested in that study it is consistent with our findings. As explained in the introduction, active transcellular ion transport processes of the gill can be studied in the simpler flat opercular membrane where the same processes are found. However, it is not known if claudin control of gill tight junction and paracellular solute flux can also be modeled in the operculum membrane. The comparable expression of claudin paralogs in these two surface epithelia is therefore an important finding that suggests opercular membranes is also an appropriate gill model when it comes to studying passive paracellular flux and sodium secretion.

Effect of Salinity on Claudin Expression in Gill and Opercular Membrane

At the start of this project, we hypothesized that killifish *cldn10* paralogs would be elevated in SW while *cldn30c* and *cldn32a* would be stimulated FW. Killifish acclimated to FW, BW, or SW for >3 weeks was used to compare *cldn* mRNA expression in gill and operculum membranes. Within the gill, *cldn10d* was the only protein that showed any significant change in relative mRNA abundance due to salinity differences. In the opercular membranes, *cldn10e*

mRNA was found at higher concentrations in the FW acclimated fish than in the BW or SW acclimated fish. In contrast, a study in Atlantic killifish by Marshall and coworkers (2018) found gill and opercular *cldn10e* mRNA abundance to be higher after the transfer to SW during FW-to-SW transfer or from SW-to-2SW (double strength SW) but on a shorter time-course 1-10 days. Nonetheless, this result was unexpected and raises a few new questions, like how long-term acclimation to environments with varying salinities affects the expression of tight junction proteins. In Japanese medaka, both short-term (1-3 days) and long-term acclimation (>3 weeks) induce elevated gill expression in all the *cldn10* paralogs examined in the present study for killifish (Bossus et al., 2015) and similar findings have been found for *cldn10e* in Atlantic salmon (Tipsmark et al., 2008) and Mozambique tilapia (Tipsmark et al., 2016), and for *cldn10d* and *cldn10e* in Japanese puffer fish (Bui and Kelly, 2014). Atlantic killifish is a truly estuarine fish with high euryhaline capacity, and it is possible that it retains constitutive expression of some genes of significance to SW acclimation. Alternatively, *cldn10* paralogs are regulated at the post-transcriptional level or different paralogs serve functions maintained by *cldn10* paralogs in other investigated species.

Killifish acclimated to FW, BW or SW for >3 weeks all had similar expression of *cldn30c* and *cldn32a* mRNA in gill and opercular membranes. In the transcriptomic killifish study by Whitehead and coworkers (2011) gill *cldn30c* and *cldn32c* mRNA peaked 24 hours after SW to FW transfer and stayed elevated after 7 days. It is possible that the transient increase in these *cldns* is not maintained after 3 weeks of transfer. This would be comparable to observations for *cldn30c* mRNA in Japanese medaka where salinity does not change its expression (Bossus et al., 2015). It is dissimilar to observations in Atlantic salmon (Tipsmark et al., 2008) and Mozambique tilapia (Tipsmark., et al., 2016), where *cldn30c* transcript levels are lower in SW

than FW, and it is possible that regulation at the mRNA level differs between euryhaline fish species depending on life cycle or evolutionary background. It is important to remember that gene expression requires transcription, translation, and turnover of mRNA and proteins. It thus cannot be excluded that Cldn30c protein abundance changes independent of absolute mRNA levels involving salinity-specific post-transcriptional regulation (see Buccitelli and Selbach, 2020; Franks et al., 2017).

The Western blot performed, demonstrated that Cldn30c protein levels increase in the gill of fish acclimated to FW as compared to BW and SW fish (Fig. 3.5). In Atlantic salmon, *cldn30c* mRNA and protein are regulated in parallel (Tipsmark et al., 2008; Engelund et al., 2012) but our data suggest that salinity-dependent post-translational control is responsible for elevated Cldn30c protein in dilute FW. We cannot extend this conclusion to the opercular epithelium since we in the salinity effect studies failed to purify enough crude membrane fraction to gain comprehensive data, however, it should be possible to optimize this in the future as we did get Cldn30c bands for this organ in the organ distribution study (Fig. 3.2). The salinity response Western blots included β-actin as a loading control and the use of this protein for normalization was validated as it was unaffected by salinity (Fig. 3.5). We found as gill Nka protein abundance to be higher in SW than in FW and BW and this is support previous findings that maximal gill Nka activity increase after transfer from FW to SW (Mancera and McCormick, 2000).

Since gill *cldn30c* mRNA and protein data for Atlantic killifish were dissimilar and a transcriptomic study suggested that FW acclimation involves an early transient increase in *cldn30c* (Whitehead et al., 2011), we also studied the initial time-course of BW-to-FW acclimation. Muscle water content was constant after 1 and 3 days but elevated after 7 days and this suggests that cellular water balance was unaffected early on. The fish in this experiment was

not able to sustain osmotic regulation in the longer term in FW or they may attain a new steady state in osmotic status when staying for 7 days in the dilute environment. In this experiment, *ncc2b* expression was elevated in both organs 24 hours after FW transfer, an effect that was transient in the gill and sustained in the opercular membranes for the duration of the experiment. This change confirms that the animal was transitioning to active ion uptake and is consistent with previous findings in Atlantic killifish opercular membrane and gill (Breves et al., 2020). The time-course experiment showed no transient increase in *cldn30c* mRNA but an increase in Cldn30c protein abundance after 3 and 7 days. This is consistent with post-transcriptional regulation. The experiment was in part performed to inform a planned *in vivo* translational knock-down experiment that combined with electrophysiological data can attain functional data. Indeed, future experiments will now be able to select a relevant time point as we identified 3 days as sufficient to observe elevated gill Cldn30c protein expression.

Cortisol and Prolactin Control of Gill Claudins

The endocrine systems control acclimation to various salinities and changes in gill function; and prolactin is known to be critical to FW acclimation, growth hormone to SW acclimation (Sakamoto and McCormick, 2006). While cortisol's role in SW acclimation is well established this hormone also seems to stimulate some aspects of FW acclimation, especially in conjunction with prolactin (McCormick et al., 2001). In this thesis endocrine control of gill claudins (*cldn30c, cldn32c, cldn10c, cldn10d, cldn10e* and *cldn10f*) and key transport proteins (*aqp3, ecac, ncc2b, nkcc1a* and *cftr*) in Atlantic killifish was examined using the gill explant *ex vivo* technique described previously for Atlantic salmon, striped bass, Mozambique tilapia and medaka (Tipsmark et al., 2009; Kiilerich et al., 2011; Inokushi et al., 2015; Bossus et al., 2017). Comparison of pre-incubation and post-incubation control showed that the lack of circulating

hormones and central innervation induced a decrease of the expression of all targets except for *cldn30c*, *cldn10e* and *nkcc1a*. This demonstrates that endocrine and possibly neuroendocrine factors are essential to maintain the level of *cldn32c, cldn10c, cldn10d, cldn10f, aqp3, ecac, ncc2b,* and *cftr* transcripts. The maintenance of *cldn30c*, *cldn10e* and *nkcc1a* may reflect maintenance by local factors or that these transcripts have longer half-life's than the others.

In the hormone combination experiment discussed below hormone concentrations were based on previous studies in other species (Tipsmark et al., 2009; Kiilerich et al., 2011; Bossus et al., 2017). *aqp3* is known to be generally upregulated in FW gill (Tipsmark et al., 2010) and was synergistically stimulated by prolactin and cortisol and the same was the case for *ncc2b* a marker for FW-type ionocytes involved in NaCl retention. A marker gene for ionocytes responsible for calcium uptake, *ecac,* was unaffected by cortisol but very significantly ($p<0.001$) stimulated by cortisol, which concurs with findings in zebrafish (Guh et al., 2015). In contrast to its role in mammals as a hypocalcemic hormone (Patschan et al., 2001), cortisol acts as a stimulator for calcium uptake in fish and in rainbow trout cortisol injection stimulates gill ECaC mRNA and protein (Shahsavarani and Perry, 2006). The two SW ionocyte markers (*cftr* and *nkcc1a*) were not significantly affected the tested hormones although cortisol-treated explants had higher *cftr* (6-fold) and *nkcc1a* (1.5-fold) levels compared to control explants. In Japanese medaka cortisol stimulates while prolactin inhibits gill *cftr* (Bossus et al., 2017).

As discussed, we saw no general trends in salinity effects on mRNA of claudins proposed to help mediate FW-acclimation (*cldn30c and cldn32a*) and SW-acclimation (*cldn10c*, *cldn10d*, *cldn10e, and cldn10f*), however, cldn30c mRNA and protein expression pattern suggests we consider a more multifaceted model for gene expression of tight junction proteins in killifish surface epithelia. It is well established that the survival of fish in FW is critically dependent on

prolactin (Manzon, 2002). These studies found that prolactin stimulates the two proposed "FW" *cldns* (30c and 32a) while "SW" *cldns* (10c, 10d, 10e, 10f) were unaffected by this hormone. While cortisol plays a role in some aspects of FW-acclimation including being a hypercalcemic hormone it seems to have a more overreaching role in SW acclimation (McCormick et al., 2001; Guh et al., 2015). In the combination experiment, a very significant stimulatory effect ($p<0.01$) was seen of cortisol on *cldn10c* and *cldn10f*. These paralogs are stimulated by *in vivo* transfer of Atlantic killifish to a hypersaline environment (Marshall et al., 2018) and it is possible this hormone is involved in increasing SW-ionocyte capability for compensatory secretion of NaCl. Under these conditions, it is thought that transcellular chloride secretion is enhanced by stimulation of Nka, Nkcc1, and Cftr chloride channel abundance. Based on the amino acid sequence in the second extracellular loop, Cldn10 paralogs are candidates for the formation of the paracellular sodium secretion pathway in ionocyte complexes (see Bossus et al., 2015 and Marshall et al., 2018).

Conclusions and Perspectives

The experiments laid out in this master thesis help to demonstrate many of the mechanisms by which the Atlantic killifish maintain ionic and osmotic homeostasis while moving between environments of drastically different salinities. We explored the mRNA and protein expression of a multitude of claudins and transport proteins and how the expression is influenced by salinity and by hormone control. However, much remains unknown about the specific mechanisms of control regarding how each hormone regulates and how the combination of prolactin and cortisol might work together to have synergistic effects on osmoregulatory protein expression. While the effect of prolactin on these transporters is well established (Bossus et al., 2017. Breves et al., 2020), the strong synergistic effect of cortisol is a novel finding, and it would be interesting to study the mechanistic background for this strong interaction.

The protein expression in the Western blots demonstrated the importance and the prevalence of Cldn30c in the gill and opercular membranes. At the mRNA level, salinity didn't seem to have an effect on the expression of *cldn30c* or any other transcripts of claudin proteins, and that might suggest that future studies may explore a multi-faceted mechanism for osmoregulatory protein and mRNA expression.

The initial plan for this paper included a vivo-morpholino knock-down of claudin-30c in determining specifically how important it is in the role of acclimation to FW. The time-course experiment was performed to determine the best length of time to allow the killifish to acclimate to the FW to see the highest claudin-30c expression. The current data showed that there was a significant increase in the expression of Cldn30c protein after 3 and 7 days, so in the future vivo knock-down experiments, a 3 day or week-long treatment of killifish in FW would be an excellent place to start.

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