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# Functional Significance of Gill Claudin Proteins in Rainbow Trout (Oncorhynchus mykiss) Osmoregulation

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## Abstract

Claudin proteins, a key element of tight junction complexes, are known to control paracellular permeability. In euryhaline fish, changes in claudin abundance and localization are critical during salinity acclimation. In seawater, a leaky paracellular pathway that facilitates sodium extrusion is hypothesized to be controlled by claudin proteins. The aim of this study was to evaluate the role of Claudin-10c, -10d -10e and Claudin-30 in gill function in freshwater (FW) and seawater (SW) rainbow trout (*Oncorhynchus mykiss*). I examined mRNA and protein abundance along with cellular localization. A tissue distribution survey showed that all of the claudins studied were predominantly expressed in gill tissue. Transcript and protein expression of Claudin-10s was significantly up-regulated after SW transfer, while no difference in Claudin-30 expressions was observed. In accordance with these expression patterns, *in silico* prediction showed that Claudin-10s could form cation-selective pores and thus be critical to sodium secretion in SW. Claudin-30 is known as a resistance forming claudin and its insensitivity to salinity suggests an epithelial barrier function in both FW and SW gills. In addition, immunofluorescence microscopy revealed that Claudin-10s are localized in association with the ionocytes in SW. Expression of Claudin-30 was restricted to intermediate cells on the gill filament. This study also suggest that claudins expression may be influenced by combination of both genetic and environmental factors.

**Acknowledgements**

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*I dedicate this thesis to my grandparents*

*who inspired me to study biology.*

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### Journal publications:

Madsen SS, **Bujak JK** and Tipsmark CK, 2014, Aquaporin expression in the Japanese medaka (*Oryzias latipes*) in freshwater and seawater: challenging the paradigm of intestinal water transport? The Journal of Experimental Biology, v. 217, p. 3108-21.

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# Chapter I

## Introduction

The ability to keep ion concentrations in the body fluids within a narrow range is essential in maintaining osmotic homeostasis in vertebrates. In the case of water-dwelling organisms, such as fish, the challenge faced when retaining ion and water homeostasis is highly influenced by the external environment. Due to osmosis, fish in freshwater (FW) constantly gain water. Simultaneously, they lose ions over the body surfaces and along with the copious urine produced by the kidney to counteract water gain from their dilute environment. In seawater (SW), the situation is reversed – fish are constantly loosing water to a concentrated environment which is counteracted by drinking SW and the salt load thus gained has to be secreted by active transport. To keep osmotic balance, fish have developed different mechanisms for ion uptake and secretion, in FW and SW, respectively. Interestingly, some fish are able to tolerate a broad range of salinity and are able to survive in both FW and SW – they are called euryhaline fish. During their lifetime, they encounter drastic changes of salinity, therefore maintaining hydro-mineral balance is demanding and involves extreme changes in physiology. Adaptations to the change include shifts in function of osmoregulatory organs such as the intestines, the kidneys, and the gills (Marshall and Grosell, 2006). The FW fish intestine is the organ where salt contained in food can be absorbed in order to supplement ion loss. In seawater, due to an increased drinking rate, osmotic loss of water can be replenished. Kidney function in a diluted environment is oriented to dispose of water excess. In a marine environment, fish use the kidney mainly to excrete divalent ions that were gained through absorption in the intestine (Marshall and Grosell, 2006).

Among the osmoregulatory organs, only the gill epithelium is in direct contact with the external environment. This versatile organ has a series of important physiological roles; it is the primary place of 1) gas exchange, 2) acid-base regulation, 3) ammonia excretion, and 4) active NaCl transport (Evans et al., 2005). The large surface area of the gills, which helps in respiration, is problematic in terms of osmosis. It is understandable, therefore, that gills are the place where water and ions can enter or escape from the body. Nevertheless, gills are also the principal location for compensatory active ion uptake and secretion to take place (Evans, 2008). Changes in external salinity level elicit dynamic rearrangement of molecular machinery in the gill epithelium. Readjustment includes proteins involved in both active and passive transport of solutes (Evans et al., 2005; Karnaky, 1998). Most of these proteins are part of the transcellular solute pathway, transport that goes through the cell. Another route leads through the space between adjacent epithelial cells and it is called the paracellular pathway. Paracellular pathways are controlled by tight junctions connecting lateral sides of epithelial cells apically and are formed by several kinds of proteins. Among them, claudins constitute the most important part when it comes to determining both general and ion-selective permeability (Tsukita et al., 2001; Van Itallie and Anderson, 2004).

Claudins are membrane proteins, characterized for the first time in 1998 by Tsukita (Furuse et al., 1998). They are the main components in tight junctions responsible for creating both paracellular barriers and charge selective pores. The claudin proteins family is represented by numerous isoforms. The number of isoforms varies from species to species, reaching the highest number in teleost fish (Loh et al., 2004). Interestingly, different claudin isoforms possess distinct properties determining permeability characteristics of the epithelium. Moreover, they exhibit a tissue-specific pattern of expression which underlines involvement in a broad range of

physiological processes in various tissues. Claudin gene knockout and mutation studies confirmed that claudins are important in many biological functions such as proper development, hearing, blood-brain barrier function, ion and water management and cell proliferation (Angelow and Yu, 2007; Chasiotis et al., 2012; Siddiqui et al., 2010; Van Itallie and Anderson, 2006; Xie et al., 2010). Claudin-based tight junctions, that create barriers and pores, are responsible for maintaining the cellular microenvironment and thus they are essential in keeping up functionality of body compartments (Tsukita et al., 2008). The dual nature of claudin-based tight junctions makes these junctions highly dynamic and plastic, able to respond to variable environmental conditions. It has been demonstrated that salinity changes and hormone treatment can affect the expression pattern of claudins and cause changes in epithelium permeability, which is crucial in managing of ions and water (Chasiotis et al., 2012; Madsen and Tipsmark, 2008; Tipsmark et al., 2008; Tipsmark et al., 2010). This fact underlines an important implication of claudins in the osmoregulation process. Research on the role of claudins in fish osmoregulation is still in its infancy, with the first paper published in 2008. Knowledge on the function of claudin proteins is of great importance since the paracellular path is a largely unexplored component of epithelial physiology. Such studies add to our understanding of basic function and have high significance for our comprehension of health and disease related to malfunction of claudin-based tight junctions.

The main goal of this research was to investigate the physiological aspects of claudins and their role in osmoregulation using rainbow trout (*Oncorhynchus mykiss*) as a model organism.

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# Chapter II

# Literature review

## A. Biology of rainbow trout (*Oncorhynchus mykiss*)

### 1. Phylogeny of rainbow trout

Rainbow trout (*Oncorhynchus mykiss*) is a ray-finned fish species, a member of the order Salmoniformes. According to the recent phylogenetic analysis, Salmoniformes is sister taxon to Esociformes and together with Osmoeriformes, forms a superorder Protacanthopterygii of the intraclass Teleostei (Osinov and Lebedev, 2004; Pavlov and Osinov, 2004). Fossil data indicates that Salmonidae fish, were present in the Eocene (McPhail, 1997). Nevertheless, current genera such as *Salmo* have appeared in early Miocene, ca 20 mya. According to Shedko (2012), genus *Oncorhynchus* must have diverged in the late Miocene ca 11.6-5.3 mya. Currently 10 genera including *Salmo, Salvelinus* and *Oncorhynchus* comprise order Salmoniformes (Osinov and Lebedev, 2004). For many years genus *Oncorhynchus* was believed to be a sister taxon with *Salmo,* however, increased taxa sampling revealed that *Oncorhynchus* is closely related to genus *Salvelinus* (Fig. 1); (Crete-Lafreniere et al., 2012; Osinov and Lebedev, 2004). Molecular phylogenetic data revealed that the dichotomy between ancestors of the genera *Oncorhynchus* and *Salvelinus* was the latest divergence that occurred in the Miocene epoch (Shedko et al., 2012).

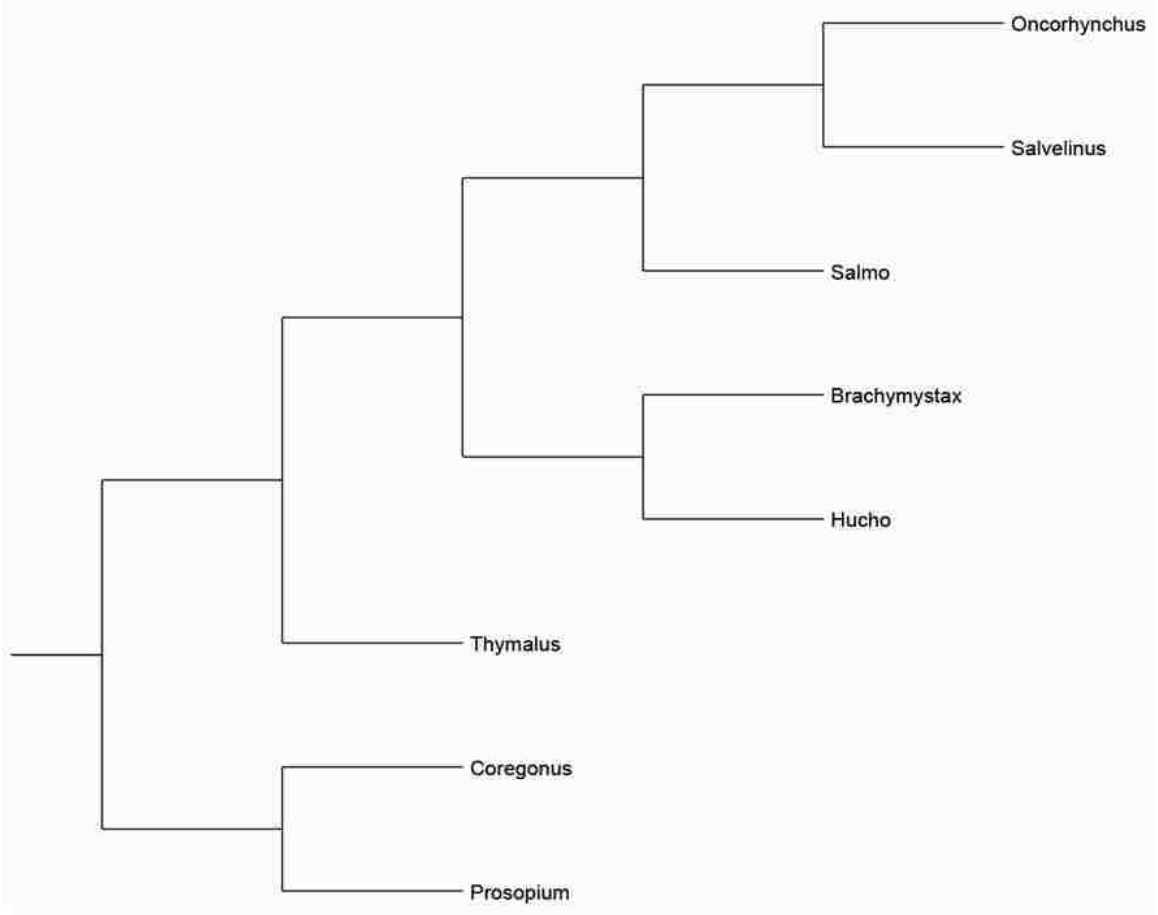


Figure 1. Cladogram representing phylogenetic relationships among genera of Salmonidae family. Based on the data by A. Crete-Lafreniere, et al. (2012).

2. Ecology and life cycle

Rainbow trout are predatory fish which can grow to maximum size of around 60 cm. They are native to the western coast of North America from California to the Gulf of Alaska and to the north eastern part of Asia, from the Bering Sea to the Sea of Okhotsk and Kamchatka peninsula (67°N - 32°N, 135°E - 117°W) (Froese and Pauly, 2000). Rainbow trout also was successfully introduced to other places outside its native range, including all of the states in the USA and another 87 locations worldwide such as European countries, Japan, New Zealand and Australia (Fausch et al., 2001). They inhabit streams, rivers and lakes, and for the migratory forms, also the marine environment. Their range of occurrence is limited mainly by temperature and oxygen

level but also by high quality of water. According to Opuszyński (1979), rainbow trout, similarly to other salmonids, are relatively stenothermal (based on Brett's thermal tolerance zone index (Opuszyński, 1979)). It is considered to be a cold water species that will not tolerate temperatures exceeding 25° C (Eaton et al., 1995). Also, they require well-oxygenated water. Drops in oxygen level to 0.8-1.2 mg/l are lethal for rainbow trout. For comparison, lethal oxygen level for crucian carp (*Carassius carassius*) is around 0.1 mg/ml (Winberg, 1956).

The regular life cycle of rainbow trout includes freshwater and seawater episodes. Rainbow trout hatch in freshwater, usually in streams. Juvenile trout, called parr, spend 1-3 years in fresh water (Suworow, 1954). After that time, trout can undergo a physiological transition called smoltification. This process prepares trout for migration and entrance to seawater. Smolts are characterized by silvering skin, higher activity of sodium-potassium pumps, and an increase of hormones involved in hypoosmoregulation in blood plasma (Sundell and Sundh, 2012). Rainbow trout spend a few years in the sea followed by reentrance to freshwater for the purpose of spawning (Fig. 2).

Interestingly, not all rainbow trout migrate to seawater. Due to its complex life history, *O. mykiss* is divided into migratory (steelheads) and non-migratory (rainbow trout) ecotypes (Docker and Heath, 2003; Meka et al., 2003). Differences in morphology and behavior were the basis for the initial incorrect classification of these two forms as two distinct species (Docker and Heath, 2003; Jordan and Evermann, 1905). Further genetic analysis revealed however, that anadromous steelheads and freshwater resident rainbow trout are the same species. Studies by Docker and Heath (2003) showed that genetic differences between steelheads and rainbow trout exist due to polyphyly and parallel evolution related to, at least in some cases, geographic isolation.

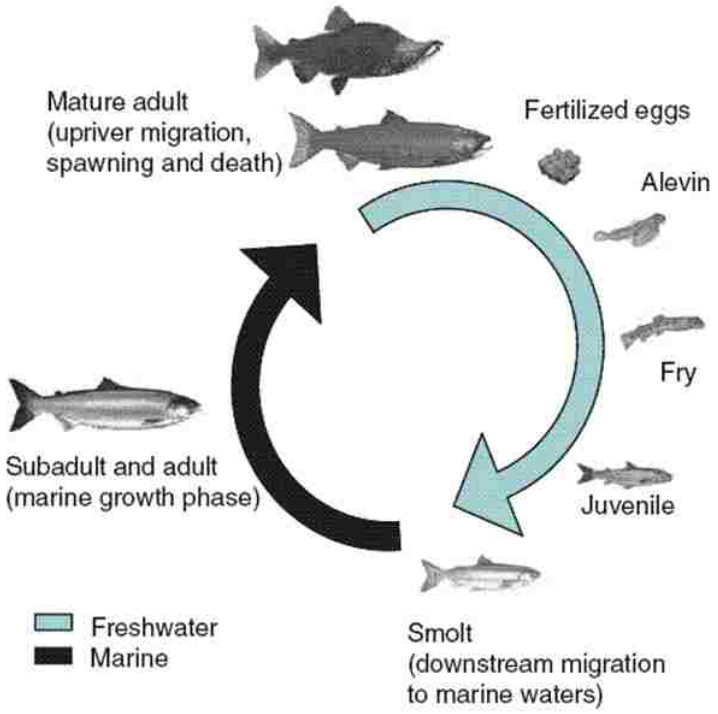


Figure 2. Life cycle of rainbow trout (*Oncorhynchus mykiss*) (Cooke et al., 2011). Reproduced with permission of Elsevier, 2015 (Appendix 6).

Distinct behavior and physiological properties also may be explained by genetic polymorphisms within the population (Currens et al., 1990). Recent studies, revealed that the migratory form of rainbow trout exhibits different transcription levels of genes that are associated with osmoregulation, compared to fresh water resident forms (Hecht et al., 2014). The physiological transition from parr stadium to smolts has a complex genetic basis and it is hypothesized that multiple QTLs are responsible for the migration decision (Hecht et al., 2014; Nichols et al., 2008). Interestingly, studies on *Salmo salar* have demonstrated that smoltification is associated with size of the fish and with influence of environmental factors such as temperature and photoperiod (Handeland et al., 2013).

Rainbow trout are considered to be euryhaline fish that exhibit a wide range of salinity tolerance which is associated with anadromy. The ability to withstand a wide range of salinity is not common in fish. It is estimated that less than 10% of Actinopterygii exhibit broad salinity tolerance (Schultz and McCormick, 2013). Euryhalinity in salmoniformes is hypothesized to be an acquired trait. Recent studies showed that Salmoniformes is a sister taxon to the exclusively freshwater Esociformes (Campbell et al., 2013).

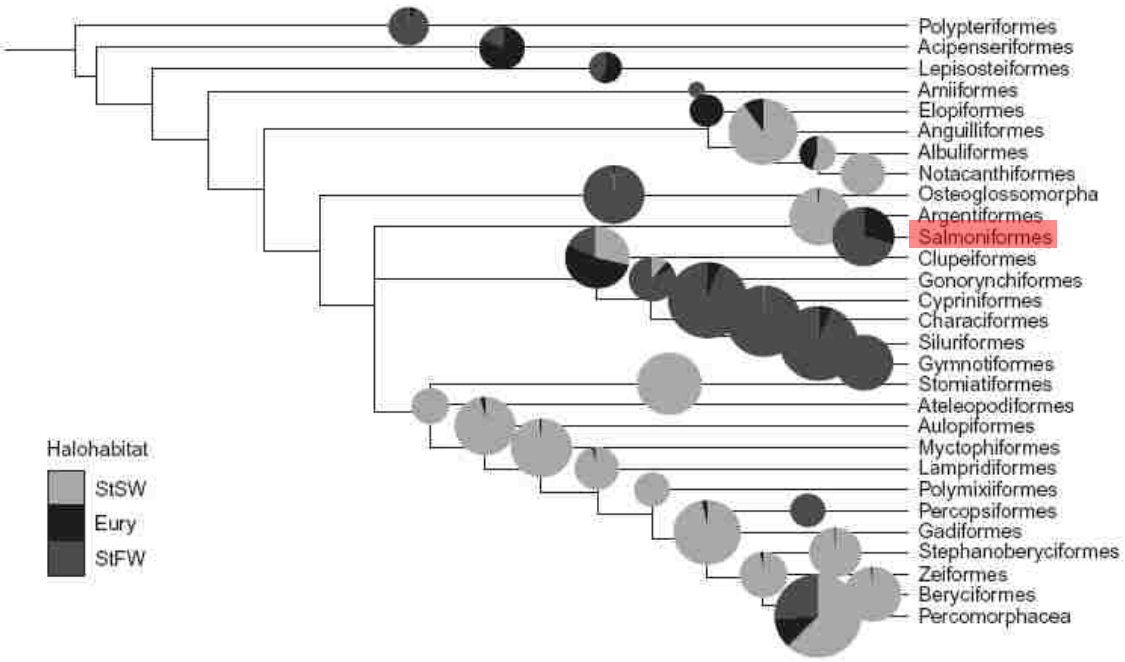


Figure 3. Halohabitat of Salmoniformes. Salmoniformes are represented by species inhabiting both exclusively freshwater environment and by anadromous euryhaline species (Schultz and McCormick, 2013). Reproduced with permission of Academic Press, 2015 (Appendix 6).

Also, Salmonidae is represented by both solely freshwater species and anadromous species but no exclusively marine species (Schultz and McCormick, 2013). According to McCormick and Schultz (2013) 70% of salmoniformes are, in fact, stenohaline freshwater species and the rest are

confined to euryhaline species (Fig.3). Furthermore, mitogenomics studies by Ramsden et al. (2003) also suggest that the salmonids' ancestor originated in freshwater.

### 3. Rainbow trout as a model organism

Rainbow trout have a high importance to industry. Farming of this species has a long history dating back to the early twentieth century (Fornshell, 2002). Currently, trout farming is widespread not only in the United States, from where it originated, but it was successfully introduced to fish farms worldwide. Besides fish farms, rainbow trout are also reared in national hatcheries. For the purpose of farming, details about biology, breeding and optimal growth conditions were developed. Currently, rainbow trout are widely used for purposes of toxicology, ecology and physiology studies (for review see Thorgaard et al., 2002). Furthermore, due to wide salinity tolerance, many of the osmoregulatory studies use rainbow trout as a model. Most of these studies were devoted to general aspects of ionoregulation and SW acclimation, impact of endocrine factors or involvement of genetic traits involved in ability to osmoregulate with respect to aquaculture (Le Bras et al., 2011; Lin et al., 1994; Prunet et al., 1985). Recent studies also showed that cell lines, derived from rainbow trout, may be used as a model for studies on hydromineral balance in fish but also on mammalian kidney function (Trubitt et al., 2015).

## B. Teleost fish osmoregulation

### 1. Principles of osmoregulation

Maintaining a proper hydromineral balance is one of the most important requirements for physiological processes and every deviation from a tolerance range may have serious consequences. Keeping hydromineral homeostasis is challenging especially for aquatic organisms, opposed to terrestrials. It is due to the fact that the aquatic environment is very diverse in terms of salinity and thus is prone to create both osmotic and ionic gradients (Opuszyński, 1979). Furthermore, fish gills possess a huge surface area, covered by a thin epithelium which is necessary for effective gas exchange but problematic for fluxes of ions and water (Evans et al., 2005). Both, freshwater and seawater teleost species maintain their body fluid composition around 1/3 of seawater (~300 mOsm), which is different from surrounding environment, having great implications for hydromineral balance (Marshall and Grosell, 2006). Fish in fresh water are hyperosmotic to the surrounding environment and because of this they must cope with salt loss and excessive water gain. Thus, they developed a mechanism of active ion uptake from their environment by the branchial epithelium. In order to cope with water gain, teleost fish secrete high volumes of hypotonic urine. The situation is reversed in seawater, where fish are exposed to high salinity concentrations in comparison to their body, which leads to dehydration and salt load. To prevent water loss, fish drink seawater and produce a small amount of isotonic urine. At the same time, they secrete sodium and chloride ions out of the body (Fig.4) (Evans, 2008; Marshall and Grosell, 2006). In order to survive in various halohabitats, teleost fish have developed effective physiological mechanisms associated with maintaining a proper

osmotic pressure of the body fluids. This process of active regulation of the osmotic pressure is called ‘osmoregulation’.

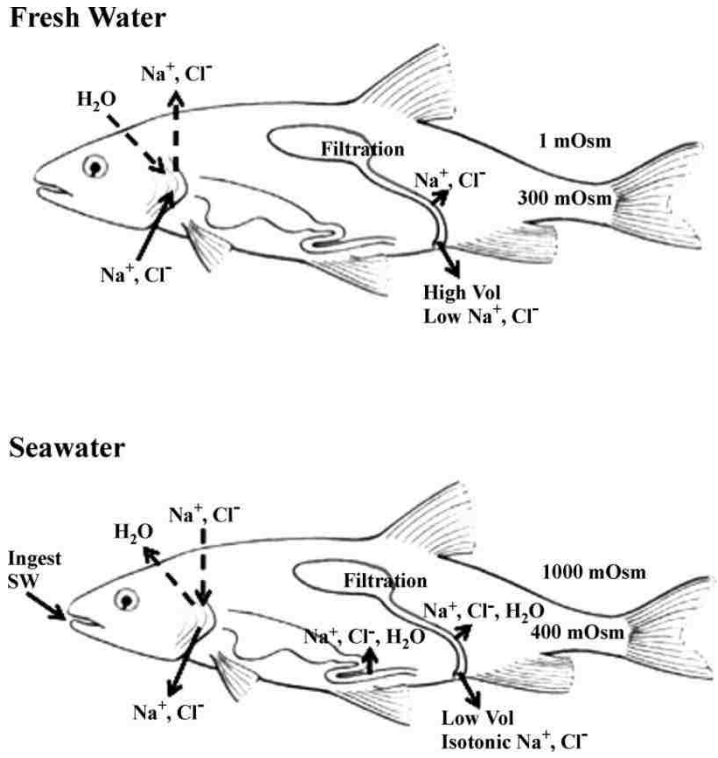


Figure 4. Osmoregulation of freshwater and seawater fish. FW species are hypertonic to external environment while SW species are hypotonic. Thus they are threatened by excessive dilution of body fluids or dehydration, respectively. That difference gave rise to distinct physiological mechanisms that maintain osmolality of body fluids at relatively stable level (Evans, 2008).

Euryhaline fish, such as rainbow trout, which can survive in both freshwater and seawater, are excellent models for osmoregulation studies. Their biology allows comparisons of FW and SW mechanisms of osmoregulation and disclose the pathways ruling transitions between these two mechanisms (Gaumet et al., 1995).

2. Gills as an osmoregulatory organ

The process of osmoregulation is associated with the functions of excretory organs. It is commonly known that gills, intestine and kidneys of teleost fish are the major organs involved in osmotic adjustment. Also, skin and urinary bladder play a role in maintaining a proper water-ion concentration (Marshall and Grosell, 2006). Among the above-mentioned organs, gills are multifunctional structures that are paramount sites for active ion transport (Fig.5).

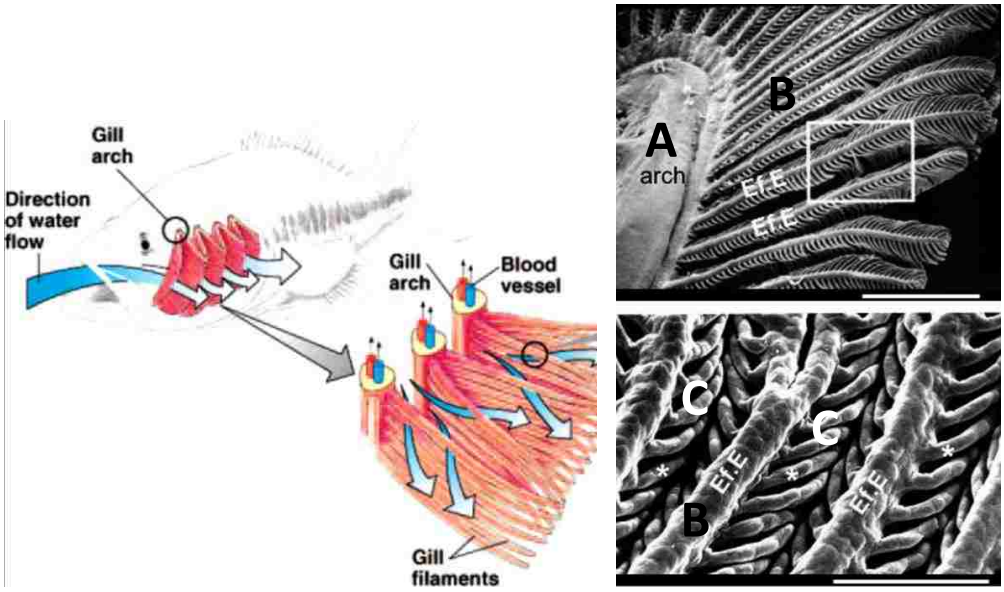


Figure 5. Fish gill anatomy. Gills are multifunctional organs that are in direct contact with the external environment. Gills consist of several gill arches (A), supporting filaments (B). Each filament possess numerous secondary lamella (C) covered by thin epithelium (Evans et al., 2005).

Gills are also a major place for gas exchange, acid-base regulation and excretion of waste products of metabolism (Evans et al., 2005). Salinity changes, in first instance, affect the gill since this organ is in direct contact with the surrounding environment. Under osmotic stress the gills epithelium undergoes structural and physiological transitions.

The gill epithelium consists of several cell types. The most abundant are pavement cells (PVC), comprising around 90% of gill epithelium. Those thin, flat cells are the primary site of gas exchange (Wilson and Laurent, 2002). Bigger ionocytes or mitochondrion-rich cells (MRC) are located on the secondary lamellae or more commonly on the base of lamellae on filaments. These cells are called NKA-reactive cells because their basolateral membrane is rich in $Na^+$, $K^+$ ATPase (Hiroi and McCormick, 2012). Ionocytes are directly involved in ionoregulation (Fig.6).

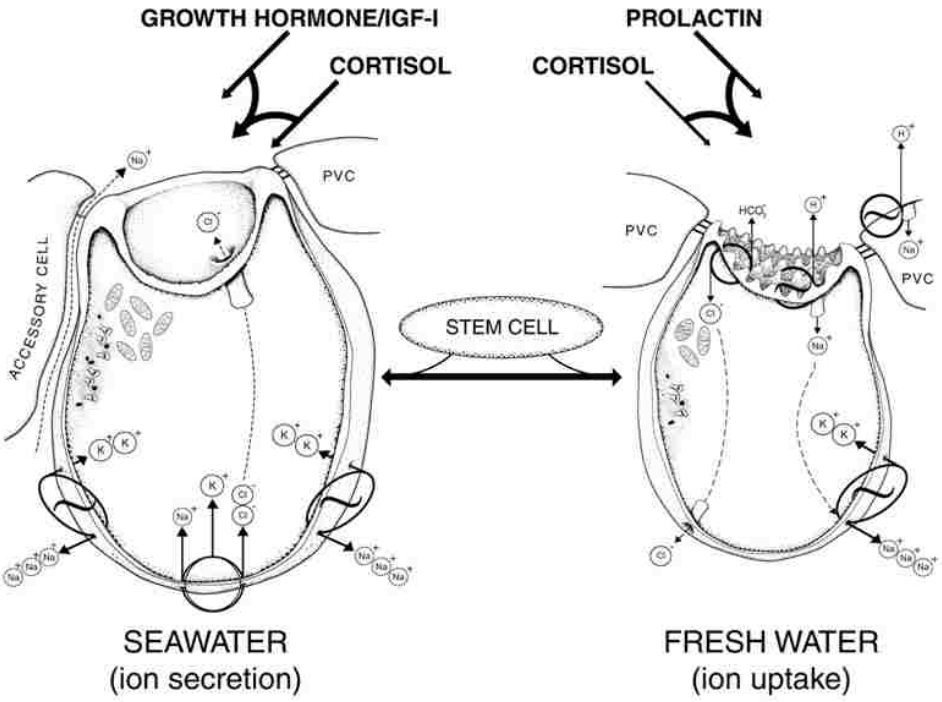


Figure 6. Mitochondrion-rich cells (ionocytes) in the gill epithelium of freshwater and seawater acclimated fish. Ionocytes are the principle cell type responsible for active ionoregulation in fish (McCormick, 2001). Reproduced with permission of Oxford University Press, 2015 (Appendix 6).

Osmotic stress triggers rearrangement of proteins involved in ion uptake or secretion on both apical and basolateral membranes of ionocytes (Evans, 2008; Karnaky, 1998). Regulation of ionic balance involves the action of a range of ion channels, co-transporters and ion-exchangers, such as NKCC ($Na^+$-$K^+$-$Cl^-$ cotransporter), CFTR (cystic fibrosis transmembrane conducntance

regulator), NHE ($Na^+/H^+$ exchanger) (Hiroi et al., 2008; Hwang and Lee, 2007; Marshall et al., 2002). The main driving force for these processes is provided by the activity of $Na^+$, $K^+$ ATPase (NKA), which maintains a proper electrochemical gradient for ion movement (Evans et al., 2005).

Depending on the osmotic pressure, MRC of euryhaline fish are able to adjust the suite of transport proteins involved in ion uptake and secretion. In SW gill epithelium mitochondrion-rich cells (MRCs) form complexes with accessory cells (ACs) that are arranged in dyads or triads (Payan et al., 1984). Tight junctions (TJs) between two MRCs and ACs were suggested to be 'leaky' in order to facilitate paracellular sodium diffusion, important in teleost hypoosmoregulation (Hootman and Philpott, 1980; Payan et al., 1984). That hypothesis was provided by electrophysiological studies on FW and SW gill epithelium. It was demonstrated that in seawater (SW) gill epithelium exhibits lower transepithelial resistance (TER) and is more permeable for sodium ions than freshwater (FW) gill epithelium (Evans et al., 2005; Zadunaisky, 1984). Transition between FW vs. SW type of MRC however, is still not clear. It has been suggested that endocrine factors may trigger the changeover (McCormick, 2001).

## C. Claudins as a key element of tight junctions

### 1. Tight junctions

Tight junctions (TJ) are protein complexes present in the most apical part of two adjacent cells. They consist of numerous proteins including membrane-spanning proteins such as claudins, occludins, tricellulins and junctional adhesion molecules along with other associated proteins including scaffolding proteins (ZO-1, -2, -3) and cytosolic proteins (Angelow et al., 2008; Gonzalez-Mariscal et al., 2003). This complex network of molecules links TJ with cell signaling, vesicle trafficking or cell polarization and proliferation (Kohler and Zahraoui, 2005; Matter et al., 2005; Schneeberger and Lynch, 2004). Tight junctions are also responsible for regulation of paracellular pathway for solute movements, by formation of barriers or selectively permeable pores (Angelow and Yu, 2007; Gumbiner, 1993; Heiskala et al., 2001). Despite the complex structure of TJ, paracellular permeability is generally thought to be controlled by claudin proteins (Angelow et al., 2008; Furuse et al., 1998; Morita et al., 1999; Tsukita and Furuse, 2000; Tsukita et al., 2001)

### 2. Claudin proteins

Claudins were discovered in 1998 by M. Furuse as a part of a tight junction complex distinct from earlier known occludin proteins (Furuse et al., 1998). Claudins are tetraspan membrane proteins with molecular weight ranging from 20 -30 kDa (Fig. 7).

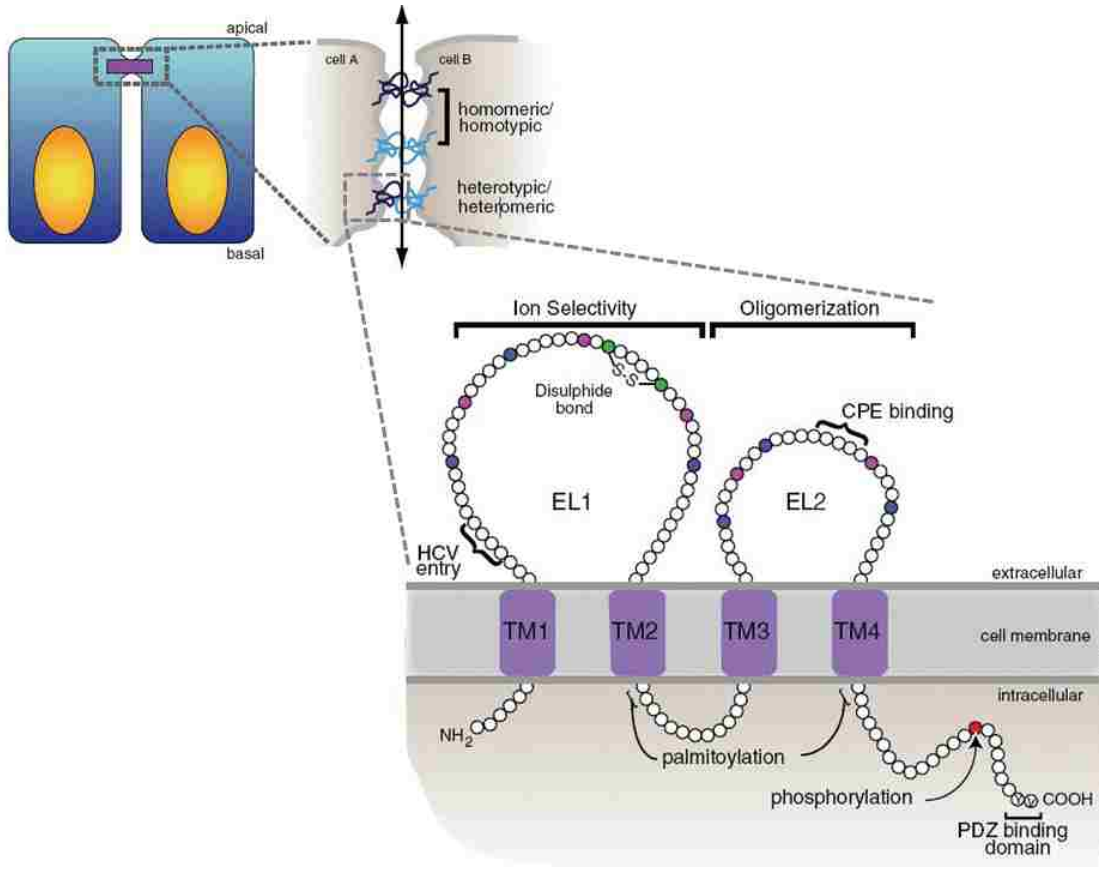


Figure 7. Claudins as an integral part of tight junction complexes of the epithelial cells. They have four transmembrane domains and two extracellular loops (ECL). The first extracellular loop was shown to be responsible for charge selectivity. The C-terminus of claudins contains putative regulatory sites (Gupta and Ryan, 2010). Reproduced with permission of John Wiley and Sons, 2015 (Appendix 6).

Claudins are represented by numerous isoforms numbering reaches 24 in mammals and up to 56 in some fish (Lal-Nag and Morin, 2009; Loh et al., 2004; Morita et al., 1999). Expression of different claudin isoforms appeared to be tissue-specific (Peppi and Ghabriel, 2004; Kolosov et al., 2013). For instance, the highest expression of Claudin-9 was found in cochlea while claudin-10a expression was restricted mainly to the kidney in mammals (Kitajiri et al., 2004; Van Itallie et al., 2006). Some claudins, however, exhibit a more ubiquitous pattern of expression. Claudin-1, similarly to Claudin-5 has been found in most epithelial tissues (Furuse et

al., 1998; Morita et al., 1999). Different claudin isoforms were shown to have distinct properties, crucial in defining permeability characteristics of epithelium (Colegio et al., 2002; Gunzel and Yu, 2013; Shen et al., 2011). Based on studies using cultured epithelial cell lines, expression of claudin-2, -10b or 15 caused an increase in epithelial permeability to sodium, leading to the conclusion that these isoforms create cation-selective pores (Amasheh et al., 2002; Van Itallie et al., 2003; Van Itallie et al., 2006). In contrast, Claudin-3, -4 or -5 were shown to form barriers that seal the epithelium layer (Milatz et al., 2010; Van Itallie et al., 2001; Wen et al., 2004). Therefore, claudin composition corresponds to tight or leaky epithelium. Recent findings demonstrated that distinct charge-selective properties of claudins are determined by the arrangement of charged amino acids on the first extracellular loop (ECL-1) (Colegio et al., 2003; Yu et al., 2009). The role of the shorter second loop is not so clear. So far, it has been shown that the second extracellular loop of some claudins contains a binding site for *Clostridium perfringens* enterotoxin and may be involved in strand formation between claudins from adjacent cells (Fujita et al., 2000; Piontek et al., 2008). Furthermore, it was reported that the cytoplasmic C-terminus tail possesses sites that can be phosphorylated or palmitoylated suggesting that it may affect the regulation of claudins (Angelow et al., 2008; Lal-Nag and Morin, 2009).

### 3. Importance of claudin proteins in physiology

Charge-selective and size-selective claudins constitute the main element of tight junctions which are responsible for regulation of the cellular microenvironment (Tsukita et al., 2008). Disruption of the claudin-based ‘barrier’ leads to several disorders and diseases in vertebrates (Gupta and Ryan, 2010). Recent studies showed that claudins are required in proper early development (Furuse and Moriwaki, 2009; Siddiqui et al., 2010). It has been shown that a knock-down of the

claudin-e and claudin-b in zebrafish embryos leads to developmental abnormalities, such as delay of epiboly, and physiological defects impairing, for instance, sodium handling (Kwong and Perry, 2013; Siddiqui et al., 2010). Furthermore, claudins were documented to be involved in the creation of body compartments, ligand-receptor segregation, immunity and tumorogenesis (Gupta and Ryan, 2010; Sawada, 2013; Singh et al., 2010; Tsukita et al., 2008). Claudin proteins were also shown to be an important element responsible for proper function of several organs including kidneys, the gastrointestinal tract, blood-brain barrier, lungs and skin (Balkovetz, 2009; Brandner, 2007; Koval, 2013; Lameris et al., 2013; Singh et al., 2010; Xie et al., 2010). Also, claudin gene mutations were associated with several diseases, such as ichtyosis (*cldn-1* mutation), nonsyndromic deafness (*cldn-14* mutations) or hypomagnesemia hypercalciuria with nephrocalcinosis (mutations of and *cldn-16* and *cldn-19* respectively) (Ben-Yosef et al., 2003; Hadj-Rabia et al., 2004; Konrad et al., 2006; Simon et al., 1999). Recent studies suggested that claudins may be involved in regulating acid-base balance in kidney and in the formation of paracellular channels permeable to water (Balkovetz, 2009; Rosenthal et al., 2009).

## D. Implications of claudins in fish osmoregulation

### 1. Claudin genes in teleost fish

Intensive studies on claudin functions in fish began with puffer fish (*Fugu rubripes*). In 2002 the puffer fish genome was sequenced, opening the door for both transcriptomic and proteomic analyses (Aparicio et al., 2002). Soon it came to light that the claudin gene family in this particular fish is extremely vast in comparison to other vertebrates and contains 56 genes (Loh et al., 2004). Results of phylogenetic analysis suggest that there was a whole genome duplication event early in the evolution of the fish lineage (Christoffels et al., 2004; Taylor et al., 2003) which was followed by retrotransposition of some genes, as well as multiple tandem duplication (Tine et al., 2011). Genome duplication can explain why some species of teleost have an extensive number of claudin genes and why some of those genes are characteristic only for the fish lineage. Interestingly, most of the known teleost genomes are very compact and contain many SEGs (single exon genes). Intronless claudin genes are specific only for fish, whereas genes containing multiple exons are relatively more or less similar to mammalian claudin (Tine et al., 2011). It has been suggested that single exon genes (SEG), that also encode claudins, have important implications in quick response to variable environmental conditions (Tine et al., 2011). These implications may validate the theory that the claudin genes' expansion was an important step in fish evolution that helped fish to maintain hydromineral balance by physiological adjustment to more demanding aquatic environments (Loh et al., 2004). If such is the case, it is not surprising that numerous claudins are specifically expressed in tissues that are directly exposed to water, and thus, take part in osmoregulation (Loh et al., 2004).

2. Osmoregulatory function of claudins in teleost

Several studies suggested that claudin proteins are important in fish osmoregulation since they control paracellular movement of solutes. This supposition is supported by the fact that some claudin isoforms are highly and specifically expressed in the osmoregulatory organs of fish. For instance, expression of claudin-15a and -3a was restricted to intestine and kidney in *Fugu rubripes* (Loh et al., 2004) while claudin-6 -10d,and -10e were found predominantly in the skin and gill of *Tetraodon nigroviridis* (Bui and Kelly, 2014; Tipsmark et al., 2008). Claudin-10c, -10d and -10e transcripts were also found in the rainbow trout gill cell line (Kolosov et al., 2014). Further, transcriptomic studies on European eel (*Anguilla anguilla*) osmoregulation provide information about regulation of claudins by salinity (Kalujnaia et al., 2007). Subsequent studies confirmed the effect of salinity on the expression level of certain claudin isoforms in different teleost species (for review see (Chasiotis et al., 2012).

The most extensive studies on osmoregulatory function of claudins were performed on fish gills, as these appear to be the major site responsible for ion and water fluxes (Chasiotis et al., 2012). In the light of recent evidences, remodeling of the gill epithelium after SW transfer includes alternations in claudin expression, which may explain changes in gill epithelium permeability. For example, claudin-10d, -10e and -6 in *Tetraodon nigroviridis* and claudin-10e in *Salmo salar* were shown to be upregulated by SW (Bui and Kelly, 2014; Tipsmark et al., 2008). Interestingly, the above mentioned isoforms were present only in MRCs-ACs and were absent from the PVCs fraction of puffer fish gill epithelium, which may underline their specific function in SW acclimation (Bui et al., 2010). Also, expression of claudin-10c, -10d and -10e was associated with the presence of MRC-AC in the cells of primary cultured rainbow trout gills (Kolosov et al., 2014).

In contrast to gill epithelium in SW, freshwater (FW) gill epithelium was described as ‘tight’, which was correlated with barrier function of claudin-based TJ. Several claudin isoforms, for example Claudin-30 in *Salmo salar* or Claudin-30c (previously Claudin-b) in *Danio rerio*, were demonstrated to form barriers that seal the epithelium and prevent from diffusive ion loss in FW (Engelund et al., 2012; Kwong and Perry, 2013). Intriguingly, claudin-30 in Atlantic salmon was shown to be associated only with pavement cells (PVC) but not with MRC (Engelund et al., 2012).

### 3. Claudin alterations in response to osmoregulatory hormones

Claudin expression was shown to be influenced by osmotic pressure. Furthermore, there is evidence that endocrine factors also influence abundance of claudin proteins in the osmoregulatory organs (Tipsmark et al., 2009).

The major osmoregulatory hormones in teleost fish are: prolactin, growth hormone and cortisol (McCormick, 2001). Prolactin in fish plays a role as freshwater-adapting hormone (Manzon, 2002). Its action not only remodels and tightens gill epithelium but also promotes ion uptake by MRC (Foskett et al., 1983; Manzon, 2002). Growth hormone, in turn, is a seawater-adapting hormone, which antagonizes prolactin (Sakamoto and McCormick, 2006). It is responsible for improvement of salinity tolerance via stimulation of salt secretion by MRC (Mancera and McCormick, 1998). The role of cortisol is more intricate. In euryhaline fish, cortisol exhibits synergistic effects with prolactin and surprisingly also with the growth hormone- IGF axis (McCormick, 2001). Cortisol acts as both freshwater and seawater-adapting hormone (Takahashi and Sakamoto, 2013). Studies on Atlantic salmon have shown that injection of growth hormone (GH) as well as prolactin (PRL) did not influence gill-specific claudin expression. Interestingly,

GH together with cortisol decrease expression of claudin-28b, whereas prolactin increased claudin-28a (Tipsmark et al., 2009). According to previously mentioned studies, claudin-28a and -28b were not affected by salinity changes in the gills of Atlantic salmon (Tipsmark et al., 2008). More relevant to osmotic adaptation, the isoforms: claudin-30, -10e, and -27a were affected by cortisol, which in turn did not change expression of claudin-28a and -28b (Tipsmark et al., 2009). Furthermore, cortisol was reported to elevate expression of some claudins in the gills of stenohaline goldfish (*Carassius aureatus*) (Chasiotis and Kelly, 2012). With reference to the above mentioned studies, these results prove that claudin-based tight junctions are highly dynamic structure regulated by a very complex net of interdependence. Other studies concerning influence of hormones on tight junctions have been performed on gill epithelia cell cultures. Primary culture of *Tetraodon nigrovirids* gill epithelium has been established for the purpose of tight junction studies (Bui and Kelly, 2011). That culture consists only of PVCs, which express almost all of the gill-specific claudins (Bui and Kelly, 2011). Exceptions are only claudin-6, claudin-10d and claudin-10e that are believed to be associated with other types cells occurring in the gills (Bui and Kelly, 2011). Cortisol treatment of such a cell culture resulted in increased expression of claudin-11a, -27a and 33b, whereas claudin-3a, -27c, and -32a were down-regulated. The rest of the isoforms present in these cells were not affected (Bui et al., 2010). The authors proposed that isoforms that were up-regulated by cortisol are forms that increase tightness of the epithelium (Bui et al., 2010). However, claudin-3a, -27c and -32a, that were decreased after cortisol action, had been described previously also as ‘tight epithelium’ isoforms (Bagherie-Lachidan et al., 2008, 2009; Bui et al., 2010). It was suggested that other factors may be involved in the regulation of these isoforms (Bui et al., 2010). Other studies that were devoted to cortisol function in regard to osmoregulation and tight junctions were performed on

euryhaline trout (*Oncorhynchus mykiss*) and stenohaline goldfish (*Carrasius aureatus*) gill epithelium cell cultures. After incubation with cortisol (500 ng/ml), claudin transcripts in cultured gill cells were measured (Chasiotis and Kelly, 2011). Results revealed that the mRNA levels of trout claudin- 30, -28a, -3a, -7, -8d and -12 were significantly elevated after cortisol treatment (Chasiotis and Kelly, 2011). These changes, however, were not observed in goldfish cultured gill epithelium cells. Only claudin-e, which is consider to be an ortholog of the trout claudin-28b, was upregulated due to the action of cortisol (Chasiotis and Kelly, 2011). Apparently, goldfish claudin-e might play the same role as the trout claudin-28b, i.e. as a barrier–building isoform (Chasiotis and Kelly, 2011). However, because of the fundamental differences between euryhaline and stenohaline fish biology, it is difficult to support this conclusion.

## E. Research Objectives

The main purpose of this study was to determine the role of claudin proteins in a process of osmoregulation. The basic knowledge about claudin biology is of high importance due to the fact that they are essential component of epithelial tissue and they are responsible for its appropriate function. Impairment of proper functioning of claudin-based tight junctions has serious consequences and may lead to severe diseases. The understanding of claudin function and regulation is still in its infancy. Thus, basic studies on claudin biology significantly contribute to this field of science.

As it is stated above, claudin-based tight junctions are crucial element of many physiological processes including osmoregulation. Based on the current state of knowledge (literature review), teleost fish possess extensive number of claudin genes and at least some of them appear to be involved in maintaining hydromineral balance. Euryhaline fish, such as rainbow trout, seems to be a perfect model to use for the purpose of claudins research and epithelial physiology. In order to investigate role of claudins in trout osmoregulation I focused on gill tissue.

The main goals of this study were as follows:

- Examine whether gill specific claudins are regulated by salinity,
- Evaluate subcellular localization of branchial claudin isoforms,
- Determine whether Claudins contribute to ‘leaky’ paracellular pathway facilitating sodium diffusion in seawater.

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# Chapter III

# Functional significance of gill claudin proteins in rainbow trout (Oncorhynchus mykiss) osmoregulation

## A. Materials and methods

### 1. Animals and experimental protocols

Rainbow trout (*Oncorhynchus mykiss*) were obtained from the Norfork National Fish Hatchery (Mountain Home, Arkansas, USA) and transported to the University of Arkansas (Fayetteville, AR, USA). Fish were in the parr stage and weighted around 2 grams. Fish were kept in 900 L tanks, with Fayetteville (AR) dechlorinated tap water, at 20°C and under constant 14:10 light-dark photoperiod. Fish were fed once a day with AquaMax fish diet pellet (Purina Mills, LLC, MO, USA). Prior to the experiments, fish were not fed for 24 hours. Experimental procedures were carried out with respect to Animal Welfare Act and were approved by University of Arkansas Institutional Animal Care & Use Committee (IACUC: #11005, #13052).

Prior to sampling, fish were euthanized using 300 mg/L of Tricaine-S (Western Chemicals Inc., Ferndale, WA, USA), buffered with 500 mg/L $NaHCO_3$ for FW and the gill apparatus was dissected carefully. Filaments were directly snap frozen on dry ice and stored at -80 °C for mRNA and Western Blot analysis. For histological analysis, gill arches were immersed in cold 4% formaldehyde (Mallinckrodt Chemicals, Phillipsburg, NJ, USA) diluted in 1X phosphate buffered saline solution (Li-Cor, Lincoln, NE, USA).

2. Study design

For the purpose of tissue distribution, fish (age group: 0+; average weight: 2.5-3.5 g) were acclimated to FW or to SW (25 ppt) for seven days (N=10). Sampling was performed after one week because after this time rainbow trout ought to acclimatize to SW and thus changes between FW and SW acclimated gill tissue should be more apparent. After that time, fish were euthanized and tissues of interest were dissected. Tissues for qPCR analysis including gills, intestine, kidney, liver and muscle were frozen immediately after dissection, on dry ice and stored at -80 ºC until processed.

In the SW challenge experiment fish (0+; 2.5-3.5 g) were transferred to FW or to SW (25 ppt). After 7 days, fish were euthanized and gill arches were dissected for the purpose of qPCR. Rainbow trout (1+; 30 g) were acclimated to FW and 25 ppt SW respectively for 30 days. On the day of sampling fish were euthanized and the gill apparatus was dissected for western blot and immunofluorescence microscopy. Samples were frozen on dry ice and stored at -80 ºC until processed. When high mortality occurred, salinity of SW was decreased to 20 ppt. For the purpose of time course experiment, fish (0+, 4 g) were transferred to FW and 20 ppt SW. Sampling was performed on days 1, 7, 15, and 30 (N=10). Gill arches were dissected and processed as previously described.

3. Analysis

- *Total RNA extraction, reverse transcription and quantitative PCR (qPCR)*

Total mRNA was extracted from gill tissue using TRI reagent (Sigma-Aldrich, Saint Louis, MO, USA). Shortly, around 50-100 mg of FW and SW gill tissue were immersed in 1 ml of TRI reagent and homogenized using VWR Power Max AHS 200 Advanced Homogenizing System

(PRO Scientific, Henry Troemner LLC, Thorofore, NJ, USA). After the addition of 100 µl of 1-Bromo-3-chloropropane and centrifugation (4 °C, 12,000 g for 15 minutes), the aqueous phase of each sample was collected into a new tube. To allow RNA precipitation, ice-cold isopropanol was added and samples were left overnight in -20 °C. The next day, samples were centrifuged at 4 °C, 12,000 g for 10 minutes and the resulting RNA pellet washed with 75 % ethanol. Following centrifugation, RNA pellets were air-dried and dissolved in 30 µl DEPC-treated pure water (VWR, West Chester, PA, USA). Concentration and purity (using A260/A280 ratio) of RNA was determined using Nano-Drop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Purity (A260/A280) ranged from 1.8-2.0. Complementary DNA synthesis was performed with 1 µg of total RNA. Reverse transcription was carried out using High Capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) in a Master Cycler Gradient thermocycler (Eppendorf, Hamburg, Germany). *Claudin-10e* and *claudin-30* mRNA expression was determined by quantitative PCR (qPCR) using SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) and CFX 96 Detection System (Bio-Rad laboratories, Hercules, CA, USA). Primers for claudin-10e and -30 have been described previously (Tipsmark et al., 2008). Primers for claudin-10c and -10d are included in the Table 1 (Appendix 1). Reaction conditions were as follows: 2 minutes of initial denaturation at 95 °C followed by 45 cycles of 30 second denaturation phase at 95 °C and 1 min annealing phases at 60 °C. A melting curve (from 65 °C to 95 °C, with an increment of 0.5 °C) analysis was performed at the end of the cycles to confirm the specificity of the amplification. Transcript quantification was normalized using three reference genes: *β-actin, ribosomal protein large P* (*rplp*) and *elongation factor* (Vandesompele et al., 2002)*1-α* (*Ef1α*) (Table 1, Appendix 1). The most stable

normalization genes were chosen based on geometric averaging of housekeeping genes (Vandesompele et al., 2002) using GeNorm software (Biogazelle, Zwijnaarde, Belgium).

- *Western blot*

Protein extraction and analysis were performed on ice to avoid protein degradation. Gill samples were homogenized in ice-cold SEID buffer (300 mM sucrose, 10 mM EDTA-$Na_2$, 50 mM imidazole and 0.1 % of sodium deoxycholate) with 1/100 protease inhibitor cocktail (Sigma-Aldrich) and using the VWR Power Max homogenizer (PRO Scientific). The homogenate was centrifuged at 4 °C, 6000 g for 10 minutes to remove cells and tissue debris; the supernatant was collected into a new tube. The protein concentration was quantified using Bradford protein assay (Bradford Reagent, Sigma-Aldrich), on a SpectraMax Plus384 Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA, USA) following manufacturer protocol. For each sample, 10 µg of proteins was used.

Samples were mixed with 4x LDS Sample Buffer (Novex, Life technologies, Carlsbad, Ca, USA) with addition of 100 mM of dithiothreitol (GE Healthcare Life Sciences, Pittsburg, PA, USA), for a final protein concentration of 1 mg/ml. Samples were incubated at 75 °C for 10 min, followed by electrophoresis separation using NuPAGE 4-12 % Bis Tris 1 mm Gels (Life technologies), NuPAGE MES SDS Running Buffer (Life Technologies) and 1 mL of NuPAGE Antioxidante (Life Technologies) in the upper chamber of the X Cell Sure Lock Mini Cell electrophoresis device (Life Technologies). Molecular weight of proteins was evaluated using Precision Plus Protein All Blue Standards (Bio-Rad laboratories, Hercules, CA, USA). Electrophoresis was carried out at constant voltage (200 V) for 35 minutes. After separation, the gel was blotted onto a nitrocellulose membrane with a 0.2 µm-pore size (Life Technologies). Electroblotting was performed using transfer buffer (Life Technologies) and 10 % X Cell II Blot

Module (Life Technologies) at constant voltage (30 V) for 1 hour. Then, the membrane was immersed in blocking buffer (5 % non-fat dried milk dissolved in 1xTBST [20 mM Tris, 140 NaCl, 0.1 % Tween-20]) and incubated for 1 hour at 4 °C under agitation. Blocking was followed by incubation with the affinity-purified polyclonal claudin-10c antibody at final concentration 0.5 µg/ml; claudin-10d at final concentration 0.8 µg/ml; claudin-10e antibody, at final concentration 0.8 µg/ml (GenScript, Piscataway, NJ, USA) or with 1.2 µg/ml of the rabbit affinity-purified polyclonal claudin-30 antibody (Table 2., Appendix 1) (GenScript). All of the anti-claudin antibodies were raised in rabbits. A monoclonal antibody, raised in mouse against the α-subunit of the $Na^+$, $K^+$-ATPase (α5) (Developmental Studies Hybridoma Bank, Iowa City, IA, USA), in a final concentration of 0.23 µg/ml. A mouse monoclonal β-actin antibody at 0.1 µg/ml (AbCam, Cambridge, MA, USA) was used as a loading control. Incubation was carried out at 4 °C overnight. Following washing with 1xTBST, membranes were incubated with anti-rabbit and anti-mouse secondary antibodies conjugated with IRDye 800 and IRDye 680, respectively (final concentration: 50 ng/mL; Li-Cor, Lincoln, NE, USA). Membranes were incubated in the dark for 45 minutes at room temperature. After incubation, membranes were washed in 1xTBST, air dried and scanned using Odyssey Infrared Imager (Li-Cor,) and bands were analyzed and quantified using Image Studio Ver. 2.0 software (Li-Cor,). Claudin-10e and Claudin-30 antibody specificity was validated with a negative control using the antibody pre-incubated with its blocking peptide. Claudin antibodies were incubated with 400 molar excess of blocking peptide dissolved in the 5% non-fat dried milk solution in 1xTBST and left to incubate overnight at 4 ºC with gentle mixing.

- *Immunofluorescence microscopy*

Dissected gill arches were fixed overnight at 4 °C. After five washes in 1xPBS, samples were transferred to O.C.T. compound (Tissue-Tek, Torrance, CA, USA) and incubated overnight at 4 °C. After the cartilage was removed, each sample was transferred into cryomolds (Tissue-Tek) and immediately frozen on dry ice. Frozen blocks were cut into 10 µm-thick slices using HM 525 Cryotom (Microm International, Walldorf, Germany). Slices were laid on a glass slide and dried at 50°C for 2 hours. Following hydration in PBS, tissue antigens were revealed using boiling citrate buffer (10 mM solution, pH 6.0) and blocked for 1 hour at room temperature with blocking solution (3% BSA, 2% Normal Goat Serum dissolved in PBS). Samples were incubated with claudin-10e antibody at final concentration of 1 µg/ml or claudin-30 at 1.5µg/ml. Tissues were co-immunostained with the α5 antibody, used as a control at a final concentration 0.4 µg/ml. Slides were washed in PBS after overnight incubation at 4°C in order to remove unbound primary antibodies, then incubated with secondary antibodies at 37 °C for 2 hours: goat anti-rabbit antibody conjugated with Cy3 at a final concentration of 3.33 µg/ml (Life technologies) and goat anti-mouse antibody conjugated with Cy5 at a final concentration of 3.33 µg/ml (Life technologies). After a final PBS washing, samples were mounted between slide and cover slide with SlowFade Gold Antifade Reagent with DAPI (Life Technologies). Immunostaining analyses were performed using a Zeiss Axio Imager M2 microscope (Zeiss, Oberkochen, Germany) equipped with AxioCam MR monochrome camera and its software Axio Vision 4.

- *Bioinformatics*

Sequences of trout/salmon claudin-10c, -10d,-10e and claudin-30 and sequences of murine claudin-2, claudin-3, claudin-4, claudin-10a and -10b proteins were retrieved from the National Center for Biotechnology Information resources. The first extracellular loop (ECL-1) of claudins

was predicted using TMPred server (Hofmann & Stoffel, 1993) on ExPASy Bioinformatics Resources Portal. The sequences corresponding to the ECL1 were processed in SeaView program (Gouy et al., 2010) and alignment was driven by MUSCLE (Edgar, 2004). Negatively charged amino acids (D – aspartic acid, E – glutamic acid) were marked with red boxes while positively charged amino acids were marked with blue boxes (K - lysine, R - arginine). Conserve cysteine (C) residues were marked with green.

- *Statistics*

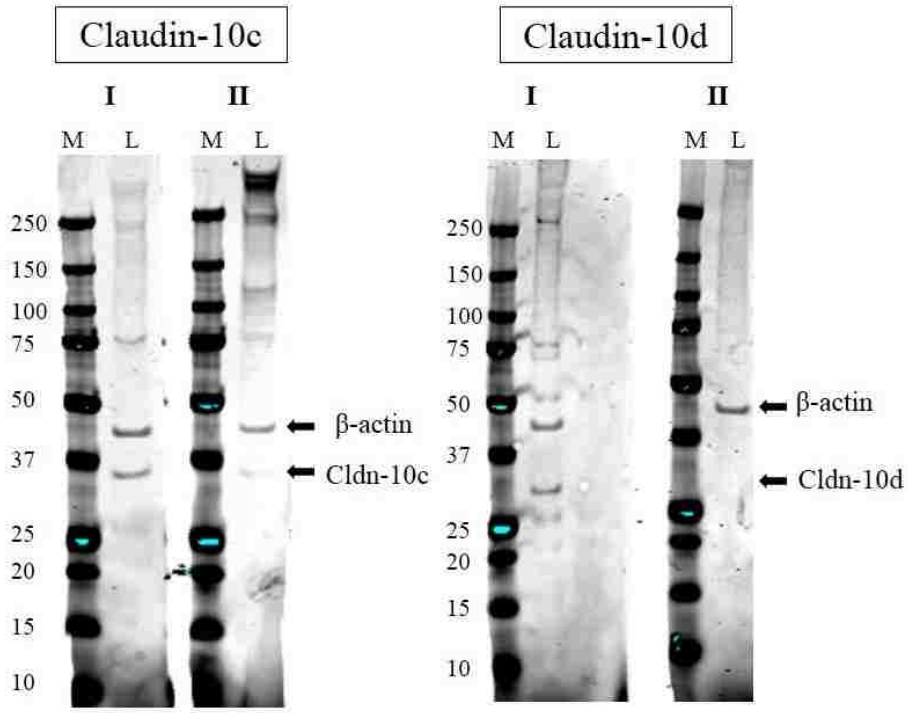
Tissue distribution data were analyzed by one-way ANOVA. For comparison of mRNA and protein expression between FW and SW, a Student's t-test was used. To meet the ANOVA assumption of homogeneity of variances (tested by Bartlett's test) logarithmic transformation of data was performed. Data are represented as the mean $\pm$ standard error of mean (SEM). Mean differences were considered significant when $P < 0.05$. Significant differences were labeled with different letter, as in the case of tissue distribution, or with asterisk: * $P<0.05$, ** $P<0.01$; *** $P<0.001$.

Statistical analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA).

## B. Results and discussion

### 1. Antibody validation

In order to perform immunolocalization studies, antibodies against Claudin-10c, -10d, -10e and -30 were generated (Table 2, Appendix 1). Claudin-10s and claudin-30 affinity purified antibodies were probed against rainbow trout gill lysate. Western blot results showed bands around 35 kDa for claudin-10c, 22 kDa for claudin-10d, 32 kDa for claudin-10e and band around 18 kDa for claudin-30 (Fig.8, strip I). Immunoreactive bands correspond to the predicted molecular weight of Claudin-10c (32 kDa), Claudin-10d (27 kDa) Claudin-10e (36 kDa) and Claudin-30 (22kDa). An additional band for claudin-10e was observed around 60 kDa and for claudin-30 around 20 kDa.



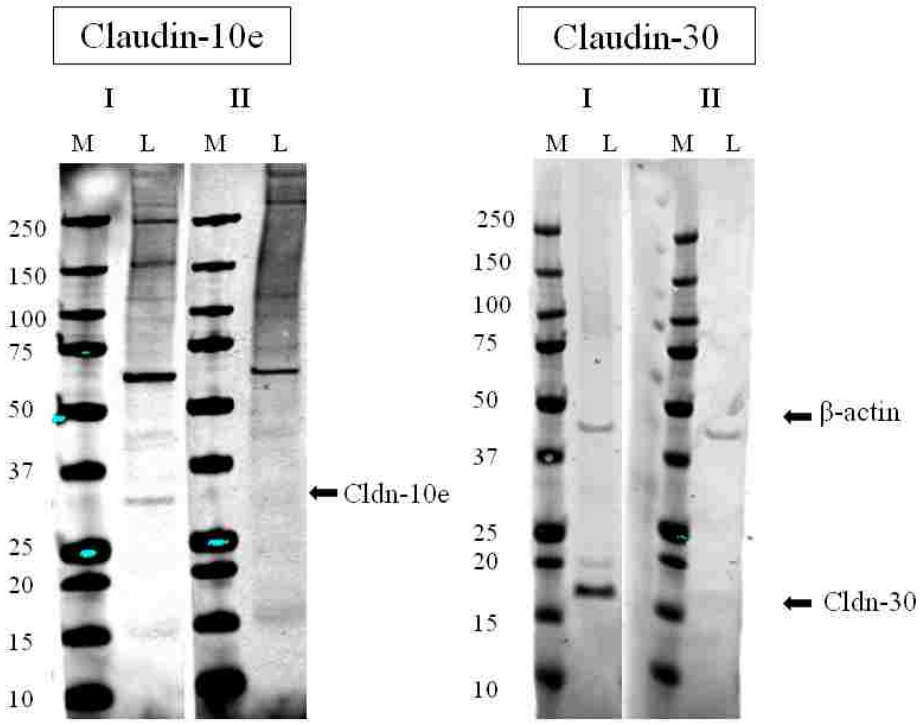



Figure 8. Immunizing peptide blocking experiment with claudin-10s and claudin-30. First strip of each blot (I) was used as positive control and was incubated with antibodies solution. Second strip of each blot (II) represents probe treated with anti-claudin antibody and blocking peptide solution. Claudin-10c corresponds to the immunoreactive band around 35kDa, Claudin-10d to the band around 27kDa and Claudin-10e to the band around 32 kDa. Claudin-30 is represented by the immunoreactive band around 18 kDa. M- marker, L- lane, I- strip probed with primary antibodies only, II- strip probed with cocktail of primary antibodies and blocking peptide.

A higher molecular band recognized by Claudin-10e antibody was approximately twice the predicted size. A possible explanation for this situation is claudin oligomerization. Several studies confirmed existence of homophilic claudin multimers. Claudin-4 was demonstrated to form hexamers in vitro (Mitic et al., 2003). Claudin-5 in turn was shown to exist in the form of mono- to pentamers in vivo and oligomers were not disrupted by SDS-PAGE (Coyne et al., 2003). Furthermore, claudin-16 and claudin-19 were proven to assemble together in order to form functional cation pores (Koval, 2013b). An additional band in the case of Claudin-30, was around 2kDa heavier than predicted, and may represent posttranslational modification such as phosphorylation. Studies on Claudin-2 showed that phosphorylation is responsible for Claudin-2

localization in cell membrane (Van Itallie et al., 2012). Subsequent studies on Claudin-4 demonstrated that phosphorylation was required for tight junction formation and regulation of barrier function (Aono and Hirai, 2008; D'Souza et al., 2007). Also, phosphorylation of Claudin-3 was linked to regulation of barrier function (D'Souza et al., 2005). Antibody specificity was tested by using a 400 molar excess of blocking peptide. As a result, specific immunoreactive bands were diminished (Fig.8, strip II).

2. *In silico* analysis of ECL-1 of trout and mouse claudins

In order to predict putative function of trout claudins, ECL-1 of trout claudin-10c, -10d, -10e and claudin-30 were aligned with mouse claudin-3, -10a, and -10b. Mouse claudins were used as a reference since their function is documented in the literature. Arrangement of charged amino acids on ECL-1 of trout claudin-10s resembles mouse claudin-10b. Trout Claudin-10s, similar to mouse Claudin-10b, have negative residues in the area of two conserved cysteines (green). Interestingly, ECL-1 of trout claudin-10s and mouse claudin-10b does not exhibit similarity to claudin-10a, which was shown to create anion-pores (Van Itallie et al., 2006). The ECL-1 of Claudin-30, in turn, exhibits a similar number and arrangement of positive and negative charges to mouse claudin-3 which were documented to have barrier function (Milatz et al., 2010) (Fig 9). Charge selectivity of claudins is determined by arrangement of charged amino acids on the ECL-1, down the conserved GLW motif (Colegio et al., 2003; Colegio et al., 2002; Van Itallie et al., 2003). Studies by Colegio et al. (2002) confirmed that charge reversing mutations within ECL-1 can change ion-selectivity of claudins.

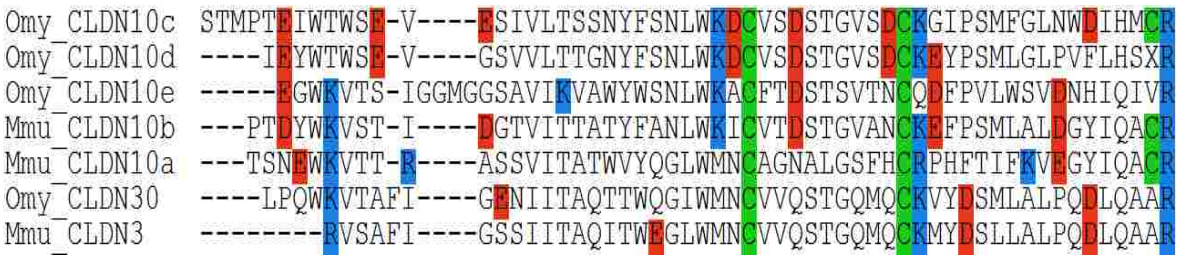



Figure 9. Alignment of the first extracellular loop (ECL-1) of trout Claudin-10s, Claudin-30 and mammalian claudins. ECL-1 of rainbow trout (Omy) claudins was compared with ECL-1 of mouse (Mmu) claudins with known function. Arrangement of positive and negative charges on the ECL-1 is associated with charge-selectivity characteristics of claudins. Residues are color-coded according to the following scheme: negative charges in red, positive in blue, conserved cysteine in green.

Subsequently, Yu et al. (2009), by neutral mutations of acidic residues, showed that the negatively charged interaction site of Claudin-2 is located down the second conserved cysteine, at the position 65 of ECL-1 . By utilizing heterologous expression systems and incorporating electrophysiological approaches, previous studies showed that Claudin-2 acts as a cation-selective isoform (Van Itallie et al., 2003). Similarly, mouse Claudin-10b is also known to be cation-selective isoform, which possesses a negatively charged residue at position 66 (Van Itallie et al., 2006). Trout Claudin-10s sequence aligned to mouse Claudin-10b exhibits similar arrangement of charged amino acids on ECL-1. All of them have negatively charged residue in the proximity of the second conserved cysteine, which may account for cation-selectivity. This is consistent with the suggestion given by Yu et al. (2009), that the molecular filter responsible for charge selectivity of claudins may be located at positions 65 and 66 (first or second position from second conserved cysteine) on ECL-1. Among Claudin-10 isoforms, only Claudin-10c does not have a negatively charged residue down the second conserved cysteine. It is possible that the cation binding site may be located in a different position. Nevertheless, the overall arrangement of negative charges on ECL-1 of Claudin-10c is similar to Claudin-10d, -10e and mouse Claudin-10b. Mouse Claudin-10a, which was shown to be anion selective, does not exhibit a

similar pattern of arrangement of charged residues. Mouse claudin-10a does not contain any negatively-charged residues in the cysteines region.

Mouse claudin-3 has a positive charge in the putative charge interaction site, down the second conserved cysteine. Claudin-3 in mammals was shown to be associated with increased epithelial resistance that is specifically associated with a drop in cation permeability (Kiuchi-Saishin et al., 2002; McLaughlin et al., 2004; Van Itallie et al., 2001). Similarly, studies by Colegio, et al. (2002) showed that charge reversing mutation within ECL-1 of Claudin-4 diminished its function as a cation barrier. The major residue responsible for charge selectivity of Claudin-4 was a lysine also located behind the second conserved cysteine of ECL-1 (Colegio et al., 2002). Alignment of mouse Claudin-3 showed that this isoform has a basic residue at the same position, which may explain a permeability characteristic similar to Claudin-4. Interestingly, trout Claudin-30, homologous to mammalian Claudin-3, contains a lysine residue at the same position as mouse Claudin-3. This may indicate similar properties of these isoforms. Indeed, studies on an ortholog of trout Claudin-30 from Atlantic Salmon (*Salmo salar*) revealed its function as a cation barrier (Engelund et al., 2012). Also, knockdown of Claudin-30c (according to the old nomenclature: claudin-b), orthologous to Claudin-30, in larval zebrafish (*Danio rerio*) resulted in decreased epithelial permeability and diffusive sodium loss, implying a barrier function of this isoform (Kwong and Perry, 2013).

3. Tissue distribution of claudin-10s and claudin-30 transcript

mRNA expression of claudin-10s and claudin-30 was analyzed in various tissues by qPCR. Results showed that mRNA expression of claudin-10s and -30 was at least 5-fold higher in the gills than in other tissues. Minor expression of claudin-10d and -10e was also detected in the intestine and in the kidney (Fig 10).

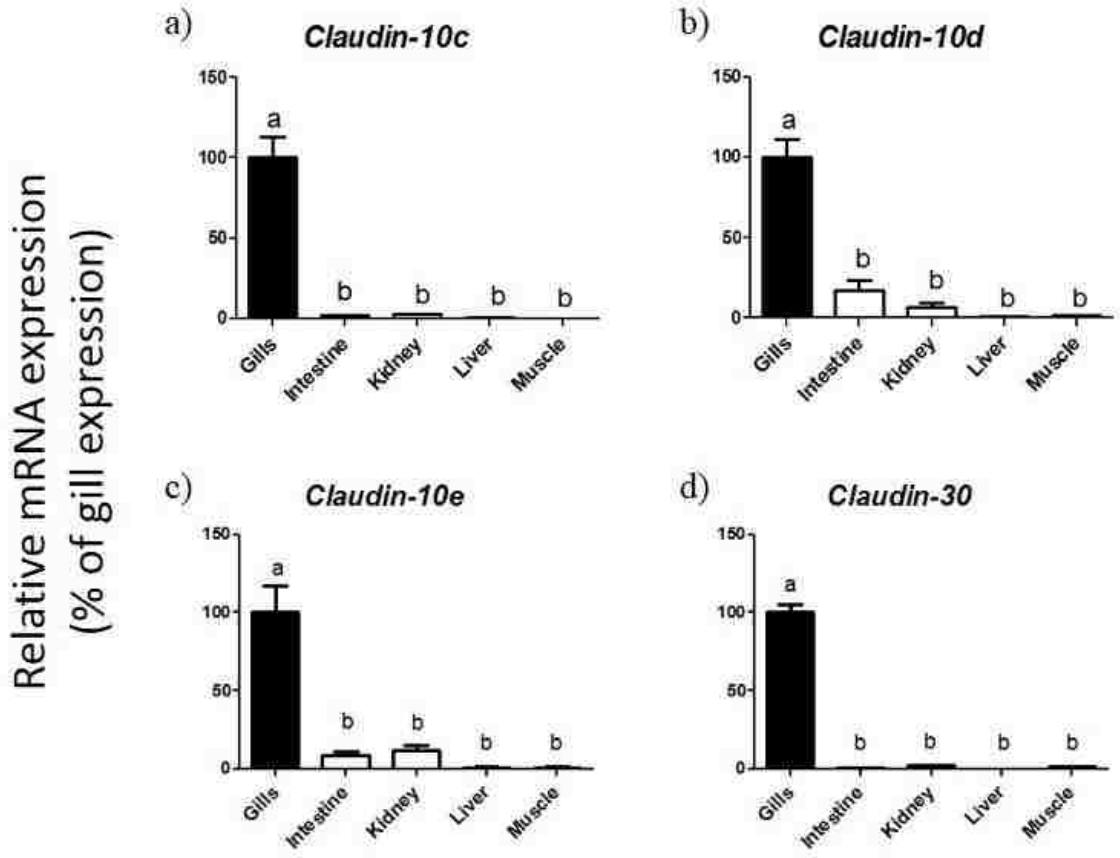



Figure 10. Claudin-10c, -10d, -10e and claudin-30 mRNA expression in tissues of rainbow trout (*Oncorhynchus mykiss*). Transcript level was evaluated in gills, intestine, kidney, liver, and muscle by quantitative PCR. mRNA level was normalized to geometric mean of three genes: *β-actin*, *Ef1a* and *rplp0*. Data are represented as mean value of pooled, FW and SW acclimated fish ± SEM (n=14). Significant differences were indicated by different letters ($P<0.05$).

Claudin-10s in rainbow trout were prominently expressed in the gill tissue, which is consistent with data about c*laudin-10e* in *Salmo salar* (Tipsmark et al., 2008), c*laudin-10d* and *-10e* in *Tetraodon nigroviridis* (Bui and Kelly, 2014) and data about c*laudin-10c, -10d* and *-10e* in gill epithelium cell lines from rainbow trout (*Oncorhynchus mykiss*) (Kolosov et al., 2014). In *Danio rerio* expression of *claudin-10c* and *-10e* was confined to gills whereas *claudin-10d* expression was found in the spleen (Baltzegar et al., 2013).

My data showed that in rainbow trout, *claudin-30* exhibited high expression in the gill tissue when compared to other tissues. It is in accordance with studies on *Salmo salar* where *claudin-*

*30* appeared to be a gill-specific isoform. In turn, the ortholog of trout *claudin-30*, *claudin-30c* from *Fugu rubripes* has a wide mRNA distribution and is most highly expressed in the external epithelia of the gill and skin (Loh et al., 2004). Similarly, in *Danio rerio claudin-30c* was found to be highly expressed in the gills, spleen and skin (Baltzegar et al., 2013). High expression of Claudin-30 in the gills and integument is not unexpected if we take into account putative barrier function of Claudin-30. Both, gills and skin, are directly exposed to environment, thus they are prone places for diffusive ion movements. Hence, proper function of claudin-based barriers appears to be an important and integral element involved in fish osmoregulation.

4. Claudin-10s, Claudin-30 and α5 expression in FW and SW

Expression of claudin-10s, claudin-30 and α5 was evaluated at both the mRNA and the protein levels. In the gill tissue of rainbow trout acclimated to 25 ppt SW transcript levels of *claudin-10c* and *-10e* were significantly upregulated when compared to FW (Fig. 11a, c). Transcript expression of *claudin-10d*, in turn, did not exhibit significant upregulation in response to salinity (Fig. 11b). Nevertheless, the data show a tendency in favor of elevation of *claudin-10d* expression in SW. On the other hand, protein quantification showed that Claudin-10s expression was significantly induced by SW (Fig.12). Induction of Claudin-10s expression during SW acclimation has been demonstrated in several species, including studies on *Salmo salar* by Tipsmark et al. (2008), *Tetraodon nigroviridis* by Bui and Kelly (2014) as well as studies on cultured gill epithelia (Bui et al., 2010). These results implie that salinity regulates expression of Claudin-10s and is responsible for adaptive modulation of claudins in the gill epithelium.

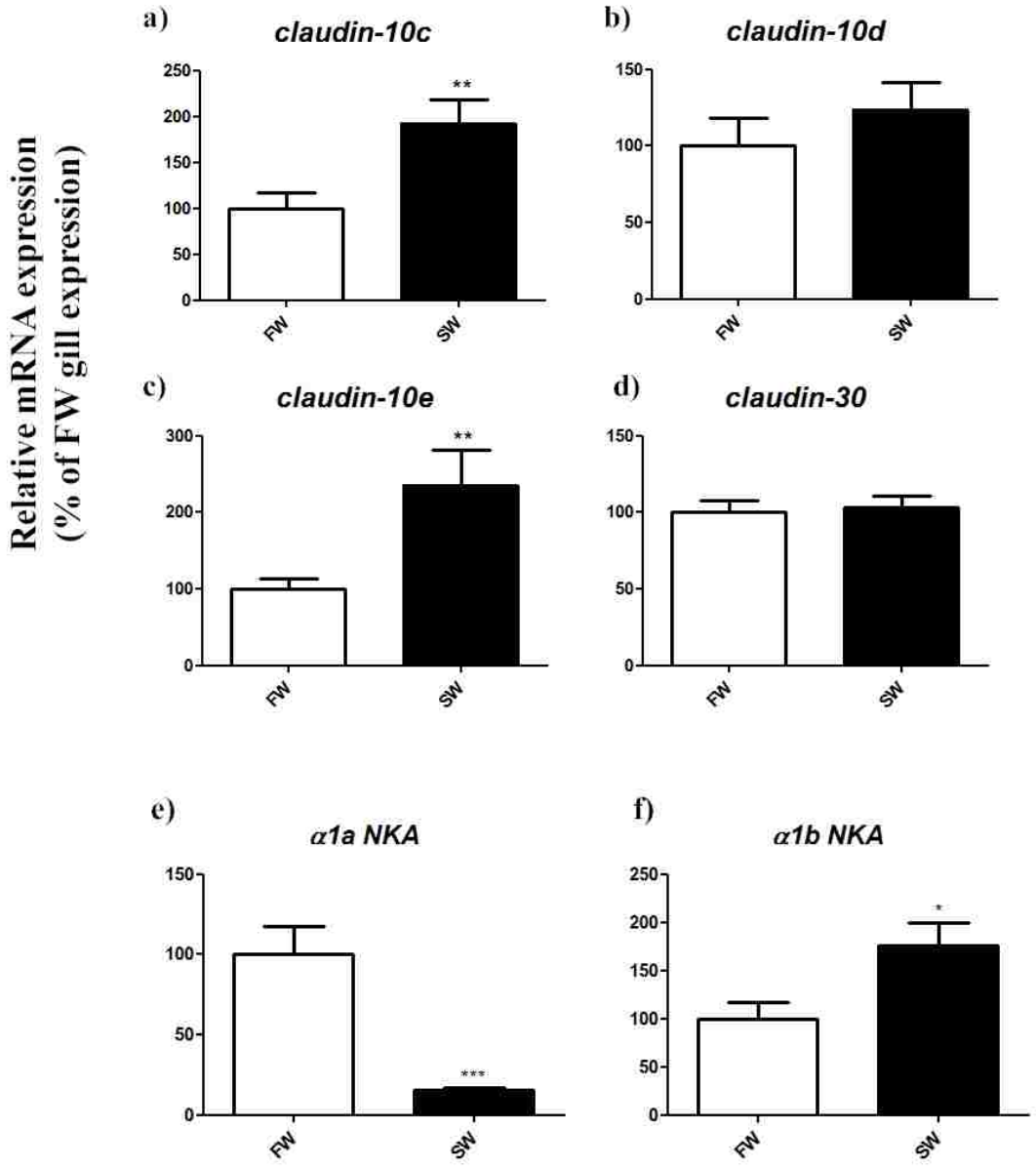
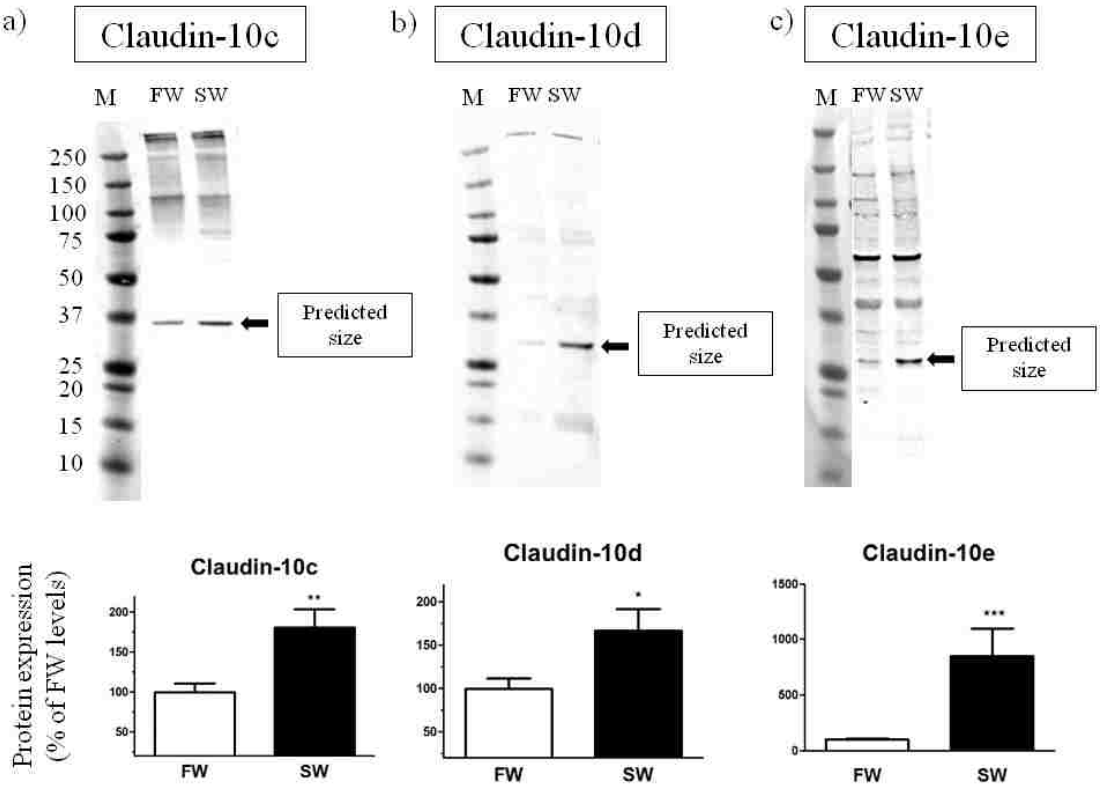



Figure 11. *Claudin-10c, -10d, -10e, claudin-30, NKA α1a* and *α1b* mRNA expression in FW and SW acclimated gills of rainbow trout. α1a and α1b subunits of $Na^+$, $K^+$-ATPase were used as a positive control of response to salinity challenge. mRNA level was normalized to geometric mean of three genes: *β-actin*, *Ef1a* and *rplp0*. Data represent mean value ± SEM (N= 7). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001).

On the contrary, claudin-30 expression was not altered by salinity as was observed on both the mRNA and protein level (Fig. 11d and Fig. 12). This result differs from what was found in a

study on *Salmo salar* where *claudin-30* was downregulated by the SW transfer (Tipsmark et al., 2008). Also, a study by Engelund, et al. (2012) suggested a role of Claudin-30 in FW adaptation in *Salmo salar*. This difference in response to SW may be explained by intraspecies variations, ensuing from differences in biology and life environment. Atlantic salmon is able to adjust to SW faster and more efficiently than rainbow trout (Bystriansky et al., 2006a), thus overall response to salinity challenge may vary between these two species. Correspondingly, studies on puffer fish, *Tetraodon nigrovirids* and *Tetraodon biocellatus*, showed differences in claudin expression arising presumably from interspecies differences (Bagherie-Lachidan et al., 2009; Duffy et al., 2011).



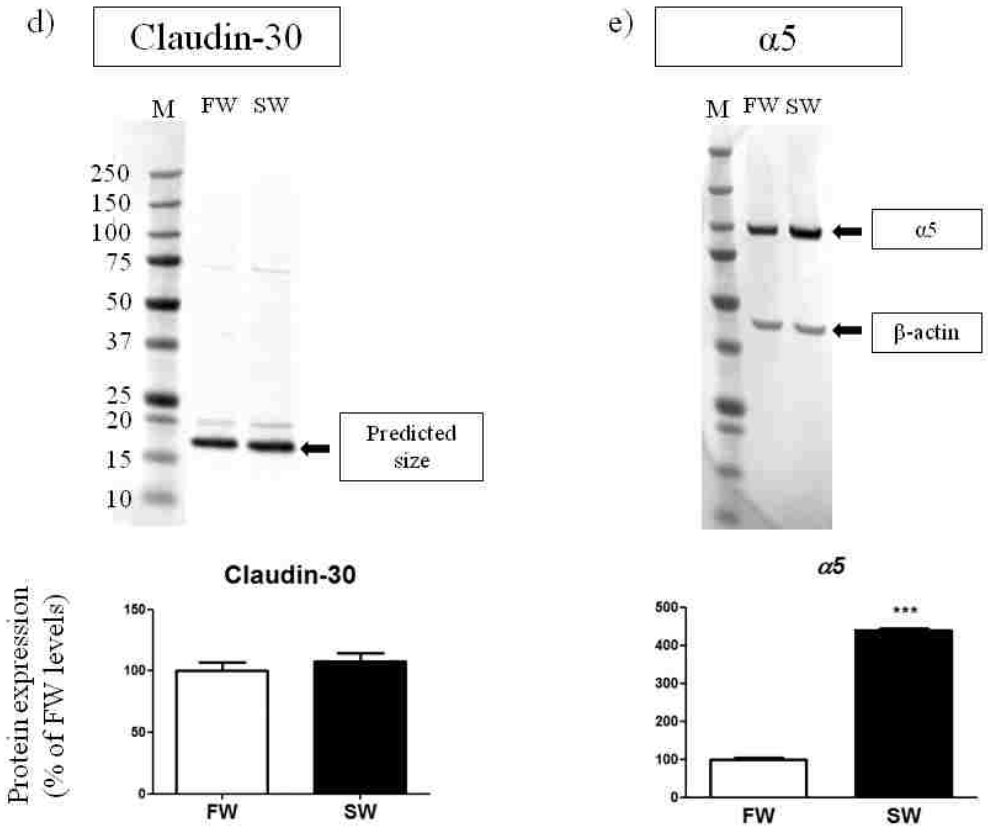



Figure 12. Protein expression of claudin-10c, -10d, -10e, claudin-30 and α5 in FW and SW acclimated gills of rainbow trout. Gill lysate from FW and SW acclimated rainbow trout was immunoblotted with Claudin-10c, -10d, -10e and Claudin-30. α5 antibodies were used as a reference. Data represent mean values ± SEM (N=5). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001).

In addition, expression of the sodium potassium pump was also taken into consideration. NKA is documented to be a major enzyme involved in osmoregulation (Hwang and Lee, 2007). Furthermore, the α-subunit of NKA exists in two isoforms: α1a, which is a FW type and α1b, responsible for SW acclimation (Jorgensen, 2008). Relative mRNA expression of α1a subunit of NKA was significantly higher in FW water when compared with SW (Fig.11e). Expression of α1b subunit was significantly upregulated in SW comparing to FW, which is in accordance with literature (Fig. 11f). SW also substantially elevated abundance of α-subunit of Na+/K+-ATPase

on protein level (Fig. 12e). Changes of NKA expression suggest that fish respond properly to the SW challenge.

5. Claudin-10s and -30 cellular localization in the gill of rainbow trout.

In order to determine the effect of salinity on cellular localization of claudins, immunofluorescence analysis was performed. Claudin-10s staining appeared to be altered by salinity. In FW gill, no strong staining of Claudin-10s was observed (Fig.13a-c). This is consistent with our findings about upregulation of Claudin-10s expression at both mRNA and protein level by SW acclimation. Studies by Bui and Kelly (2014), however, showed that claudin-10d and-10e staining was also present in FW gill of *Tetraodon nigroviridis*. We have not found the same results in rainbow trout where claudin-10d and-10e staining in FW gill was comparable with background fluorescence. One of the explanations may be that expression of Claudin-10s in FW was too low to give a strong staining as in the case of SW gill. This is in line with our qPCR and Western Blot results that showed lower expression of claudin-10s in FW than in SW. Interestingly, cellular localization of Claudin-10s in SW acclimated gill was associated mainly with MRC-AC complex. It is important to note that only in SW MRCs group together with other MRCs and ACs, and form dyadic or triadic complexes (Edwards and Marshall, 2013; Zydlewski and Wilkie, 2013). Claudin-10d and -10e in the gill of *Tetraodon nigroviridis* were found to be associated with ionocytes (Bui and Kelly, 2014). Studies by Kolosov, et al. (2014) also showed association of *claudin-10c, -10d* and *-10e* with presence of the MRCs in the primary cultured rainbow trout gill cells. Also, studies on the cultured gill epithelium of *Tetraodon nigroviridis*, demonstrated that *claudin-10d* and *-10e* were expressed in the whole gill tissue but was absent in the culture consisting only of PVC (Bui et al., 2010; Bui and Kelly, 2011). Similar

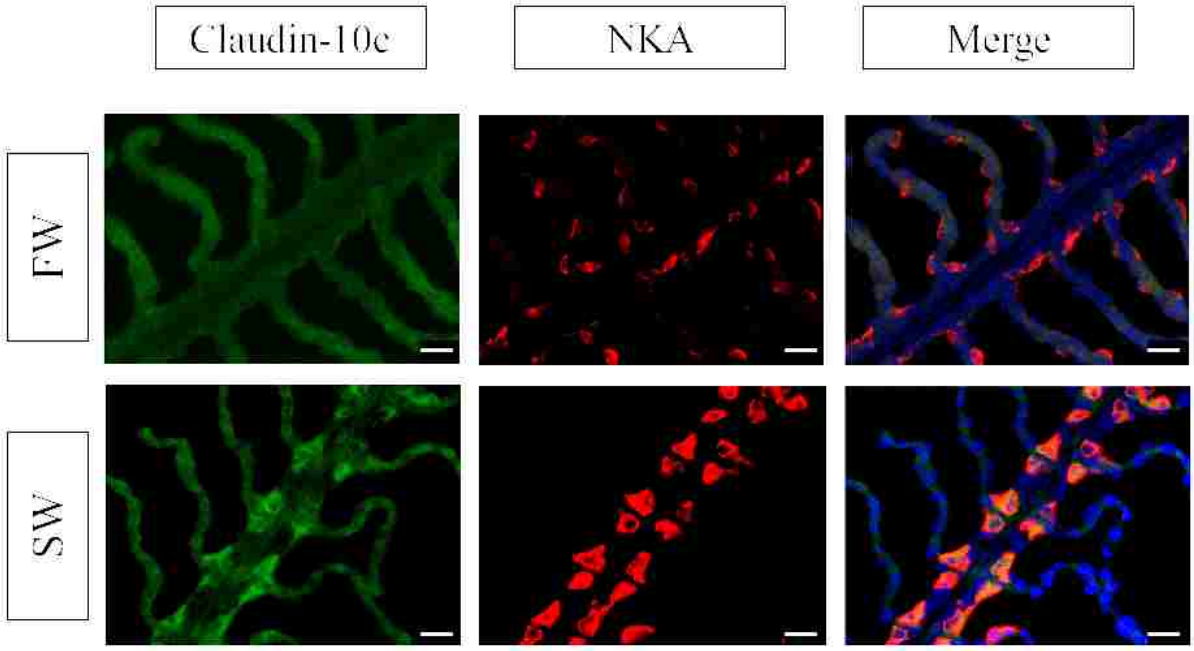
findings were demonstrated in a study on goldfish gill epithelium, where PVC exhibit a different set of claudins than MRC and AC (Chasiotis et al., 2012). It is thus reasonable, to think that the difference in expression and localization of Claudin-10s in FW and SW gill is related to alterations of gill epithelium permeability, which is necessary for proper handling of salinity changes. Electrophysiological studies demonstrated that SW gill epithelium of teleost fish is more permeable than FW gill when correlated with the presence of leaky junctions between MRC and AC complexes (Hootman and Philpott, 1980; Sardet et al., 1979). In contrast, FW gill epithelium appeared to be tighter and less permeable to prevent diffusive ion loss. Also, in FW, MRCs do not form complexes with MRCs and ACs, which are hypothesized to create leaky pathway for sodium diffusion (Zydlewski and Wilkie, 2013). In the light of recent studies, it is clear that changes in gill epithelium permeability in response to salinity are influenced by changes in claudin isoforms expression. Consequently, association of claudin-10s with MRC-AC cells as well as upregulation of claudin-10s expression in SW supports the hypothesis that claudin-10s create 'leaky' junctions facilitating Na+ excretion in SW.

In the case of Claudin-10s, weak staining in gill cells other than MRCs was also observed. Yet, their function has to be determined. It is important that one claudin isoform does not determine overall epithelial permeability. Several studies demonstrated that some claudins have to heterooligomerize and interact with distinct claudin isoforms to create functional pores or barriers (Koval, 2013a; Koval, 2013b). Thus, the presence of Claudin-10s aside from MRC could be potentially related to their distinct functions arising from different interaction partners.

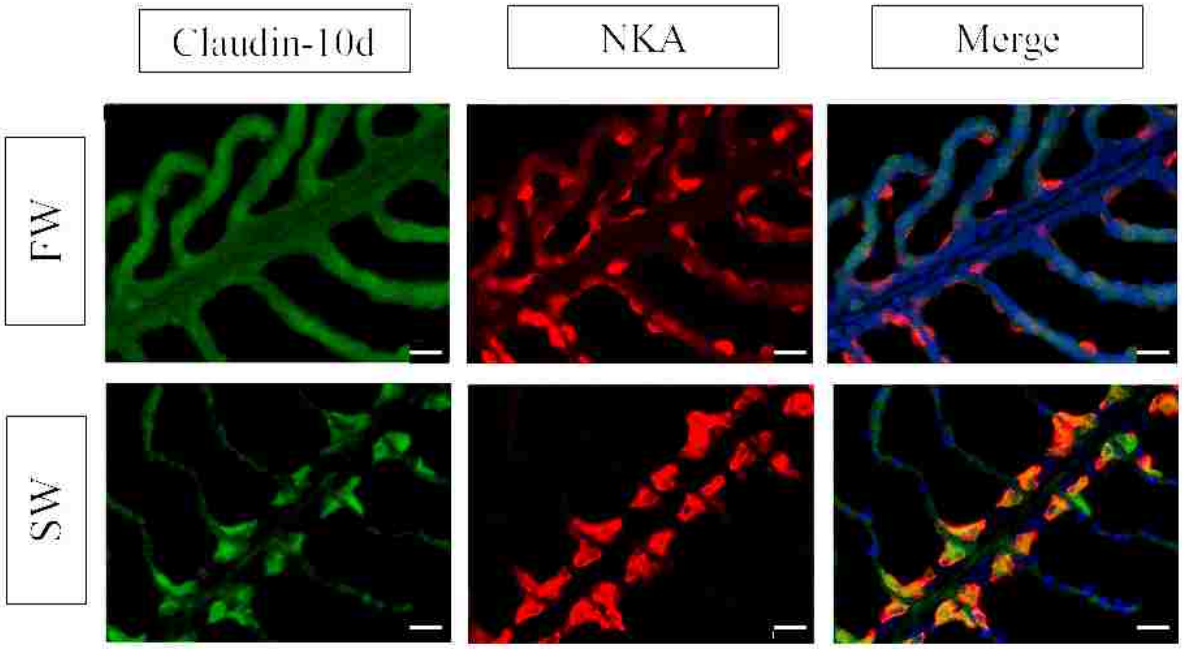
My data focus on four claudin isoforms that are encoded by four distinct genes (Appendix 2). However, it is known that some claudin isoforms exist as spliced variants. Van Itallie, et al. (2006) initially found that Claudin-10 in human and mouse have two spliced variants, each with

completely different characteristics. Nonetheless, further studies revealed that Claudin-10 possesses not only two but six alternatively spliced variants (Guenzel et al., 2009). Interestingly, some of the claudin-10 splice variants were shown to localize not only in TJ but also in the cytoplasmic regions of the cell. Similarly, claudin-10s associated with MRC also exhibit staining that corresponds to cytosolic regions of the cells.

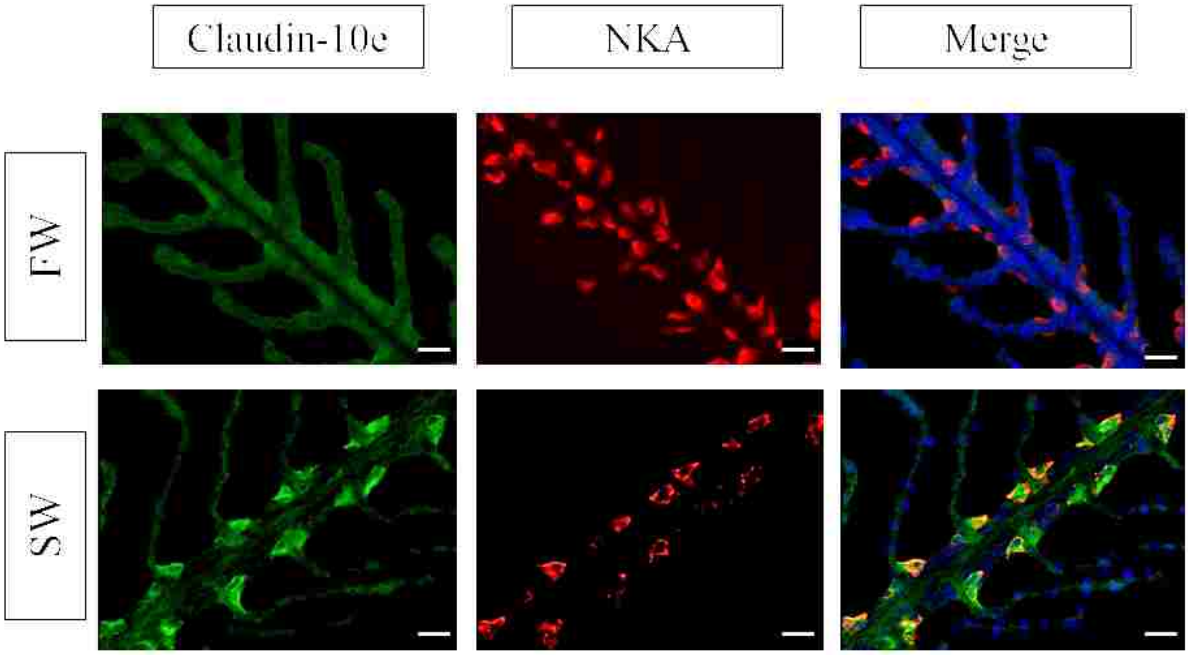
a)



b)




c)



d)

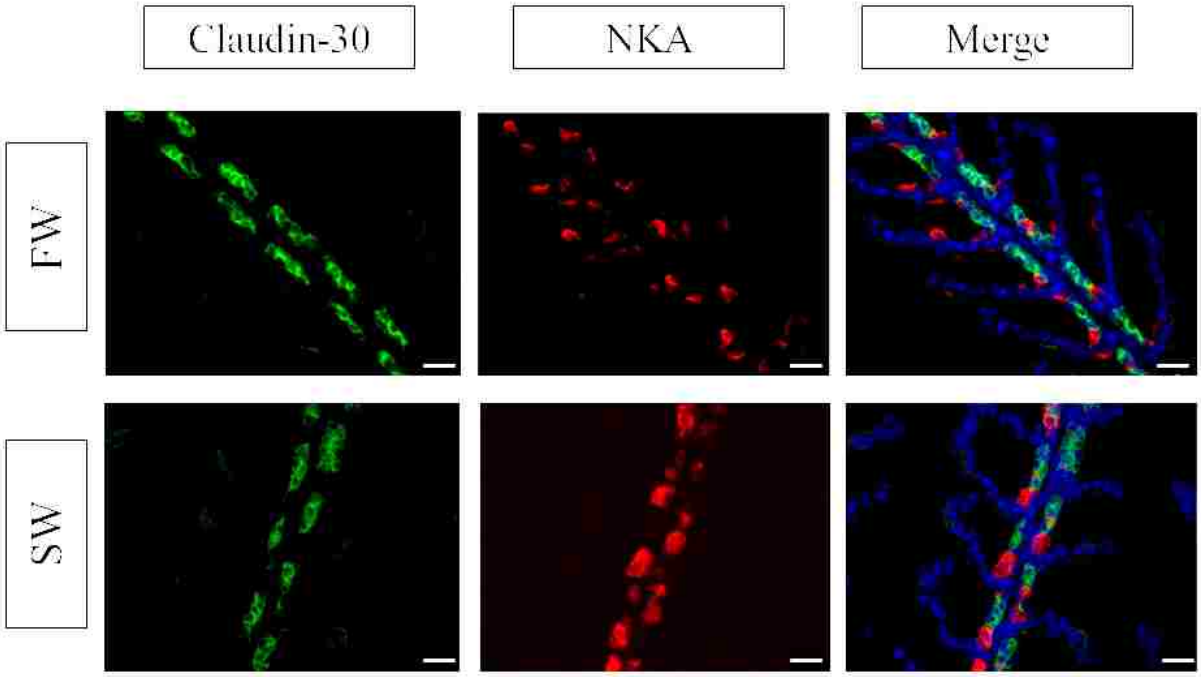



Figure 13. Immunofluorescence images of claudin-30 and claudin-10c, 10-d, -10e in the FW and SW rainbow trout gill. Gills cross sections were probed with Claudin-10c, -10d, -10e or Claudin-30 antibodies (green). α5 antibodies (red) were used in order to indicate position of mitochondrion-rich cells. Cells nuclei were stained with DAPI (blue). Scale bars: 20 μm

Differential centrifugation test of claudin-10e, showed that this isoform was present in the gill lysate but the predicted claudin-10e band was absent in the cell membrane enriched fraction. These results are surprising, especially since claudin-10e, as a part of TJ, should be more abundant in the membranous fraction. Western blot results of the centrifugation test revealed also that the pellet (enriched in plasma membrane) exhibited stronger staining of claudin-10e-reactive band that molecular weight was estimated to be double the predicted size of claudin-10e. It is too early to draw too far-forwarded conclusions, but one of the explanations might be that functional claudin-10e, recruited into TJ, is strongly associated with other proteins. However, in order to

evaluate whether it is a band of unknown origin, or an actual dimer or heterodimer of claudin-10e, more data need to be generated.

Immunofluorescence analysis showed no differences in expression and cellular localization of Claudin-30 between FW and SW gills (Fig.13d). This is in line with mRNA and western blot data. Claudin-30 appeared to be associated mainly with tight junctions between intermediate or basal cells located deeper in the filament epithelium. According to Wilson & Laurent (2002), these undifferentiated cells share some common features with PVC and are thus believed to be PVC progenitors. Claudin-30 did not colocalize with α5, which means that there is no association of Claudin-30 with MRC. This is in accordance with studies on *Salmo salar by* Engelund et al. (2012). Similar findings were also demonstrated in studies on primary cultured gill epithelium showing that transcript expression of orthologous *claudin-30c* from *Danio rerio* was significantly higher in the PVC fraction compared to MRC (Chasiotis et al., 2012). Presence of Claudin-30 between PVC or PVC progenitors on the gill filament is in line with the assumption that this isoform is responsible for epithelium tightening. A study on cultured gill epithelia, consisting only of PVC, demonstrated involvement of claudin-30 and its ortholog claudin-b in gill tightening in trout and goldfish respectively (Chasiotis and Kelly, 2011). Also, a study on zebrafish demonstrated that orthologous claudin-30c is involved in epithelium tightening and prevents excessive loss of Na+ in FW and ion-poor water (Kwong et al., 2013; Kwong and Perry, 2013).

The occurrence of intercellular strands between PVC cells, responsible for sealing epithelium and preventing ion loss, is documented in the literature (Evans et al., 2005). In the light of current research, Claudin-30 seems to be an important part of these strands. However, the possibility of heterophilic interaction of claudin-30 with other gill-specific claudins in barrier

formation since tight junction strands may be formed by several different claudin isoforms, has to be taken into account (Furuse et al., 1999). Therefore, future studies should also take into consideration the presence of other gill-specific claudins and their contribution in sealing gill epithelium. No difference in Claudin-30 expression between FW and SW could be explained by involvement of this particular isoform in gill tightening in SW, predominantly in the PVCs and intermediate cells but not in the MRCs. The possibility of a similar scenario has also been proposed by Bagherie-Lachidan (2008) in a study on *Tetraodon nigroviridis*, where expression of *claudin-3*, associated with barrier function, was elevated in the skin after SW acclimation. Additionally, freeze-fracture replica studies on lamprey gills demonstrated that SW acclimation caused a decrease in the number of *zonula occludentes* strands between MRCs but not between MRC and PVC (Bartels and Potter, 1991). Similar findings were presented in the study on gill epithelium of SW acclimated teleost fish where TJ between associated MRCs and ACs were shallow while junctions between PVCs remained deep (Sardet et al., 1979). Payan and Girard (1984) also pointed out that PVCs do not rearrange TJ under influence of SW. Thus, despite the fact that gill epithelium in SW is described as 'leaky', due to the existence of a paracellular sodium diffusion pathway between MRC-AC cells, tight junctions between other types of cells remain tight. Also, gill epithelial cell lines derived from SW acclimated fish actually exhibit higher TER values than FW-derived epithelium (Bagherie-Lachidan et al., 2009).

6. Osmoregulation capacity in relation to size, salinity and expression of α5

An understanding of the regulation of claudin expression is still in its infancy. Aside from salinity, claudin expression was shown to be influenced by the action of osmoregulatory hormones such as prolactin, growth hormone or cortisol (McCormick, 2001). Little is known

about other endogenous or exogenous factors that may regulate claudins. Nevertheless, some of my results, indicate that expression of claudins and their function in osmoregulation may be dependent on fish size, salt concentration, or even on expression of catalytic subunits of NKA. Fish transferred from FW to 20 ppt SW and acclimated for one week did not exhibit significant changes in transcript abundance of claudin-10e, which is not consistent with the previous data (Fig.14c). Claudin-30 expression was also not changed (Fig.14d).

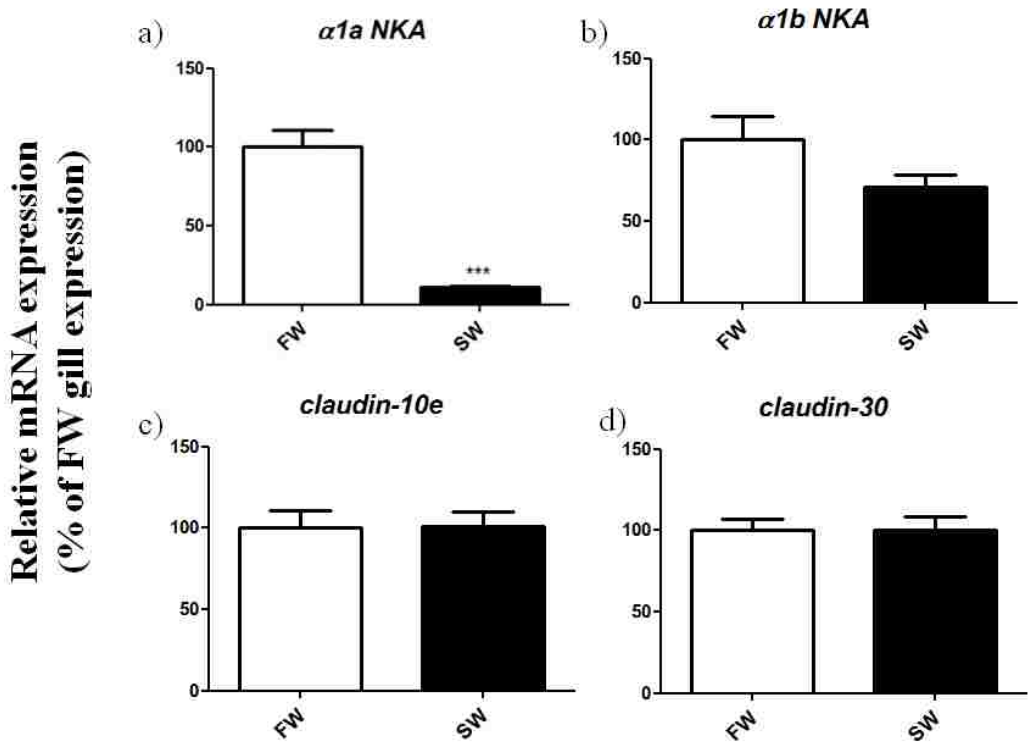



Figure 14. Relative transcript abundance in the gills of rainbow trout acclimated to 20 ppt SW for a one week. Only expression of *α1a*, a freshwater isoform of NKA, was changed after SW acclimation. Surprisingly, *claudin-10e* and *α1b* were not elevated by SW. Data represent mean values ± SEM (N=10). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001).

Interestingly, also α1b, an isoform of α-subunit of NKA responsible for SW acclimation, was not elevated by SW transfer (Fig.14b). However, α1a, a typical FW isoform, significantly decreased after SW transfer, which is what I expected (Fig.14a).

Western blot analysis and band quantification also reveal that in the gills of small fish (around 4 grams) acclimated for one week to 20 ppt, no differences between FW and SW expression of claudin-10e were observed (Fig.15). One of the explanations for this situation may be salinity level. Based on a study by Flores and Shrimpton (2012), 24‰ is an optimal salinity level for osmoregulation studies on rainbow trout. They suggested that higher salinity may result in a high rate of mortality while lower level of salinity may not be sufficient to trigger significant physiological and biochemical response. Thus, salinity of 20‰ may be too low to upregulate expression of claudin-10e. On the other hand, higher salinity was problematic due to mortality; not all of the rainbow trout, which were transferred to 25 ppt SW, were able to survive. Limited seawater tolerance may be related to the fish size. It was demonstrated in several studies that ability to withstand higher changes in salinity level is associated with size of the fish. Partially, tis effect may be explained by SA/vol (surface to volume) ratio (Allen et al., 2009; Zydlewski and Wilkie, 2013). Smaller fish have bigger SA/vol ratio, meaning that they have relatively larger diffusion surface, which is problematic in terms of osmoregulation. McCormick and Naiman (1984) suggested that SW tolerance is linked with ion transport ability, which may be size-dependent. Similarly, studies by Johnston and Cheverie (1985) on rainbow trout showed that the ablity to ionoregulate is associated not only with fish size but also with activity of NKA, which plays a crucial role in ion transport.

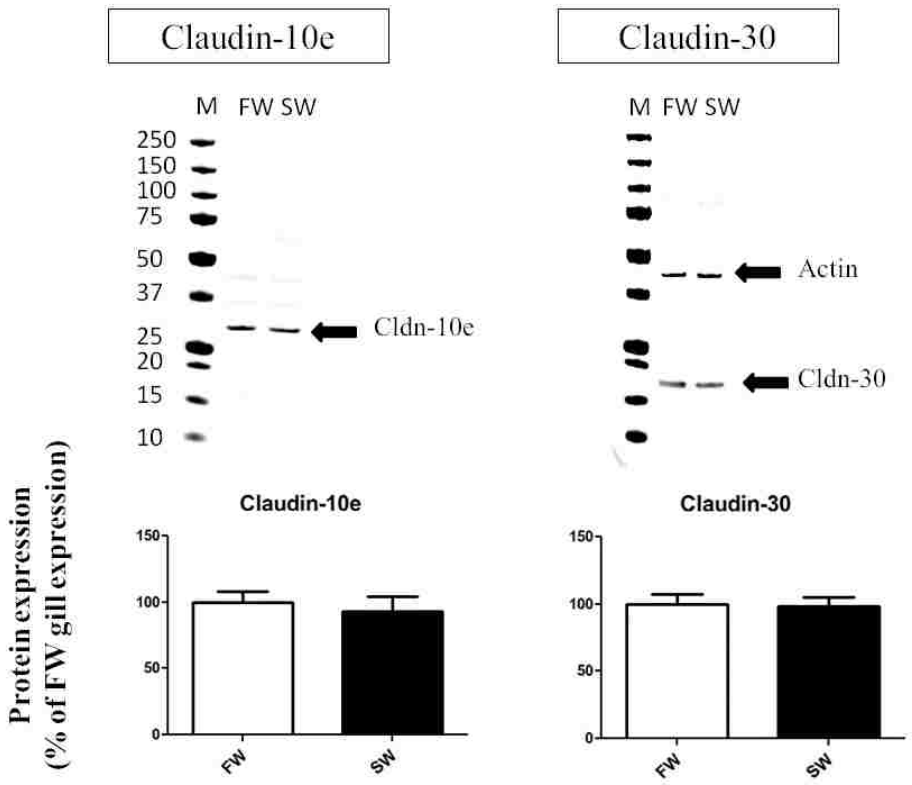



Figure 15. Claudin-10e and claudin-30 protein expression in the gills of rainbow trout acclimated to FW and 20 ppt SW for one week. Expression of Claudin-10e, supposedly SW isoforms, was not altered by SW transfer. Expression of Claudin-30 remained unchanged. Data represents mean value ±SEM (N=5).

Further studies revealed that SW tolerance may not be associated with NKA activity solely but with differential expression of NKA α-isoforms (Richards et al., 2003). Isoform α1a was demonstrated to be upregulated in the FW, whereas expression of α1b was significantly induced by SW acclimation in rainbow trout gills (Richards et al., 2003). When the fish is not able to upregulate expression of α1b after SW transfer it may not be able to acclimatize and survive. Bystriansky et al. (2007) confirmed that statement in their study on land-locked populations of Arctic char (*Salvelinus alpinus*). More importantly, Bystriansky et al. (2007) proved that α1a expression was decreased after exposure to SW but no changes were observed in regard to α1b, which may explain high mortality rates of Arctic char in response to salinity. Results of the time

course experiment are in accordance with the studies by Bystriansky et al. (2007). Expression of α1a was significantly decreased in SW while level of α1b transcript remained stable (Fig.16a-b). Moreover, no changes in mRNA levels of *claudin-10e* were detected (Fig.16c). *Claudin-30* levels were equal in both FW and SW (Fig. 16d). These results suggest that there may be a link between NKA and claudins. It is indicated that NKA is strongly associated with tight junctions proteins and is responsible for TJ assembly and proper function (Rajasekaran, 2009). Moreover, a low level of intracellular $Na^+$, maintained by NKA, is necessary for assemblage of TJ (Rajasekaran and Rajasekaran, 2009). These findings may have serious implications for fish osmoregulation. When a fish is not able to upregulate α1b in SW, not only will it be incapable of excreting excess $Na^+$ ions but it also will not have properly functioning TJs. Consequently, that will lead to impairment of the ability to osmoregulate, disruption of hydromineral balance, and finally, death.

Recent studies suggest that the ability to osmoregulate in rainbow trout has a complex genetic background (Le Bras et al., 2011; Nichols et al., 2008). So far, several QTLs coupled with salinity tolerance traits were annotated in the genome of rainbow trout, arctic char and atlantic salmon (Le Bras et al., 2011; Nichols et al., 2008; Norman et al., 2011; Norman et al., 2012). Deviation in the salinity tolerance of Arctic char (*Salvelinus alpinus*) was associated with allelic variation of ATPα1b locus (Norman et al., 2011). The same study suggests that both *ATPα1b* and *cldn-10e* loci are localized at the same very conserved linkage group, which is characteristic in salmonids but not in other teleost such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) (Norman et al., 2011). These findings may partially explain possible correlations between variations in salinity tolerance and expression of *α1b* and *cldn-10e* in rainbow trout.

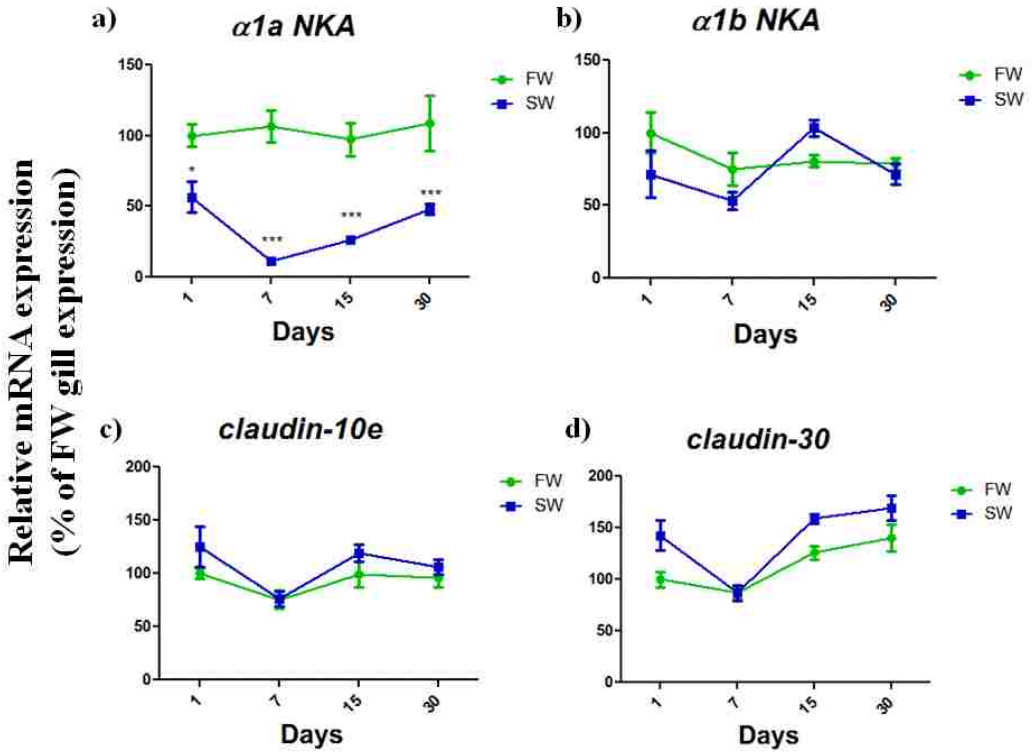



Figure 16. mRNA expression of NKA *α1a, α1b, claudin-10e* and *claudin-30* time course experiment. Only *α1a* exhibited significant downregulation after SW-transfer. No changes in expression of *claudin-10e, -30* and *α1b* were observed. Data represent mean values ± SEM (N=10). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001).

It is widely known that *Salmo salar* is able to adjust to SW challenge faster and more efficiently than *Oncorhynchus mykiss* and *Salvelinus alpinus* (Bystriansky et al., 2006b). These interspecific variations in salinity tolerance result in part from different life histories. Nevertheless, it is important to keep in mind that pronounced differences in salinity tolerance exist also between various populations of the same species. Comparative studies on *Oncorhynchus* showed that two ecotypes such as resident rainbow trout and anadromous steelhead, at least in some cases, are genetically different but the divergence arises mostly from the geography (Docker and Heath, 2003). Studies by Hecht et al. (2014) showed differences in gene expression, related to the

process of smoltification, between the resident and anadromous forms of rainbow trout. Another source of divergent phenotypes in rainbow trout might be related to domestication. Selective breeding affects gene expression and leads to apparent changes in morphology and physiology of rainbow trout (White et al., 2013). Studies on a karyotype of *Salmon salar* also disclosed that fish from hatchery might exhibit not only an abnormal phenotype but also a deviant karyotype (Brenna-Hansen et al., 2012). Variable number of chromosomes were shown to be associated with differences in physiology such as growth rate, maturation, hypoxia tolerance and performance in rainbow trout (Scott et al., 2015).

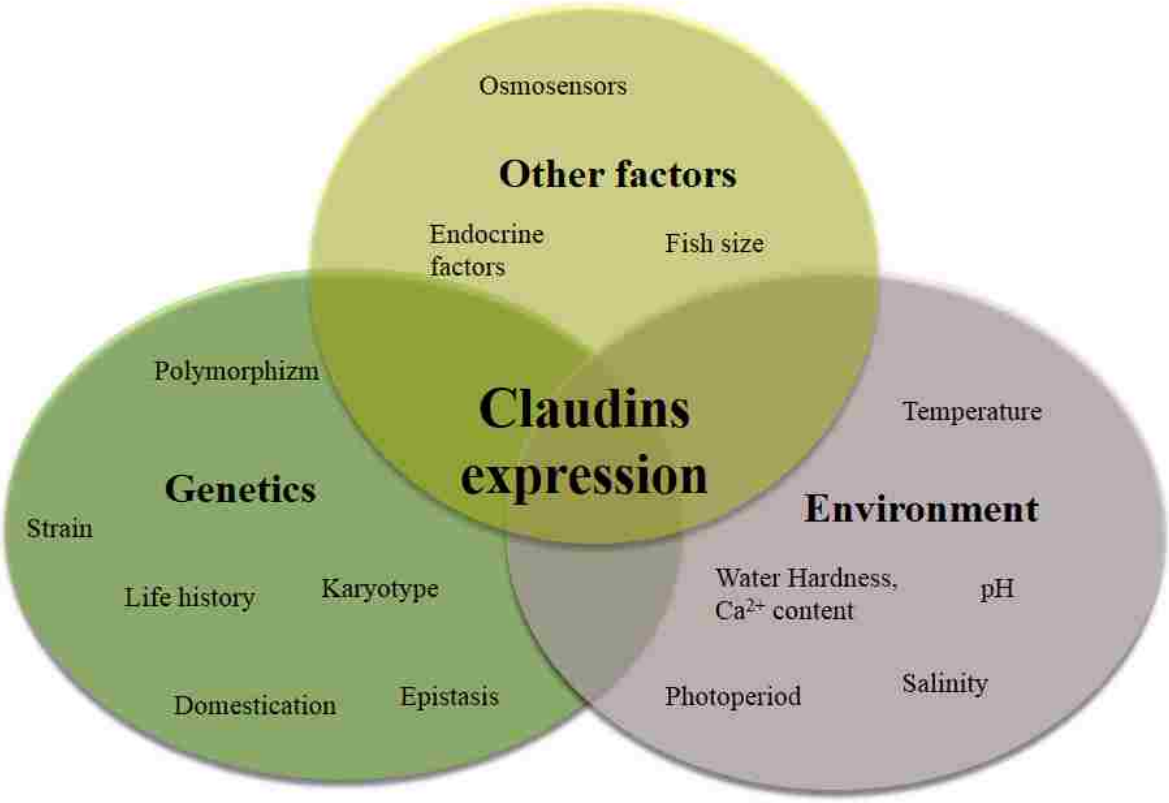



Figure 17. Influence of genetics, environment and other factors on claudin expression.

In view of these aspects, it is clear that expression of claudins and their function in osmoregulation might be under the control of multiple complex factors (Fig. 17). The current

state of knowledge is rudimentary and requires further investigation. Also, it is important to remember that physiology of biological systems, especially fish, is intricate and convoluted, thus it requires a careful approach. Nevertheless, gaps in the knowledge pave the way for further studies on claudin contribution in fish osmoregulation.

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# Chapter IV

## Conclusions and Perspectives

The present study indicates that claudin proteins contribute to the osmoregulation process of euryhaline teleosts. I demonstrated a significant upregulation of Claudin-10s by SW and their association with MRC-AC complexes. Claudin-10e, except MRC, also exhibited a weak staining of other cell types in the gill epithelium. The results strongly suggest that Claudin-10s are involved in SW acclimation probably by providing a leaky paracellular pathway between MRC and AC. One question that can be addressed in future studies is whether differential expression of claudin-10e is related to the existence of splice variants. It is known that claudin splice variants may exhibit completely different properties (Van Itallie et al., 2006). Thus, it is important to evaluate this hypothesis not only in relation to specific function and properties of the splice variant but also in relation to developmental stage of the fish and influence of environmental factors. I also showed that Claudin-30 in the gill of rainbow trout is constantly expressed regardless of salinity changes. Its cellular localization is restricted to filamentous intermediate cells of the gill epithelium. The role of claudin-30 appears to be related with tightening of the epithelium in both FW and SW. Nevertheless, the fact that claudin-30 was not highly expressed on secondary lamella compels me to think that putative function of claudin-30 could be different. Several studies demonstrated that permeability properties of epithelium depend on the suite of different claudin isoforms within tight junction (Gonzalez-Mariscal et al., 2003; Gunzel and Yu, 2013). Charge-selectivity of TJ is thus based on the combination and interaction of several claudin proteins. Therefore, it is essential to investigate further the interplay of different claudin isoforms in the gill epithelium of teleosts.

An unexplored and potentially fruitful area for further studies concerns correlation between claudin expression and activity of TRP (transient receptor potential) ion channels or CaSR (Calcium sensing receptors). TRP channels are a large family of proteins involved in sensing different types of stimuli including osmotic signals (Yue et al., 2015). Some studies demonstrated that TRPV4 is involved in maintaining barrier function of TJ in keratinocytes (Akazawa et al., 2013). Also, studies by Reiter et al. (2006) showed that epithelial permeability and claudin expression can be regulated by TRPV4. Hence, knowledge of the contribution of TRP in TJ regulation is essential for better understanding of claudin expression changes in response to salinity. Similarly, CaSR were demonstrated to play an important role in fish osmosensing (Loretz, 2008; Nearing et al., 2002). In addition, CaSR appeared to be involved in regulation of TJ and claudin protein expression in MDCK cells (Jouret et al., 2013). Further studies should address the function of TRP and CaSR in aspects of fish osmoregulation and claudin expression.

Another important question is the issue related to regulation of claudin function. My data showed that control of claudin expression is a very complex process that may be influenced by multiple factors. Salinity tolerance and indirect expression of certain claudin isoforms depend on crosstalk between genes and the environment. Further studies should address questions about the genetic basis of osmoregulation in aspects of distinct life histories, allelic variations and epistasis of genes associated with osmoregulation. Also, the impact of environmental factors alone and in combination should be taken into account. Effects of different levels of salinity, temperature, pH and even oxygen level on claudin expression in fish is undoubtedly a potential area for further studies.

Claudin-based TJs can also be regulated by post-translational modifications such as phosphorylation and palmitoylation. It is known that the intracellular C-terminus of claudin proteins contains phosphorylation sites that can take part in regulation of claudin functions but nothing is known about such regulation in fish (Heiskala et al., 2001; Van Itallie et al., 2012). Claudin isoforms investigated in this study possess several putative phosphorylation sites (see Appendix 3) and thus, in the light of recent studies it is promising to evaluate the contribution of phosphorylation in regulation of claudin functions in response to salinity or endocrine factors. Another post-translational modification that might be involved in regulation of claudin function, is palmitoylation. According to Shen et al. (2011), palmitoylation affects claudin trafficking and half-life. Yet, nothing is known about the possible role of palmitoylation in regulating claudin function in fish osmoregulation.

Our knowledge about claudin isoforms in rainbow trout is rudimentary. My study focused only on a small fraction of claudin isoforms. Therefore, future studies should address questions about other claudin isoforms and their functions. My study provided an initial but valuable insight into claudin function in aspects of rainbow trout osmoregulation. It is obvious that claudin proteins, as a key element of epithelial tight junction complexes, are essential factors that restrict the movement of water and ions. For this reason, they appear to be an important component involved in fish osmoregulation. Nevertheless, there are numerous areas in this field of science that need to be clarified and explained. Data inconsistencies emphasize the complexity of evaluating the physiology of such a dynamic structure as claudin-based tight junctions.

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# Appendices

## Appendix 1:

Table A1.1. Sequences of primers used for quantitative PCR of Rainbow trout claudins and normalization genes.

| Target name | Forward primer | Reverse primer | Accession number (GenBank) |
|---|---|---|---|
| ***claudin-10c* [Ssa]** | AGTGGATGGATCCTGGTCTG | TCCTTCCAGAGGTTGGAGAA | BK006389 |
| ***claudin-10d* [Ssa]** | ACTGGACCTGGTCTGAGGTG | TGGAGGGGTACTCTTTGCAG | BK006390 |
| ***claudin-10e* [Ssa]** | ATCAAGGTGGCCTGGTACTG | GACCAGAGCACAGGGAAGTC | BK006391 |
| ***claudin-30* [Ssa]** | TGATCATTGGAGGAGGGTTC | AACATAGTCCCTGGGTGCTG | BK006405 |
| ***Ef1α* [Omy]** | AGAACCATTGAGAAGTTCGAGAAG | GCACCCAGGCATACTTGAAAG | AF498320 |
| ***β-actin* [Omy]** | TCCTCGGTATGGAGTCTTGC | AGCACTGTGTTGGCGTACAG | AJ438158 |
| ***rplp0* [Ssa]** | GTTCCTGGAGGGTGTTCGTA | TCCGTTGATGATGGTGTGAG | BT060322 |

Omy: *Oncorhynchus mykiss*; Ssa: *Salmo salar*

Table A1.2. Antibodies used for Western blot and immunofluorescence with predicted molecular weight of target proteins

| Target | Epitope | Molecular weight of protein |
|---|---|---|
| Claudin-10c | CIRAEFQDPNFRAQK | 32kDa |
| Claudin-10d | CQGSRGGRKIRKTRT | 28 kDa |
| Claudin-10e | CGRSSRSRSHGSVDS | 32 kDa |
| Claudin-30 | CKYSAARSTAPKDYV | 20 kDa |

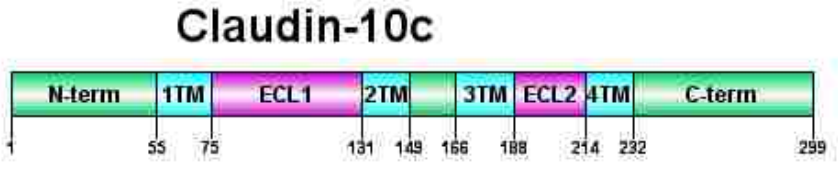
**Appendix 2:**

Protein sequences of claudin-10c, -10d, -10e and -30 and their graphical representations. Sequences were retrieved from GenBank. Prediction of transmembrane domains was done using TMpred program (Hofmann and Stoffel, 1993) on ExPasy platform and Phobius webserver (Käll, 2007). Protein domains were represented graphically using DOG 1.0: Illustrator of Protein Domain Structures (Jian Ren et al., 2009).

1. Claudin-10c

GenBank: DAA06154

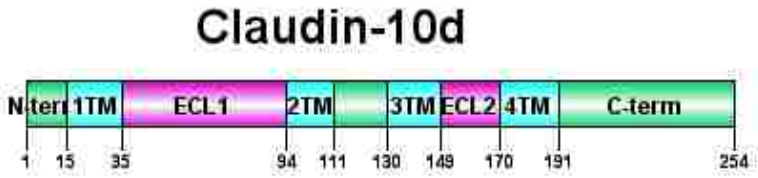
>Ssa_cldn10c
MGQQTPCIFFHQCGARAGNSSTAIFPPSCLVSLHTEAAMNYRTVVMYMEIGCFVVCVSGWIL
VCSTMPTEIWTWSEVESIVLTSSNYFSNLWKDCVSDSTGVSDCKGIPSMFGLNWDIHMCRAL
IIISIILGFFGAILVLVGMKCTKIGGSEVANARVTFAGGMNYLVSGLCSMIAFSYYGNKIRAEF
QDPNFRAQKFEIGVAVYIGWGGSTLLVIGGLIYSVFAGKEACQSSSKNKHMPVYKLPDADVA
PPAKPTYRPVSTALTEGGESRESKTSRVSSETRRSGSSSKTLSSLNAYV
**299 aa**



2. Claudin-10d

GenBank: DAA06155.1

> Ssa_cldn10d
MKHRTVMMYMEIGCFVSCLAGWILVSSTLAIEYWTWSEVGSVVLTTGNYFSNLWKDCVSD
STGVSDCKEYPSMLGLPVFLHSVRALSICSVILGFFAGVLTLIGMKCTKIGGSELANARVTFA
GGITYLASGFAGLIVYSWWGNKVRSEFVDPNFKAQKFEIGAAVFIGWGGSILLITGGFVLSFF
SGKEGLRSTSKKRPRRPNSYATARTRRTYMMPNSSRVTPMPQLVQGSRGGRKIRKTRTTGTY
SRDDFV
**254 aa**




3. Claudin-10e

GenBannk: DAA06156

>SSa_cldn10e
MKIRVMQIWGFLMTVLGWIFVACTMAMEGWKVTSIGGMGGSAVIKVAWYWSNLWKACF
TDSTSVTNCQDFPVLWSVDNHIQIVRGLLMGALSVGMLGFVLSLIGMECTFLGGKDKAKHR
KLFTGGVCHIISGFLAASGYAVYAKYVSGEYFNPYFDGLKFDLGTPLFLGWVGSAFHMTGG
WFYLVSVCKLLCGDKSKTIVIPELPEVERDQAKSTTAQYPVSPITSKIMVSSASKISSKA
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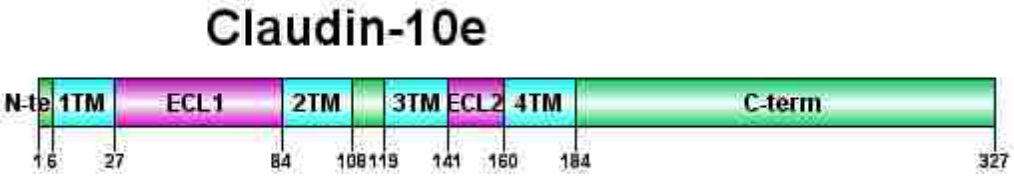
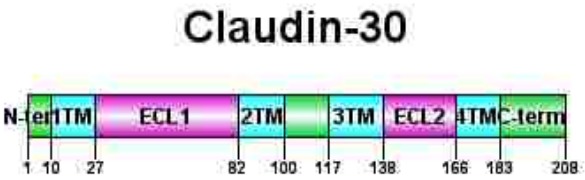
SGSSSTVSSLSSGSRRRERKPFIKNSYI
**327aa**



4. Claudin-30

GenBank: NP_001233202.1

>Omy_cldn30
MASAGFQMLGTALGIIGWIGAIVVCALPQWKVTAFIGENIITAQTTWQGIWMNCVVQSTGQ
MQCKVYDSMLALPQDLQAARALIIISIMMGLVGILLSVAGGKCTNCVEDERAKSRIGVGSGV
VFIIAGILCLIPVCWSANTIIRDFYNPMLMSSQKMELGAALYIGWGAAALMIMGGGFLCANCP
PKEDNYPTKYSAARSTAPKDYV
**208 aa**

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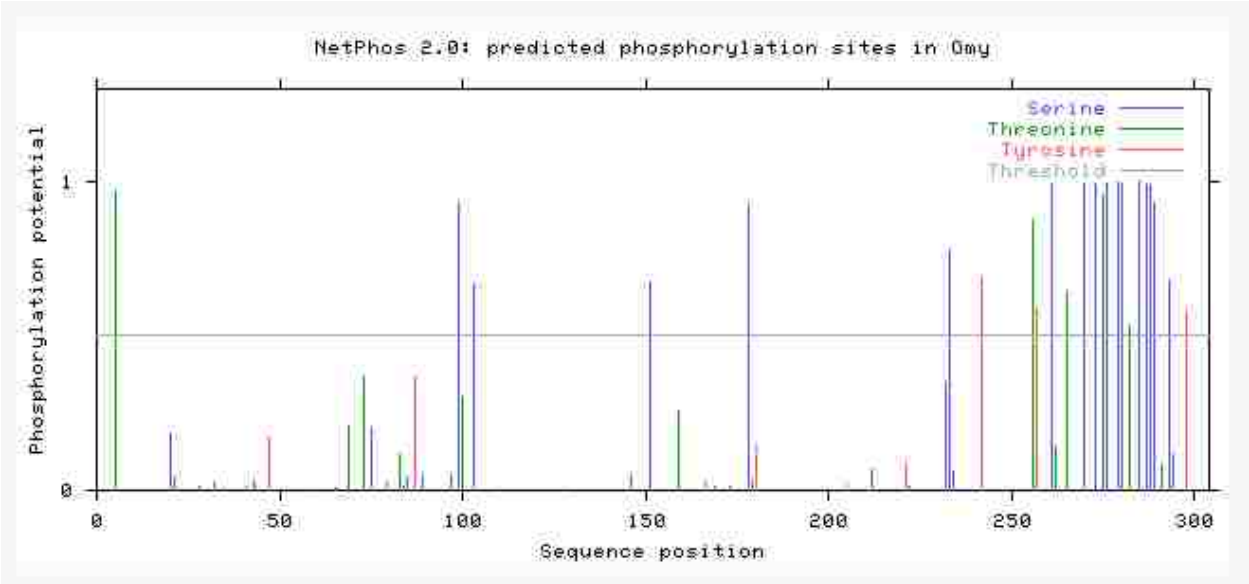
Jian Ren, Longping Wen, Xinjiao Gao, Changjiang Jin, Yu Xue and Xuebiao Yao, 2009. DOG 1.0: Illustrator of Protein Domain Structures, *Cell Research* 19:271–273.

Käll L., Krogh A., Sonnhammer E.L.L., 2007. Advantages of combined transmembrane topology and signal peptide prediction--the Phobius web server. *Nucleic Acids Res.*, 35:W429-32.

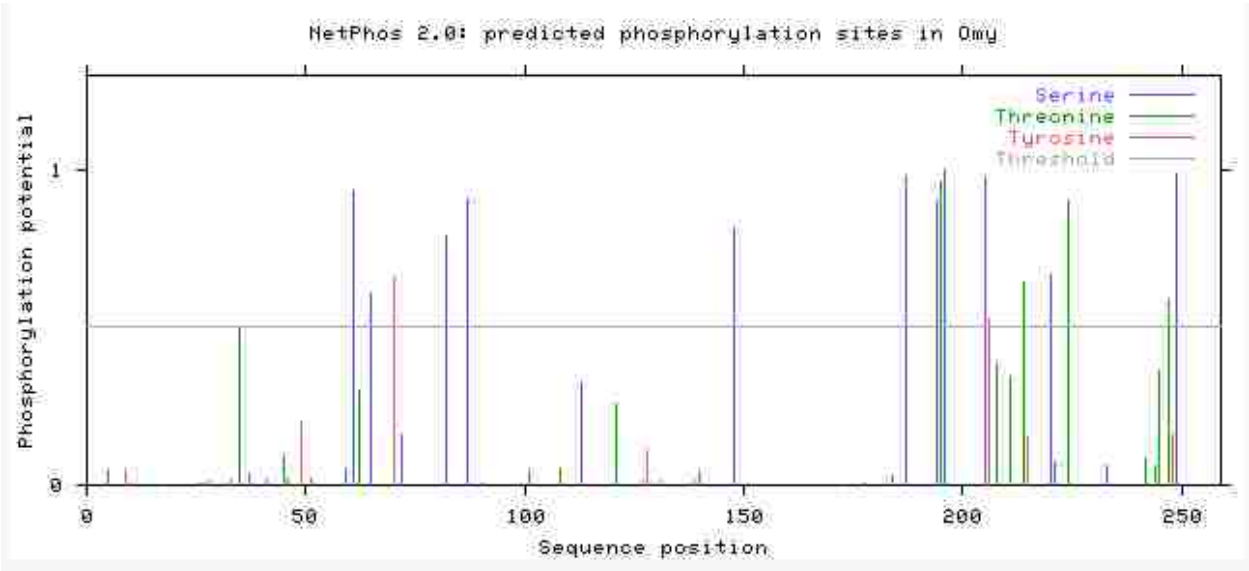
**Appendix 3:**

Prediction of phosphorylation sites of Claudin-10s and Claudin-30 was performed using NetPhos.2.0. server

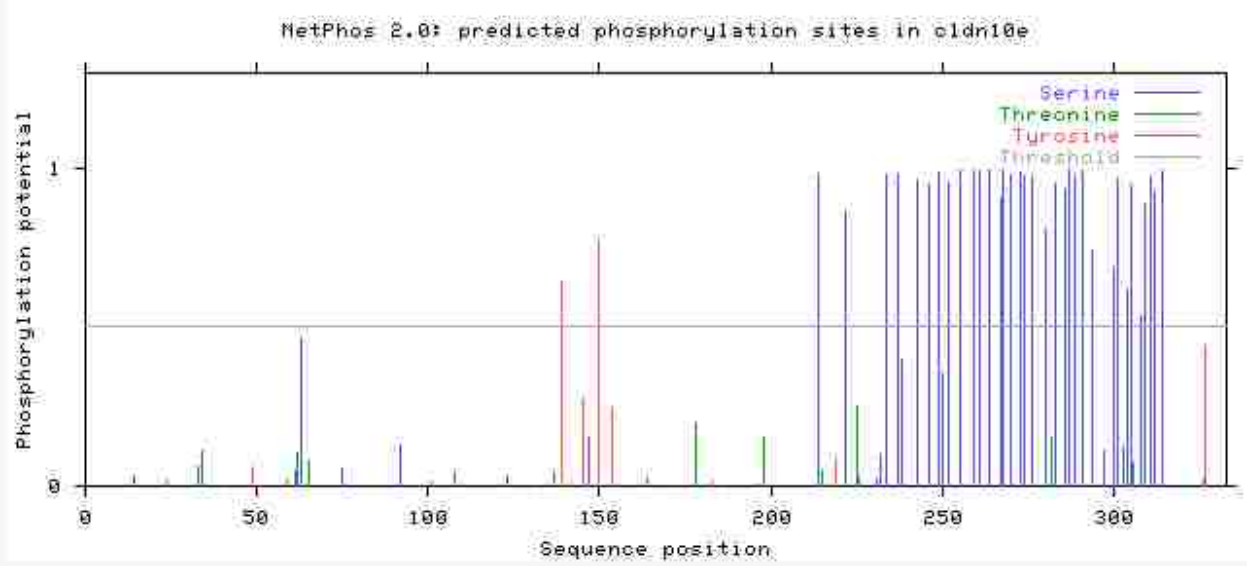
1. Claudin-10c



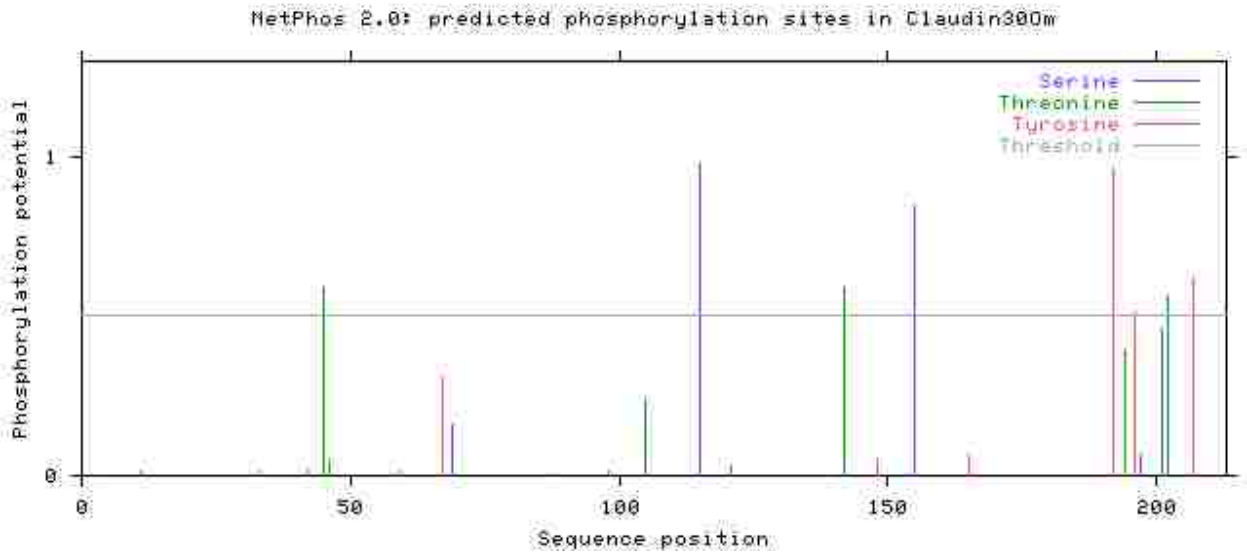

2. Claudin-10d



3. Claudin-10e




4. Claudin-30



**Appendix 4:**

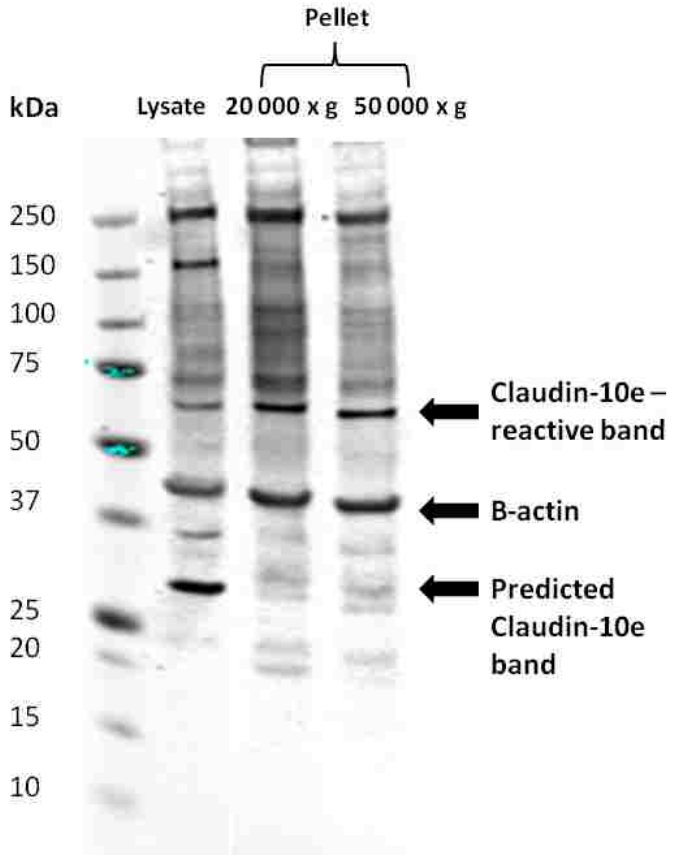



Figure A4.1 Differential centrifugation test for Claudin-10e. Lysate and pellet, obtained by lysate centrifugation at 20000 x g for 90 minutes and 50000 x g for 60 minutes, were used for detection and comparison of enrichment in Claudin-10e on western blot.

**Appendix 5:**

IACUC approval for research.

UNIVERSITY OF ARKANSAS

Office of Research Compliance

*MEMORANDUM*

TO: Christian Tipsmark

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: May 9, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **May 8, 2016**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13052 - "Functional Regulation of Claudins in the Fish Gill Model"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **05-08-2016** you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

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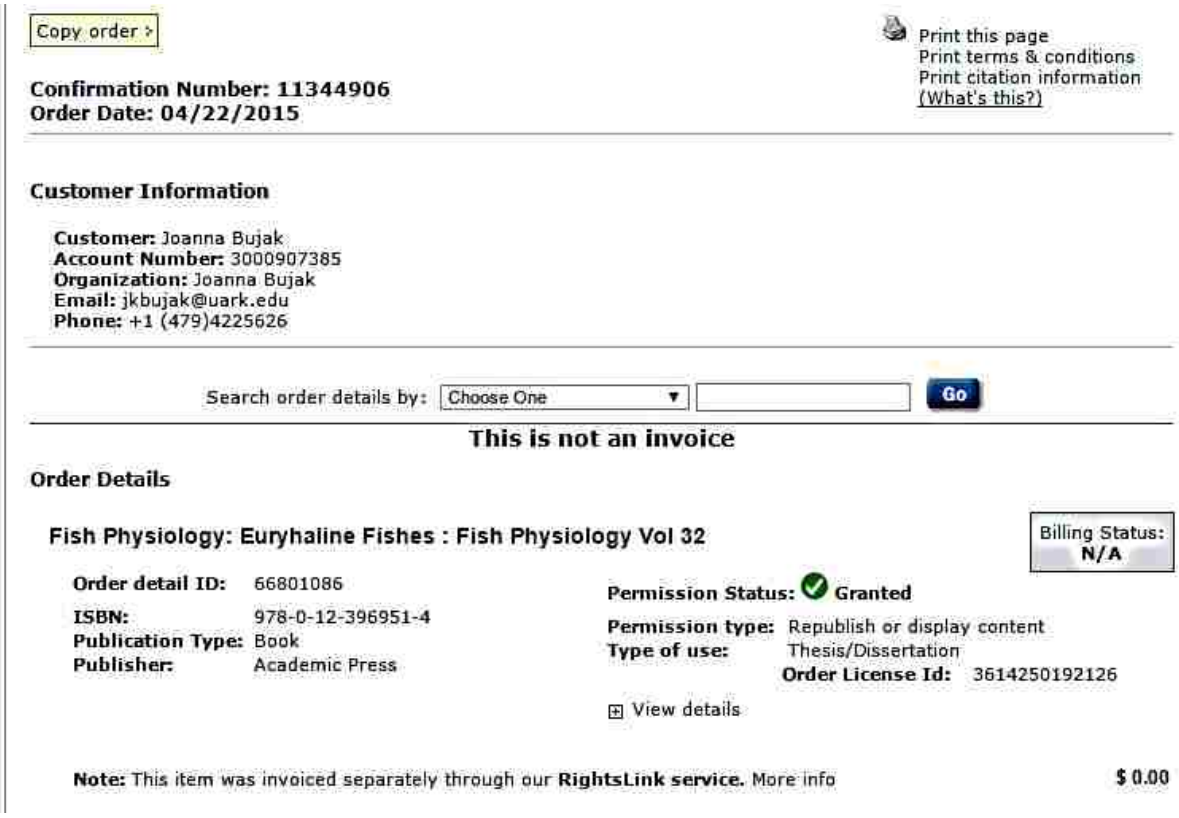

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