

5-2015

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

Joanna Katarzyna Bujak
University of Arkansas, Fayetteville

Follow this and additional works at: <http://scholarworks.uark.edu/etd>

 Part of the [Cellular and Molecular Physiology Commons](#), and the [Molecular Biology Commons](#)

Recommended Citation

Bujak, Joanna Katarzyna, "Functional Significance of Gill Claudin Proteins in Rainbow Trout (*Oncorhynchus mykiss*) Osmoregulation" (2015). *Theses and Dissertations*. 1135.
<http://scholarworks.uark.edu/etd/1135>

This Thesis is brought to you for free and open access by ScholarWorks@UARK. It has been accepted for inclusion in Theses and Dissertations by an authorized administrator of ScholarWorks@UARK. For more information, please contact scholar@uark.edu, ccmiddle@uark.edu.

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

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

A thesis submitted in partial fulfillment
of the requirements for degree of
Master of Science in Biology

by

Joanna Katarzyna Bujak
University of Opole
Master of Sciences in Biology, 2010

May 2015
University of Arkansas

This thesis is approved for Recommendation to the Graduate Council

Dr. Michelle Evans-White
Thesis Director

Dr. Steven Beaupre
Committee Member

Dr. Charles Rosenkrans
Committee Member

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.

©2015 by Joanna Katarzyna Bujak
All Rights Reserved

Acknowledgements

I would like to acknowledge all people who help me to succeed. I am grateful to my committee members, Dr. Charles Rosenkrans, Dr. Steven Beaupre and Dr. Michelle Evans-White for their comments and constructive criticism that helped me to improve my thesis. I wish to express my thanks to my lab mates, especially Rebecca Bollinger, for cooperation and help in conducting experiments. I place on record my sincere thanks to all my friends for being with me and supporting me in the worst days of my life. I am also grateful to all “people of good will” for good advises and encouragement. I am extremely thankful to small but extremely warm and friendly Polish community of Northwest Arkansas for their hospitality and care – you were my family here in Fayetteville.

I take this opportunity to express my gratitude to Dr. Marites Sales, for her valuable suggestions, corrections of my thesis and the most importantly for her help, guidance and big heart. I also thank my family for encouragement, support and all the love they give me. I am also grateful to Piotr Gorecki, for being with me “for good and for bad” and for his patience and unconditional love – I could not have done this without you.

*I dedicate this thesis to my grandparents
who inspired me to study biology.*

Table of Contents

Table of Contents	1
List of Figures.....	
List of Tables	
Introduction.....	1
Literature cited.....	4
Literature review	6
A. Biology of rainbow trout (<i>Oncorhynchus mykiss</i>)	6
1. Phylogeny of rainbow trout.....	6
2. Ecology and life cycle	7
3. Rainbow trout as a model organism.....	11
B. Teleost fish osmoregulation.....	12
1. Principles of osmoregulation.....	12
2. Gills as an osmoregulatory organ.....	14
C. Claudins as a key element of tight junctions	17
1. Tight junctions.....	17
2. Claudin proteins	17
3. Importance of claudin proteins in physiology.....	19
D. Implications of claudins in fish osmoregulation.....	21
1. Claudin genes in teleost fish	21
2. Osmoregulatory function of claudins in teleost	22
3. Claudin alterations in response to osmoregulatory hormones	23
E. Research Objectives.....	26
Literature Cited	27
Functional significance of gill claudin proteins in rainbow trout (<i>Oncorhynchus mykiss</i>) osmoregulation	38
A. Materials and methods	38
1. Animals and experimental protocols.....	38
2. Study design	39
3. Analysis.....	39
B. Results and discussion	45
1. Antibody validation.....	45
2. <i>In silico</i> analysis of ECL-1 of trout and mouse claudins	47
3. Tissue distribution of claudin-10s and claudin-30 transcript	49
4. Claudin-10s, Claudin-30 and $\alpha 5$ expression in FW and SW	51
5. Claudin-10s and -30 cellular localization in the gill of rainbow trout.	55
6. Osmoregulation capacity in relation to size, salinity and expression of $\alpha 5$	61
Literature cited.....	69
Conclusions and Perspectives	76
Literature cited.....	79
Appendices.....	81

List of Figures

- Figure 1. Cladogram representing phylogenetic relationships among genera of Salmonidae family. Based on the data by A. Crete-Lafreniere, et al. (2012). 7
- Figure 2. Life cycle of rainbow trout (*Oncorhynchus mykiss*) (Cooke et al., 2011). Reproduced with permission of Elsevier, 2015 (Appendix 6). 9
- 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). 10
- 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). 13
- 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). 14
- 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). 15
- 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). 18
- 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. 46
- 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. 48

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, *Efla* and *rplp0*. Data are represented as mean value of pooled, FW and SW acclimated fish \pm SEM (n=14). Significant differences were indicated by different letters (P<0.05)..... 50

Figure 11. *Claudin-10c, -10d, -10e, claudin-30, NKA α 1a* and *α 1b* mRNA expression in FW and SW acclimated gills of rainbow trout. α 1a and α 1b 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, *Efla* and *rplp0*. Data represent mean value \pm SEM (N= 7). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001). 52

Figure 12. Protein expression of claudin-10c, -10d, -10e, claudin-30 and α 5 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. α 5 antibodies were used as a reference. Data represent mean values \pm SEM (N=5). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001)..... 54

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). α 5 antibodies (red) were used in order to indicate position of mitochondrion-rich cells. Cells nuclei were stained with DAPI (blue). Scale bars: 20 μ m 59

Figure 14. Relative transcript abundance in the gills of rainbow trout acclimated to 20 ppt SW for a one week. Only expression of *α 1a*, a freshwater isoform of NKA, was changed after SW acclimation. Surprisingly, *claudin-10e* and *α 1b* were not elevated by SW. Data represent mean values \pm SEM (N=10). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001)..... 62

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 \pm SEM (N=5)..... 64

Figure 16. mRNA expression of NKA *α 1a*, *α 1b*, *claudin-10e* and *claudin-30* time course experiment. Only *α 1a* exhibited significant downregulation after SW-transfer. No changes in expression of *claudin-10e*, -30 and *α 1b* were observed. Data represent mean values \pm SEM (N=10). Significant differences between FW and SW were indicated with stars (* = P<0.05, **=P<0.01, ***= P<0.001)..... 66

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. 87

List of Tables

Table A1.1. Sequences of primers used for quantitative PCR of rainbow trout claudins and normalization genes.	81
Table A1.2. Antibodies used for Western blot and immunofluorescence with predicted molecular weight of target proteins	81

List of Published Papers

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.

Trubitt RT, Rabeneck DB, **Bujak, JK**, Bossus MC, Madsen SS, Tipsmark CK, 2014, Transepithelial resistance and claudin expression in trout Rt gill-W1 cell line: effects of osmoregulatory hormones, *Comparative Biochemistry and Physiology, Part A, Molecular and Integrative Physiology*, v. 182, p. 45-52.

Conference publications:

Bujak JK, Madsen SS, Bomane-Bossus MC, Tipsmark CK, Abundance and localization of branchial claudins in rainbow trout (*Oncorhynchus mykiss*) and implications in hypoosmoregulation, APS Intersociety Meeting: Comparative Approaches to Grand Challenges in Physiology, San Diego, USA, 2014.

Bujak JK, Bollinger RJ, Madsen SS, Tipsmark CK, Effects of salinity and hormone treatment on gill claudins expression and localization in rainbow trout (*Oncorhynchus mykiss*), Meeting of the Society for Integrative and Comparative Biology, Austin, USA 2014

Trubitt RT, **Bujak JK**, Madsen SS, Rabeneck DB, Tipsmark CK, Osmoregulatory hormones modulate tight junction protein expression and transepithelial resistance in an epithelial gill cell line from rainbow trout, II Meeting of the North American Society for Comparative Endocrinology, Queretaro, Mexico, 2013.

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 losing 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.

Literature cited

Angelow, S., and A. S. L. Yu, 2007, Claudins and paracellular transport: an update: *Current Opinion in Nephrology and Hypertension*, v. 16, p. 459-464.

Chasiotis, H., D. Kolosov, P. Bui, and S. P. Kelly, 2012, Tight junctions, tight junction proteins and paracellular permeability across the gill epithelium of fishes: A review: *Respiratory Physiology & Neurobiology*, v. 184, p. 269-281.

Evans, D. H., 2008, Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys(v 295, pg R704, 2008): *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 295, p. R1359-R1359.

Evans, D. H., P. M. Piermarini, and K. P. Choe, 2005, The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste: *Physiological Reviews*, v. 85, p. 97-177.

Furuse, M., K. Fujita, T. Hiragi, K. Fujimoto, and S. Tsukita, 1998, Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin: *Journal of Cell Biology*, v. 141, p. 1539-1550.

Karnaky, K. J., Jr., 1998, *Osmotic and ionic regulation: The physiology of fishes*. Second edition., p. 157-176.

Loh, Y. H., A. Christoffels, S. Brenner, W. Hunziker, and B. Venkatesh, 2004, Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*: *Genome Research*, v. 14, p. 1248-1257.

Madsen, S. S., and C. K. Tipsmark, 2008, Changes in claudin isoform expression in the gill during salinity shifts and smoltification of Atlantic salmon: *Faseb Journal*, v. 22, p. 1200.2.

Marshall, W. S., and M. Grosell, 2006, *Ion transport, osmoregulation, and acid-base balance: The physiology of fishes*. Third edition. [Marine Biology Series.]. p. 177-230.

Siddiqui, M., H. Sheikh, C. Tran, and A. E. E. Bruce, 2010, The Tight Junction Component Claudin E is Required for Zebrafish Epiboly: *Developmental Dynamics*, v. 239, p. 715-722.

Tipsmark, C. K., D. A. Baltzegar, O. Ozden, B. J. Grubb, and R. J. Borski, 2008, Salinity regulates claudin mRNA and protein expression in the teleost gill: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 294, p. R1004-R1014.

Tipsmark, C. K., K. J. Sorensen, K. Hulgard, and S. S. Madsen, 2010, Claudin-15 and-25b expression in the intestinal tract of Atlantic salmon in response to seawater acclimation, smoltification and hormone treatment: *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, v. 155, p. 361-370.

Tsukita, S., M. Furuse, and M. Itoh, 2001, Multifunctional strands in tight junctions: *Nature Reviews Molecular Cell Biology*, v. 2, p. 285-293.

Tsukita, S., Y. Yamazaki, T. Katsuno, and A. Tamura, 2008, Tight junction-based epithelial microenvironment and cell proliferation: *Oncogene*, v. 27, p. 6930-6938.

Van Itallie, C. M., and J. M. Anderson, 2004, The role of claudins in determining paracellular charge selectivity: *Proceedings of the American Thoracic Society*, v. 1, p. 38-41.

Van Itallie, C. M., and J. M. Anderson, 2006, Claudins and epithelial paracellular transport: *Annual Review of Physiology*, v. 68, p. 403-429.

Xie, J., E. Farage, M. Sugimoto, and B. Anand-Apte, 2010, A novel transgenic zebrafish model for blood-brain and blood-retinal barrier development: *Bmc Developmental Biology*, v. 10, p.76.

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).

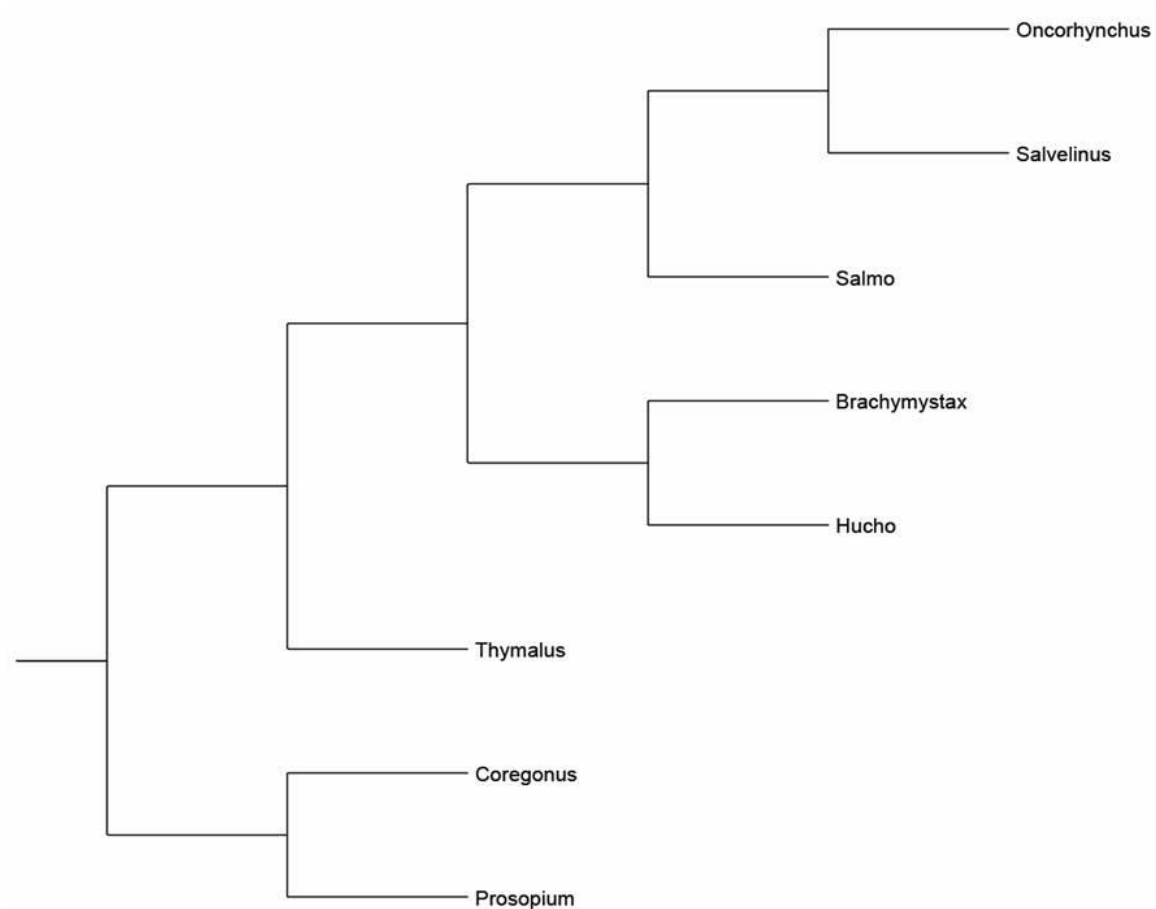


Figure 1. Cladogram representing phylogenetic relationships among genera of Salmonidae family. Based on the data by A. Crete-Lafreniere, et al. (2012).

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.

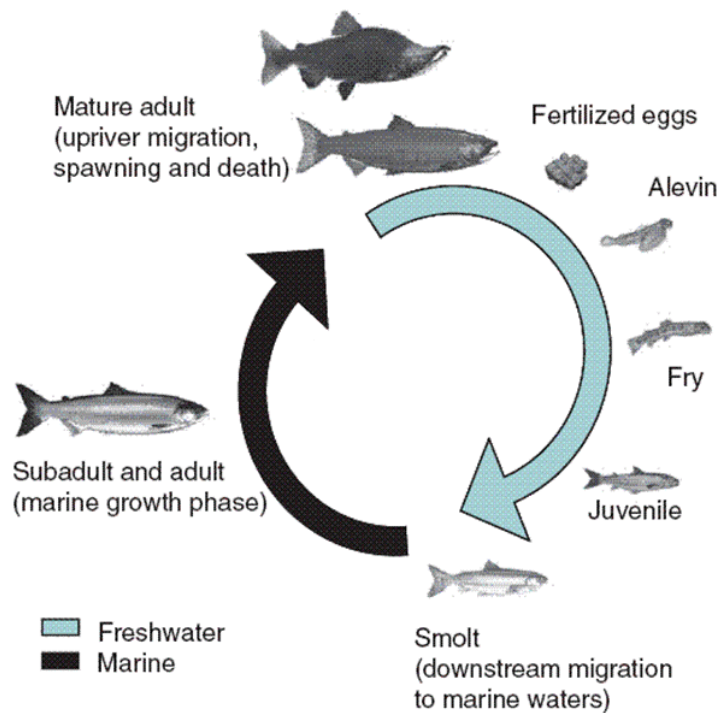


Figure 2. Life cycle of rainbow trout (*Oncorhynchus mykiss*) (Cooke et al., 2011). Reproduced with permission of Elsevier, 2015 (Appendix 6).

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).

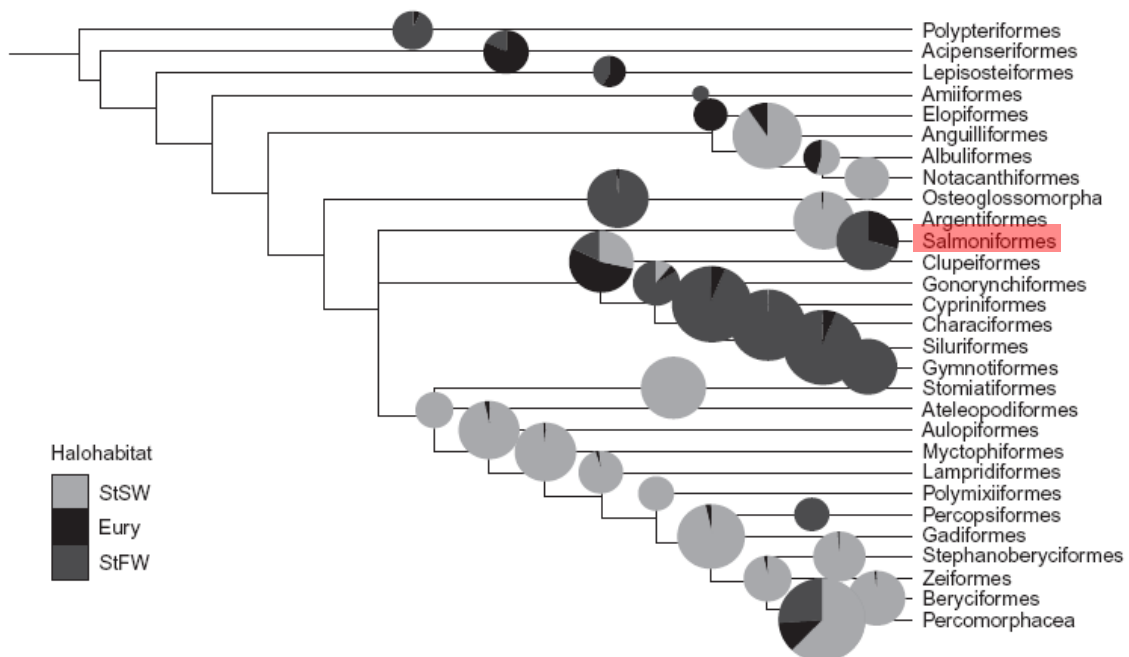


Figure 3. Halohabitat of Salmoniformes. Salmoniformes are represented by species inhabiting both exclusively freshwater environment and by anadromous euryhaline species (Schultz and McCormick, 2013). Reproduced with permission of Academic Press, 2015 (Appendix 6).

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’.

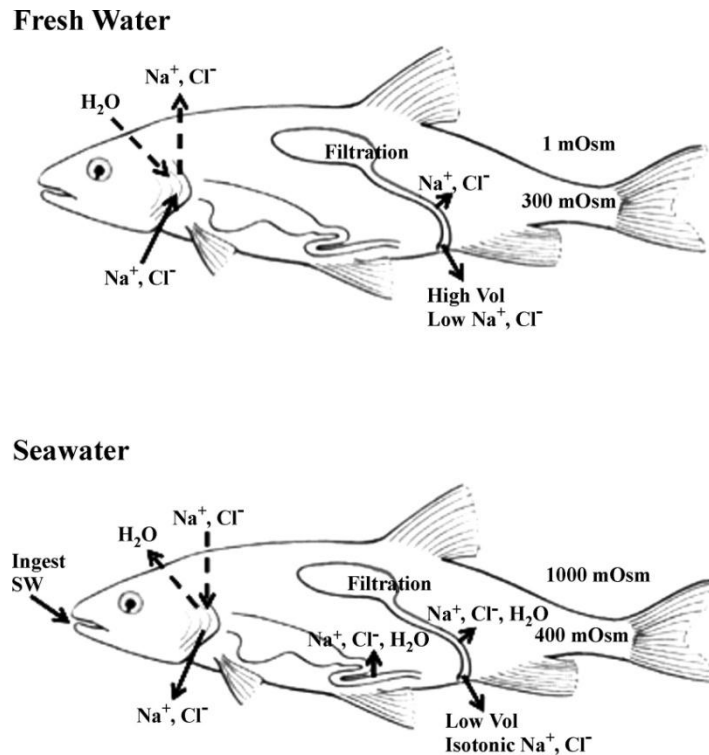


Figure 4. Osmoregulation of freshwater and seawater fish. FW species are hypertonic to external environment while SW species are hypotonic. Thus they are threatened by excessive dilution of body fluids or dehydration, respectively. That difference gave rise to distinct physiological mechanisms that maintain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

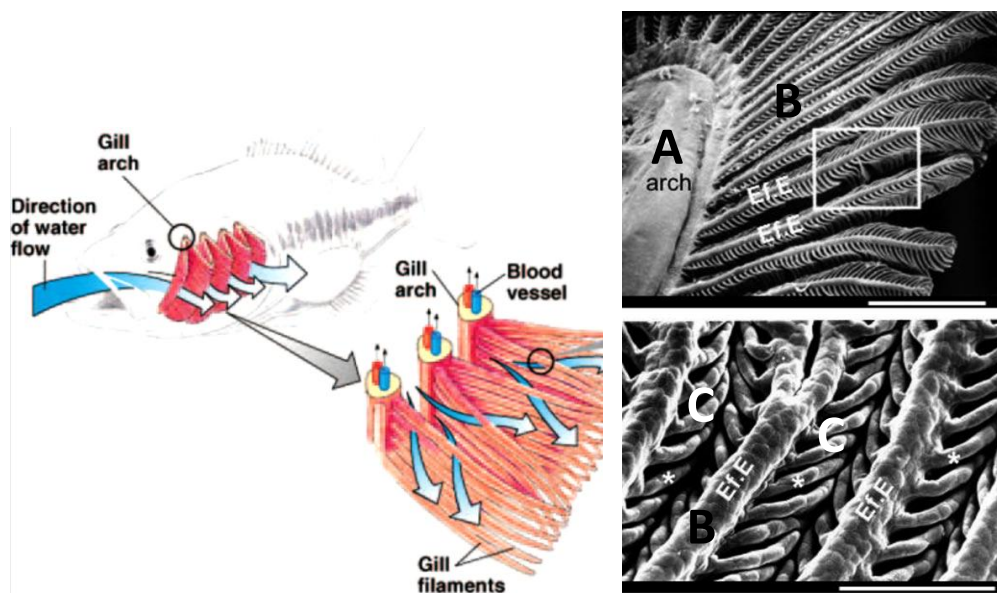


Figure 5. Fish gill anatomy. Gills are multifunctional organs that are in direct contact with the external environment. Gills consist of several gill arches (A), supporting filaments (B). Each filament possess numerous secondary lamella (C) covered by thin epithelium (Evans et al., 2005).

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).

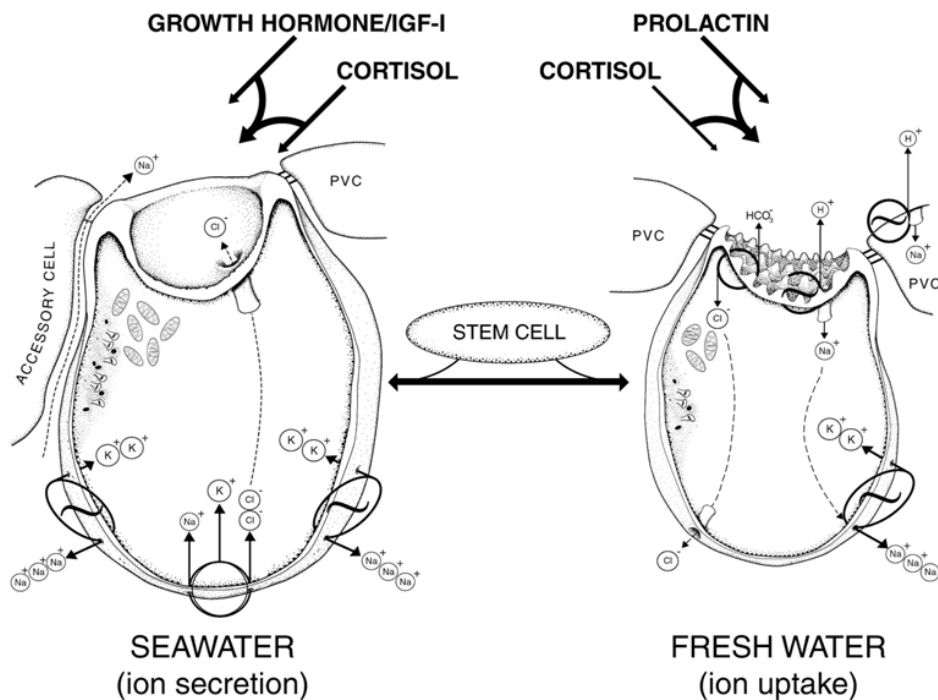


Figure 6. Mitochondrion-rich cells (ionocytes) in the gill epithelium of freshwater and seawater acclimated fish. Ionocytes are the principle cell type responsible for active ionoregulation in fish (McCormick, 2001). Reproduced with permission of Oxford University Press, 2015 (Appendix 6).

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 conductance

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).

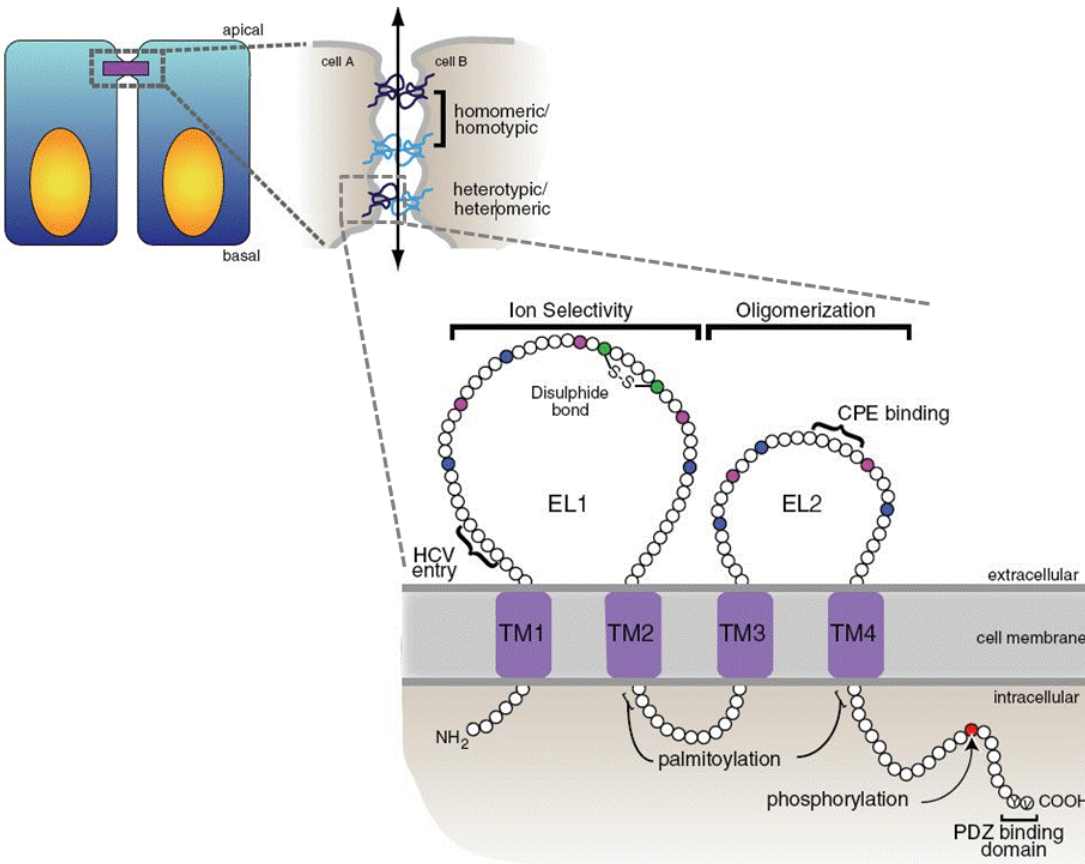


Figure 7. Claudins as an integral part of tight junction complexes of the epithelial cells. They have four transmembrane domains and two extracellular loops (ECL). The first extracellular loop was shown to be responsible for charge selectivity. The C-terminus of claudins contains putative regulatory 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 tumorigenesis (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 ichthyosis (*cldn-1* mutation), nonsyndromic deafness (*cldn-14* mutations) or hypomagnesemia hypercalciuria with nephrocalcinosis (mutations of *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 auratus*) (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 nigroviridis* 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.

Literature Cited

Amasheh, S., N. Meiri, A. H. Gitter, T. Schoneberg, J. Mankertz, J. D. Schulzke, and M. Fromm, 2002, Claudin-2 expression induces cation-selective channels in tight junctions of epithelial cells, *Journal of Cell Biology*, p. 4969-4976.

Angelow, S., R. Ahlstrom, and A. S. L. Yu, 2008, Biology of claudins: *American Journal of Physiology-Renal Physiology*, v. 295, p. F867-F876.

Angelow, S., and A. S. L. Yu, 2007, Claudins and paracellular transport: an update: *Current Opinion in Nephrology and Hypertension*, v. 16, p. 459-464.

Aparicio, S., J. Chapman, E. Stupka, N. Putnam, J. Chia, P. Dehal, A. Christoffels, S. Rash, S. Hoon, A. Smit, M. D. S. Gelpke, J. Roach, T. Oh, I. Y. Ho, M. Wong, C. Detter, F. Verhoef, P. Predki, A. Tay, S. Lucas, P. Richardson, S. F. Smith, M. S. Clark, Y. J. K. Edwards, N. Doggett, A. Zharkikh, S. V. Tavtigian, D. Pruss, M. Barnstead, C. Evans, H. Baden, J. Powell, G. Glusman, L. Rowen, L. Hood, Y. H. Tan, G. Elgar, T. Hawkins, B. Venkatesh, D. Rokhsar, and S. Brenner, 2002, Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*: *Science*, v. 297, p. 1301-1310.

Bagherie-Lachidan, M., S. I. Wright, and S. P. Kelly, 2008, Claudin-3 tight junction proteins in *Tetraodon nigroviridis*: cloning, tissue-specific expression, and a role in hydromineral balance: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 294, p. R1638-R1647.

Bagherie-Lachidan, M., S. I. Wright, and S. P. Kelly, 2009, Claudin-8 and-27 tight junction proteins in puffer fish *Tetraodon nigroviridis* acclimated to freshwater and seawater: *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, v. 179, p. 419-431.

Balkovetz, D. F., 2009, Tight junction claudins and the kidney in sickness and in health: *Biochimica Et Biophysica Acta-Biomembranes*, v. 1788, p. 858-863.

Ben-Yosef, T., I. A. Belyantseva, T. L. Saunders, E. D. Hughes, K. Kawamoto, C. M. Van Itallie, L. A. Beyer, K. Halsey, D. J. Gardner, E. R. Wilcox, J. Rasmussen, J. M. Anderson, D. F. Dolan, A. Forge, Y. Raphael, S. A. Camper, and T. B. Friedman, 2003, Claudin 14 knockout mice, a model for autosomal recessive deafness DFNB29, are deaf due to cochlear hair cell degeneration: *Human Molecular Genetics*, v. 12, p. 2049-2061.

Brandner, J. M., 2007, Pores in the epidermis: aquaporins and tight junctions: *International Journal of Cosmetic Science*, v. 29, p. 413-422.

Bui, P., M. Bagherie-Lachidan, and S. P. Kelly, 2010, Cortisol differentially alters claudin isoforms in cultured puffer fish gill epithelia: *Molecular and Cellular Endocrinology*, v. 317, p. 120-126.

Bui, P., and S. P. Kelly, 2011, Claudins in a primary cultured puffer fish (*Tetraodon nigroviridis*) gill epithelium: *Methods in molecular biology (Clifton, N.J.)*, v. 762, p. 179-94.

Bui, P., and S. P. Kelly, 2014, Claudin-6, -10d and -10e contribute to seawater acclimation in the euryhaline puffer fish *Tetraodon nigroviridis*: *The Journal of experimental biology*, v. 217, p. 1758-67.

Campbell, M. A., J. A. Lopez, T. Sado, and M. Miya, 2013, Pike and salmon as sister taxa: Detailed intraclade resolution and divergence time estimation of Esociformes plus Salmoniformes based on whole mitochondrial genome sequences: *Gene*, v. 530, p. 57-65.

Chasiotis, H., and S. P. Kelly, 2011, Effect of cortisol on permeability and tight junction protein transcript abundance in primary cultured gill epithelia from stenohaline goldfish and euryhaline trout: *General and Comparative Endocrinology*, v. 172, p. 494-504.

Chasiotis, H., and S. P. Kelly, 2012, Effects of elevated circulating cortisol levels on hydromineral status and gill tight junction protein abundance in the stenohaline goldfish: *General and Comparative Endocrinology*, v. 175, p. 277-283.

Chasiotis, H., D. Kolosov, P. Bui, and S. P. Kelly, 2012, Tight junctions, tight junction proteins and paracellular permeability across the gill epithelium of fishes: A review: *Respiratory Physiology & Neurobiology*, v. 184, p. 269-281.

Christoffels, A., E. G. L. Koh, J. M. Chia, S. Brenner, S. Aparicio, and B. Venkatesh, 2004, Fugu genome analysis provides evidence for a whole-genome duplication early during the evolution of ray-finned fishes: *Molecular Biology and Evolution*, v. 21, p. 1146-1151.

Colegio, O. R., C. Van Itallie, C. Rahner, and J. M. Anderson, 2003, Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture, *Am J Physiol Cell Physiol*, p. C1346–C1354.

Colegio, O. R., C. M. Van Itallie, H. J. McCrea, C. Rahner, and J. M. Anderson, 2002, Claudins create charge-selective channels in the paracellular pathway between epithelial cells: *American Journal of Physiology-Cell Physiology*, v. 283, p. C142-C147.

Cooke, S. J., G. T. Crossin, and S. G. Hinch, 2011, Pacific Salmon Migration: Completing the Cycle, *in* F. A.P., ed., *Encyclopedia of Fish Physiology: From Genome to Environment*, San Diego: Academic Press, p. 1945–1952.

Crete-Lafreniere, A., L. K. Weir, and L. Bernatchez, 2012, Framing the Salmonidae Family Phylogenetic Portrait: A More Complete Picture from Increased Taxon Sampling: *Plos One*, v. 7.

Currens, K. P., C. B. Schreck, and H. W. Li, 1990, Allozyme and morphological divergence of rainbow trout (*Oncorhynchus mykiss*) above and below waterfalls in the Deschutes River, Oregon: *Copeia*, p. 730-746.

Docker, M. F., and D. D. Heath, 2003, Genetic comparison between sympatric anadromous steelhead and freshwater resident rainbow trout in British Columbia, Canada: *Conservation Genetics*, v. 4, p. 227-231.

Eaton, J. G., J. H. McCormick, B. E. Goodno, D. G. O'Brien, H. G. Stefany, M. Hondzo, and R. M. Scheller, 1995, A field information-based system for estimating fish temperature tolerances: *Fisheries*, v. 20, p. 10-18.

Engelund, M. B., A. S. L. Yu, J. Li, S. S. Madsen, N. J. Faergeman, and C. K. Tipsmark, 2012, Functional characterization and localization of a gill-specific claudin isoform in Atlantic salmon: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 302, p. R300-R311.

Evans, D. H., 2008, Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys(vol 295, pg R704, 2008): *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 295, p. R1359-R1359.

Evans, D. H., P. M. Piermarini, and K. P. Choe, 2005, The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste: *Physiological Reviews*, v. 85, p. 97-177.

Fausch, K. D., Y. Taniguchi, S. Nakano, G. D. Grossman, and C. R. Townsend, 2001, Flood disturbance regimes influence rainbow trout invasion success among five holarctic regions: *Ecological Applications*, v. 11, p. 1438-1455.

Fornshell, G., 2002, Rainbow trout - Challenges and solutions: Reviews in Fisheries Science, v. 10, p. 545-557.

Foskett, J. K., H. A. Bern, T. E. Machen, and M. Conner, 1983, Chloride cells and the hormonal control of teleost fish osmoregulation: Journal of Experimental Biology, v. 106, p. 255-&.

Froese, R., and D. Pauly, 2000, FishBase 2000: concepts, design and data sources., ICLARM, Los Baños, Laguna, Philippines., p. 344.

Fujita, K., J. Katahira, Y. Horiguchi, N. Sonoda, M. Furuse, and S. Tsukita, 2000, Clostridium perfringens enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein: Febs Letters, v. 476, p. 258-261.

Furuse, M., K. Fujita, T. Hiiiragi, K. Fujimoto, and S. Tsukita, 1998, Claudin-1 and -2: Novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin: Journal of Cell Biology, v. 141, p. 1539-1550.

Furuse, M., and K. Moriwaki, 2009, The Role of Claudin-Based Tight Junctions in Morphogenesis: Molecular Structure and Function of the Tight Junction: from Basic Mechanisms to Clinical Manifestations, v. 1165, p. 58-61.

Gaumet, F., G. Boeuf, A. Severe, A. Leroux, and N. Mayergonstan, 1995, Effect of salinity on the ionic balance and growth of juvenile turbot: Journal of Fish Biology, v. 47, p. 865-876.

Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B. E. Jaramillo, 2003, Tight junction proteins, Progress in Biophysics & Molecular Biology, p. 1-44.

Gumbiner, B. M., 1993, Breaking through the tight junction barrier: Journal of Cell Biology, v. 123, p. 1631-1633.

Gunzel, D., and A. S. L. Yu, 2013, Claudins and the modulation of tight junction permeability: Physiological reviews, v. 93, p. 525-69.

Gupta, I. R., and A. K. Ryan, 2010, Claudins: unlocking the code to tight junction function during embryogenesis and in disease: Clinical Genetics, v. 77, p. 314-325.

Hadj-Rabia, S., L. Baala, P. Vabres, D. Hamel-Teillac, E. Jacquemin, M. Fabre, S. Lyonnet, Y. De Prost, A. Munnich, M. Hadchouel, and A. Smahi, 2004, Claudin-1 gene mutations in neonatal sclerosing cholangitis associated with Ichthyosis: A tight junction disease: *Gastroenterology*, v. 127, p. 1386-1390.

Handeland, S. O., A. K. Imsland, B. T. Bjornsson, and S. O. Stefansson, 2013, Long-term effects of photoperiod, temperature and their interaction on growth, gill Na⁺, K⁺-ATPase activity, seawater tolerance and plasma growth-hormone levels in Atlantic salmon *Salmo salar*: *Journal of Fish Biology*, v. 83, p. 1197-1209.

Hecht, B. C., M. E. Valle, F. P. Thrower, and K. M. Nichols, 2014, Divergence in Expression of Candidate Genes for the Smoltification Process Between Juvenile Resident Rainbow and Anadromous Steelhead Trout: *Marine Biotechnology*, v. 16, p. 638-656.

Heiskala, M., P. A. Peterson, and Y. Yang, 2001, The roles of claudin superfamily proteins in paracellular transport: *Traffic*, v. 2, p. 92-98.

Hiroi, J., and S. D. McCormick, 2012, New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish: *Respiratory Physiology & Neurobiology*, v. 184, p. 257-268.

Hiroi, J., S. Yasumasu, S. D. McCormick, P.-P. Hwang, and T. Kaneko, 2008, Evidence for an apical Na-Cl cotransporter involved in ion uptake in a teleost fish: *Journal of Experimental Biology*, v. 211, p. 2584-2599.

Hootman, S., and C. Philpott, 1980, Accessory cells in teleost branchial epithelium, *American Journal of Physiology*, p. 199-206.

Hwang, P.-P., and T.-H. Lee, 2007, New insights into fish ion regulation and mitochondrion-rich cells: *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, v. 148, p. 479-497.

Jordan, D. S., and B. W. Evermann, 1905, *American Food and Game Fishes*, Doubleday, Page & Co. New York.

Kalujnaia, S., I. S. McWilliam, V. A. Zaguinaiko, A. L. Feilen, J. Nicholson, N. Hazon, C. P. Cutler, and G. Cramb, 2007, Transcriptomic approach to the study of osmoregulation in the European eel *Anguilla anguilla*: *Physiological Genomics*, v. 31, p. 385-401.

Karnaky, K. J., Jr., 1998, Osmotic and ionic regulation: The physiology of fishes. Second edition., p. 157-176.

Kitajiri, S., M. Furuse, K. Morita, Y. Saishin-Kiuchi, H. Kido, J. Ito, and S. Tsukita, 2004, Expression patterns of claudins, tight junction adhesion molecules, in the inner ear, *Hearing Research*, p. 25-34.

Kohler, K., and A. Zahraoui, 2005, Tight junction: a co-ordinator of cell signalling and membrane trafficking, *Biology of the Cell*, p. 659-665.

Kolosov, D., H. Chasiotis, and S. P. Kelly, 2014, Tight junction protein gene expression patterns and changes in transcript abundance during development of model fish gill epithelia: *Journal of Experimental Biology*, v. 217, p. 1667-1681.

Kolosov, D., P. Bui, H. Chasiotis, S. P. Kelly, 2013, Claudins in teleost fishes, *Tissue Barriers*, v.1(3),

Konrad, M., A. Schaller, D. Seelow, A. V. Pandey, S. Waldegger, A. Lesslauer, H. Vitzthum, Y. Suzuki, J. M. Luk, C. Becker, K. P. Schlingmann, M. Schmid, J. Rodriguez-Soriano, G. Ariceta, F. Cano, R. Enriquez, H. Juppner, S. A. Bakkaloglu, M. A. Hediger, S. Gallati, S. C. F. Neuhauss, P. Nurnberg, and S. Weber, 2006, Mutations in the tight-junction gene claudin 19 (CLDN19) are associated with renal magnesium wasting, renal failure, and severe ocular involvement: *American Journal of Human Genetics*, v. 79, p. 949-957.

Koval, M., 2013, Claudin Heterogeneity and Control of Lung Tight Junctions, *in* D. Julius, ed., *Annual Review of Physiology*, Vol 75: *Annual Review of Physiology*, v. 75: Palo Alto, Annual Reviews, p. 551-567.

Kwong, R. W. M., and S. F. Perry, 2013, The tight junction protein claudin-b regulates epithelial permeability and sodium handling in larval zebrafish, *Danio rerio*: *American journal of physiology. Regulatory, integrative and comparative physiology*, v. 304, p. R504-13.

Lal-Nag, M., and P. J. Morin, 2009, The claudins: *Genome Biology*, v. 10, p. 235

Lameris, A. L., S. Huybers, K. Kaukinen, T. H. Makela, R. J. Bindels, J. G. Hoenderop, and P. I. Nevalainen, 2013, Expression profiling of claudins in the human gastrointestinal tract in health and during inflammatory bowel disease: *Scandinavian Journal of Gastroenterology*, v. 48, p. 58-69.

Le Bras, Y., N. Dechamp, F. Krieg, O. Filangi, R. Guyomard, M. Boussaha, H. Bovenhuis, T. G. Pottinger, P. Prunet, P. Le Roy, and E. Quillet, 2011, Detection of QTL with effects on osmoregulation capacities in the rainbow trout (*Oncorhynchus mykiss*): *Bmc Genetics*, v. 12, p. 14.

Lin, H., D. C. Pfeiffer, A. W. Vogl, J. Pan, and D. J. Randall, 1994, Immunolocalization of H⁺-ATPase in the gill epithelia of rainbow trout: *Journal of Experimental Biology*, v. 195, p. 169-183.

Loh, Y. H., A. Christoffels, S. Brenner, W. Hunziker, and B. Venkatesh, 2004, Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*: *Genome Research*, v. 14, p. 1248-1257.

Mancera, J. M., and S. D. McCormick, 1998, Osmoregulatory actions of the GH/IGF axis in non-salmonid teleosts: *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, v. 121, p. 43-48.

Manzon, L. A., 2002, The role of prolactin in fish osmoregulation: A review: *General and Comparative Endocrinology*, v. 125, p. 291-310.

Marshall, W. S., and M. Grosell, 2006, Ion transport, osmoregulation, and acid-base balance: *The physiology of fishes*. Third edition. [Marine Biology Series.]. p. 177-230.

Marshall, W. S., J. A. Howard, R. R. F. Cozzi, and E. M. Lynch, 2002, NaCl and fluid secretion by the intestine of the teleost *Fundulus heteroclitus*: involvement of CFTR: *Journal of Experimental Biology*, v. 205, p. 745-758.

Matter, K., S. Aijaz, A. Tsapara, and M. S. Balda, 2005, Mammalian tight junctions in the regulation of epithelial differentiation and proliferation, *Current Opinion in Cell Biology*, p. 453-458.

McCormick, S. D., 2001, Endocrine control of osmoregulation in teleost fish: *American Zoologist*, v. 41, p. 781-794.

McPhail, J. D., 1997, The origin and speciation of *Oncorhynchus* revisited: Pacific salmon & their ecosystems: status and future options., p. 29-38.

Meka, J. M., E. E. Knudsen, D. C. Douglas, and R. B. Benter, 2003, Variable migratory patterns of different adult rainbow trout life history types in a southwest Alaska watershed: Transactions of the American Fisheries Society, v. 132, p. 717-732.

Milatz, S., S. M. Krug, R. Rosenthal, D. Guenzel, D. Mueller, J.-D. Schulzke, S. Amasheh, and M. Fromm, 2010, Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes, *Biochimica et Biophysica Acta - Biomembranes*, p. 2048-2057.

Morita, K., M. Furuse, K. Fujimoto, and S. Tsukita, 1999, Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands: Proceedings of the National Academy of Sciences of the United States of America, v. 96, p. 511-516.

Nichols, K. M., A. F. Edo, P. A. Wheeler, and G. H. Thorgaard, 2008, The genetic basis of smoltification-related traits in *Oncorhynchus mykiss*: *Genetics*, v. 179, p. 1559-1575.

Opuszyński, K., 1979, *Podstawy biologii ryb*: Warszawa, Państwowe Wydawnictwo Rolnicze i Leśne.

Osinov, A. G., and V. S. Lebedev, 2004, Salmonid fish (Salmonidae, Salmoniformes): Position in the suborder protacanthopterygii. Basic evolutionary history, molecular dating: *Voprosy Ikhtiologii*, v. 44, p. 738-765.

Pavlov, D. A., and A. G. Osinov, 2004, Main features of early ontogeny in salmoniforms (Salmoniformes) and other representatives of the protacanthopterygii in relation to the phylogeny: *Voprosy Ikhtiologii*, v. 44, p. 293-312, CP1.

Payan, P., J. Girard, and N. Mayergostan, 1984, Branchial ion movements in teleosts - the roles of respiratory and chloride cells, *Fish Physiology*, Academic Press Inc., p. 39-63.

Peppi, M. and M. N. Ghabriel, 2004, Tissue-specific expression of the tight junction proteins claudins and occludin in the rat salivary glands, *Journal of Anatomy*, v. 205, p. 257-266.

Piontek, J., L. Winkler, H. Wolburg, S. L. Muller, N. Zuleger, C. Piehl, B. Wiesner, G. Krause, and I. E. Blasig, 2008, Formation of tight junction: determinants of homophilic interaction between classic claudins: *Faseb Journal*, v. 22, p. 146-158.

Prunet, P., G. Boeuf, and L. M. Houdebine, 1985, Plasma and pituitary prolactin levels in rainbow trout during adaptation to different salinities: *Journal of Experimental Zoology*, v. 235, p. 187-196.

Ramsden, S. D., H. Brinkmann, C. W. Hawryshyn, and J. S. Taylor, 2003, Mitogenomics and the sister of Salmonidae: *Trends in Ecology & Evolution*, v. 18, p. 607-610.

Rosenthal, R., S. Milatz, S. M. Krug, D. Guenzel, B. Oelrich, S. Amasheh, and M. Fromm, 2009, The tight junction protein claudin-2 forms a paracellular water channel: *Faseb Journal*, v. 23.

Sakamoto, T., S.D. McCormick, 2006, Prolactin and growth hormone in fish osmoregulation, *General and Comparative Endocrinology*, v. 147, p. 24-30

Sawada, N., 2013, Tight junction-related human diseases: *Pathology International*, v. 63, p. 1-12.

Schneeberger, E. E., and R. D. Lynch, 2004, The tight junction: a multifunctional complex: *American Journal of Physiology-Cell Physiology*, v. 286, p. C1213-C1228.

Schultz, E. T., and S. D. McCormick, 2012, Euryhalinity in an evolutionary context: *Fish Physiology*, v. 32, p. 477-533.

Shedko, S. V., I. L. Miroshnichenko, and G. A. Nemkova, 2012, Phylogeny of salmonids (Salmoniformes: Salmonidae) and its molecular dating: Analysis of nuclear RAG1 gene: *Russian Journal of Genetics*, v. 48, p. 575-579.

Shen, L., C. R. Weber, D. R. Raleigh, D. Yu, and J. R. Tumer, 2011, Tight, Junction Pore and Leak Pathways: A Dynamic Duo: *Annual Review of Physiology*, Vol 73, v. 73, p. 283-309.

Siddiqui, M., H. Sheikh, C. Tran, and A. E. E. Bruce, 2010, The Tight Junction Component Claudin E is Required for Zebrafish Epiboly: *Developmental Dynamics*, v. 239, p. 715-722.

Simon, D. B., Y. Lu, K. A. Choate, H. Velazquez, E. Al-Sabban, M. Praga, C. Casari, A. Bettinelli, C. Colussi, J. Rodriguez-Soriano, D. McCredie, D. Milford, S. Sanjad, and R. P. Lifton, 1999, Paracellin-1, a renal tight junction protein required for paracellular Mg²⁺ resorption: *Science*, v. 285, p. 103-106.

Singh, A. B., A. Sharma, and P. Dhawan, 2010, Claudin family of proteins and cancer: an overview: *Journal of oncology*, v. 2010, p. 541957-541957.

Sundell, K. S., and H. Sundh, 2012, Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins: *Frontiers in physiology*, v. 3, p. 388-388.

Suworow, E., 1954, *Podstawy ichtiologii*, Państwowe Wydawnictwo Naukowe.

Takahashi, H., and T. Sakamoto, 2013, The role of 'mineralocorticoids' in teleost fish: Relative importance of glucocorticoid signaling in the osmoregulation and 'central' actions of mineralocorticoid receptor: *General and Comparative Endocrinology*, v. 181, p. 223-228.

Taylor, J. S., I. Braasch, T. Frickey, A. Meyer, and Y. Van de Peer, 2003, Genome duplication, a trait shared by 22,000 species of ray-finned fish: *Genome Research*, v. 13, p. 382-390.

Thorgaard, G. H., G. S. Bailey, D. Williams, D. R. Buhler, S. L. Kaattari, S. S. Ristow, J. D. Hansen, J. R. Winton, J. L. Bartholomew, J. J. Nagler, P. J. Walsh, M. M. Vijayan, R. H. Devlin, R. W. Hardy, K. E. Overturf, W. P. Young, B. D. Robison, C. Rexroad, and Y. Palti, 2002, Status and opportunities for genomics research with rainbow trout: *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology*, v. 133, p. 609-646.

Tine, M., H. Kuhl, A. Beck, L. Bargelloni, and R. Reinhardt, 2011, Comparative analysis of intronless genes in teleost fish genomes: Insights into their evolution and molecular function: *Marine Genomics*, v. 4, p. 109-119.

Tipsmark, C. K., C. Jorgensen, N. Brande-Lavridsen, M. Engelund, J. H. Olesen, and S. S. Madsen, 2009, Effects of cortisol, growth hormone and prolactin on gill claudin expression in Atlantic salmon: *General and Comparative Endocrinology*, v. 163, p. 270-277.

Tipsmark, C. K., P. Kiilerich, T. O. Nilsen, L. O. E. Ebbesson, S. O. Stefansson, and S. S. Madsen, 2008, Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 294, p. R1563-R1574.

Trubitt, R. T., D. B. Rabeneck, J. K. Bujak, M. C. Bossus, S. S. Madsen, and C. K. Tipsmark, 2015, Transepithelial resistance and claudin expression in trout RT gill-W1 cell line: Effects of somoregulatory hormones., *Comparative Biochemistry and Physiology, Part A, Molecular and Integrative Physiology*, p. 45-52.

Tsukita, S., and M. Furuse, 2000, Pores in the wall: Claudins constitute tight junction strands containing aqueous pores: *Journal of Cell Biology*, v. 149, p. 13-16.

Tsukita, S., M. Furuse, and M. Itoh, 2001, Multifunctional strands in tight junctions: *Nature Reviews Molecular Cell Biology*, v. 2, p. 285-293.

Tsukita, S., Y. Yamazaki, T. Katsuno, and A. Tamura, 2008, Tight junction-based epithelial microenvironment and cell proliferation: *Oncogene*, v. 27, p. 6930-6938.

Van Itallie, C., C. Rahner, and J. Anderson, 2001, Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability, *Journal of Clinical Investigation*, p. 1319-1327.

Van Itallie, C. M., A. S. Fanning, and J. M. Anderson, 2003, Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins: *American Journal of Physiology-Renal Physiology*, v. 285, p. F1078-F1084.

Van Itallie, C. M., S. Rogan, A. Yu, L. S. Vidal, J. Holmes, and J. M. Anderson, 2006, Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities: *American Journal of Physiology-Renal Physiology*, v. 291, p. F1288-F1299.

Wen, H. J., D. D. Watry, M. C. G. Marcondes, and H. S. Fox, 2004, Selective decrease in paracellular conductance of tight junctions: role of the first extracellular domain of claudin-5, *Molecular and Cellular Biology*, p. 8408-8417.

Wilson, J., and P. Laurent, 2002, Fish gill morphology: Inside out, *Journal Of Experimental Zoology*, p. 192-213.

Winberg, G., 1956, *Intensivnost' obmiena i pishchewyje potrebnosti ryb*, Izdat. Bielgosuniwersiteta im. W. I. Lenina.

Xie, J., E. Farage, M. Sugimoto, and B. Anand-Apte, 2010, A novel transgenic zebrafish model for blood-brain and blood-retinal barrier development: *Bmc Developmental Biology*, v. 10.

Yu, A. S. L., M. H. Cheng, S. Angelow, D. Günzel, S. A. Kanzawa, E. E. Schneeberger, M. Fromm, and R. D. Coalson, 2009, Molecular basis for cation selectivity in claudin-2 – based paracellular pores: identification of an electrostatic interaction site, *Journal of General Physiology*, p. 111-127.

Zadunaisky, J. A., 1984, The Chloride Cell: The active transport of chloride and the paracellular pathways, *Fish Physiology*, p. 129-176.

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 Norfolk 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 NaHCO₃ 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 °C 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 °C 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 μ l of 1-Bromo-3-chloropropane and centrifugation (4 $^{\circ}$ 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 $^{\circ}$ C. The next day, samples were centrifuged at 4 $^{\circ}$ 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 μ l 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 μ g 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 $^{\circ}$ C followed by 45 cycles of 30 second denaturation phase at 95 $^{\circ}$ C and 1 min annealing phases at 60 $^{\circ}$ C. A melting curve (from 65 $^{\circ}$ C to 95 $^{\circ}$ C, with an increment of 0.5 $^{\circ}$ 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)*I- α (Efl α)* (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 (α 5) (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 °C 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). Conserved cysteine (C) residues were marked with green.

- *Statistics*

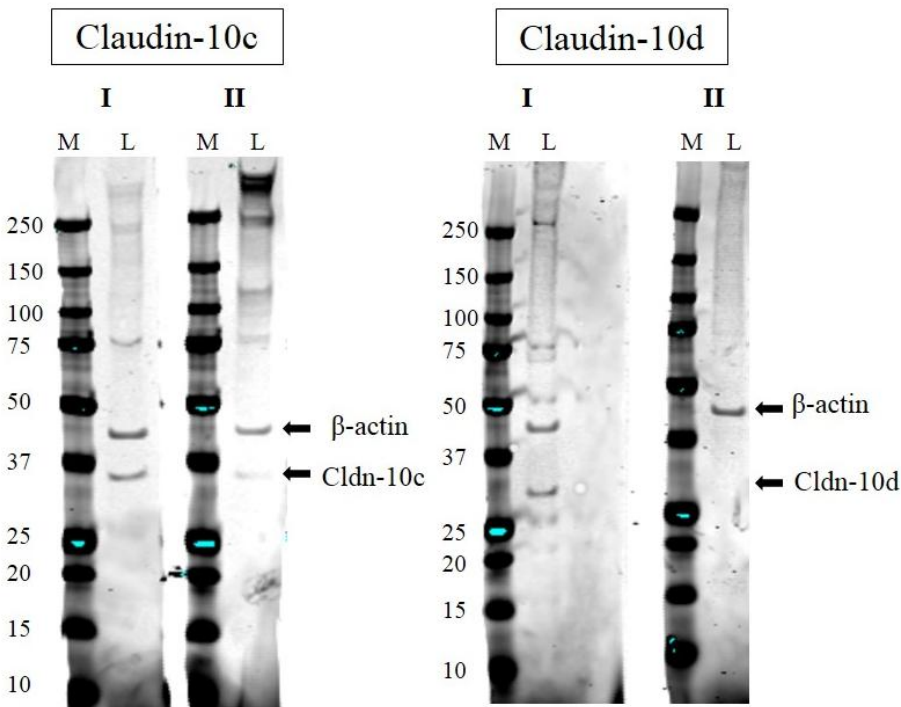
Tissue distribution data were analyzed by one-way ANOVA. For comparison of mRNA and protein expression between FW and SW, a Student's t-test was used. To meet the ANOVA assumption of homogeneity of variances (tested by Bartlett's test) logarithmic transformation of data was performed. Data are represented 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.



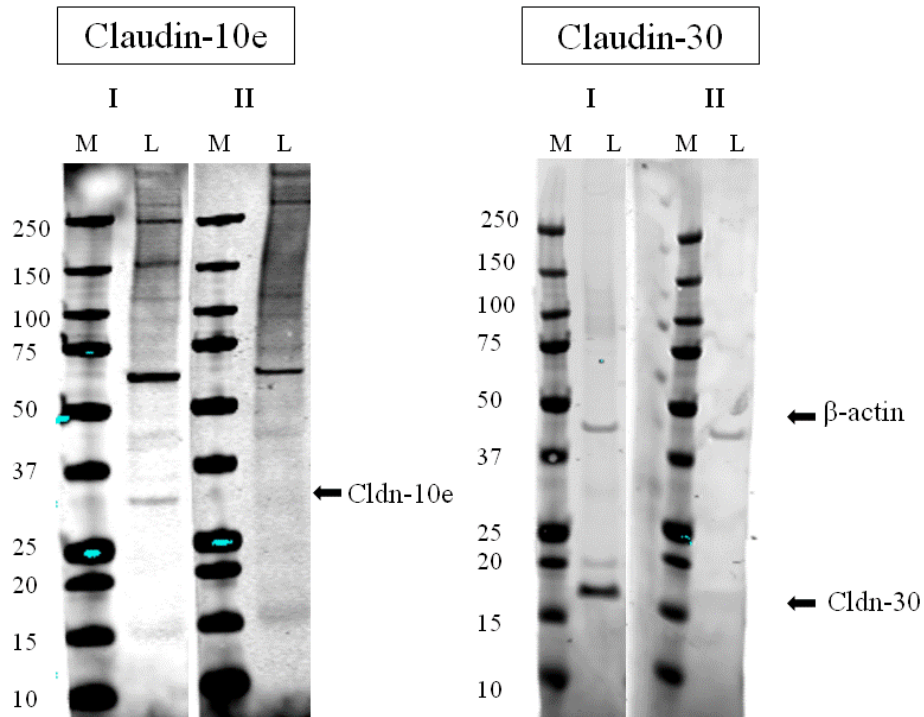


Figure 8. Immunizing peptide blocking experiment with claudin-10s and claudin-30. First strip of each blot (I) was used as positive control and was incubated with antibodies solution. Second strip of each blot (II) represents probe treated with anti-claudin antibody and blocking peptide solution. Claudin-10c 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.

```

Omy_CLDN10c STMPTEIWTWSE-V---ESIVLTSSNYFSNLWKDCVSDSTGVSDCKGIPSMFGLNWDIHMCR
Omy_CLDN10d ----IEYWTWSE-V---GSVVLTTGNYFSNLWKDCVSDSTGVSDCKEYPSMLGLPVFLHSXR
Omy_CLDN10e -----EGWKVTS-IGGMGGSAAVKKVAWYWSNLWKACFTDSTSVTNQDFPVLWSVDNHIQIVR
Mmu_CLDN10b ---PTDYWKVST-I----IGTVITTATYFANLWKICVTDSTGVANCKEFPFMLALDGYIQACR
Mmu_CLDN10a ---TSNEWKVTT-R----ASSVITATWVYQGLWMNCAGNALGSFHRPHFTIFKVEGYIQACR
Omy_CLDN30 ----LPQWKVTAFI----GENIITAQTTWQGIWMNCVVQSTGQMCKVYDSMLALPQDLQAAR
Mmu_CLDN3  -----RVSAFI----GSSIITAQITWEGLWMNCVVQSTGQMCKMYDSSLALPQDLQAAR

```

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).

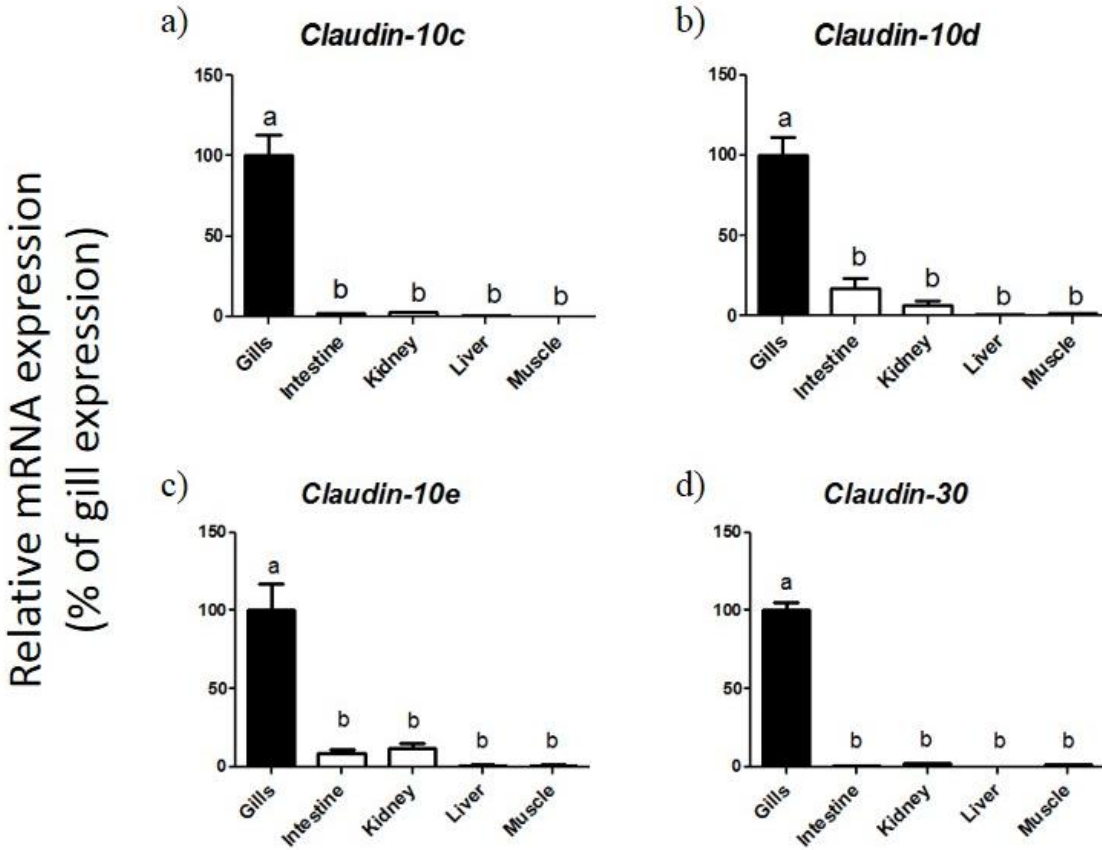


Figure 10. Claudin-10c, -10d, -10e and claudin-30 mRNA expression in tissues of rainbow trout (*Oncorhynchus mykiss*). Transcript level was evaluated in gills, intestine, kidney, liver, and muscle by quantitative PCR. mRNA level was normalized to geometric mean of three genes: β -actin, *Ef1a* and *rplp0*. Data are represented as mean value of pooled, FW and SW acclimated fish \pm 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 *claudin-10e* in *Salmo salar* (Tipsmark et al., 2008), *claudin-10d* and *-10e* in *Tetraodon nigroviridis* (Bui and Kelly, 2014) and data about *claudin-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 $\alpha 5$ expression in FW and SW

Expression of claudin-10s, claudin-30 and $\alpha 5$ 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 implicate that salinity regulates expression of Claudin-10s and is responsible for adaptive modulation of claudins in the gill epithelium.

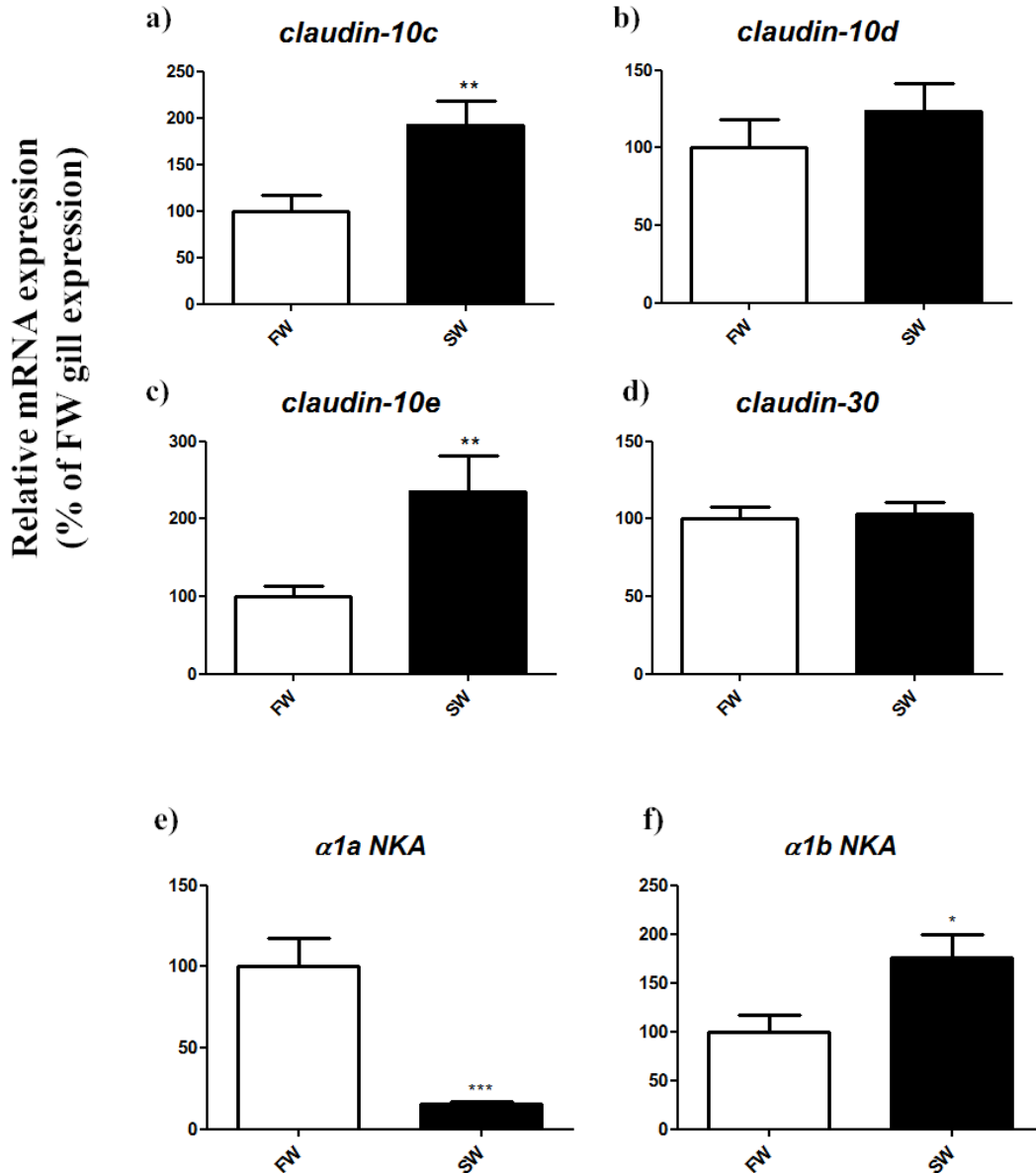
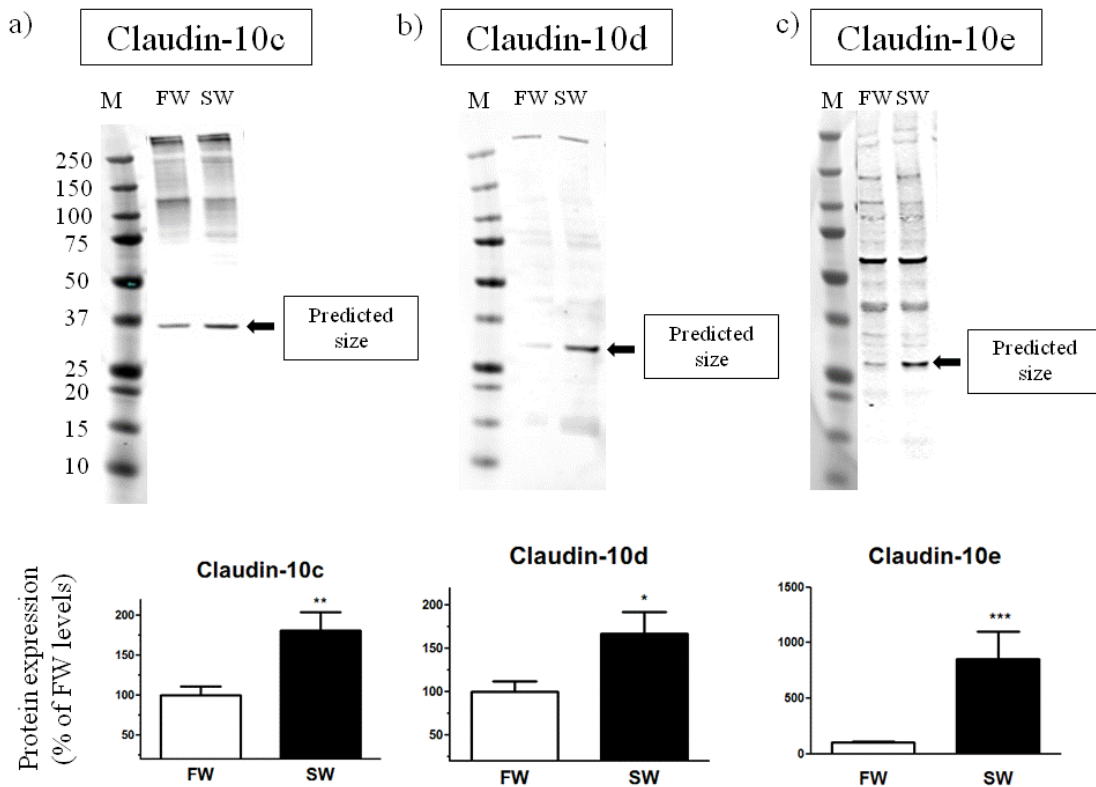


Figure 11. *Claudin-10c*, *-10d*, *-10e*, *claudin-30*, *NKA $\alpha 1a$* and *$\alpha 1b$* mRNA expression in FW and SW acclimated gills of rainbow trout. $\alpha 1a$ and $\alpha 1b$ subunits of Na^+ , K^+ -ATPase were used as a positive control of response to salinity challenge. mRNA level was normalized to geometric mean of three genes: *β -actin*, *Ef1a* and *rplp0*. Data represent mean value \pm 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 nigroviridis* and *Tetraodon biocellatus*, showed differences in claudin expression arising presumably from interspecies differences (Bagherie-Lachidan et al., 2009; Duffy et al., 2011).



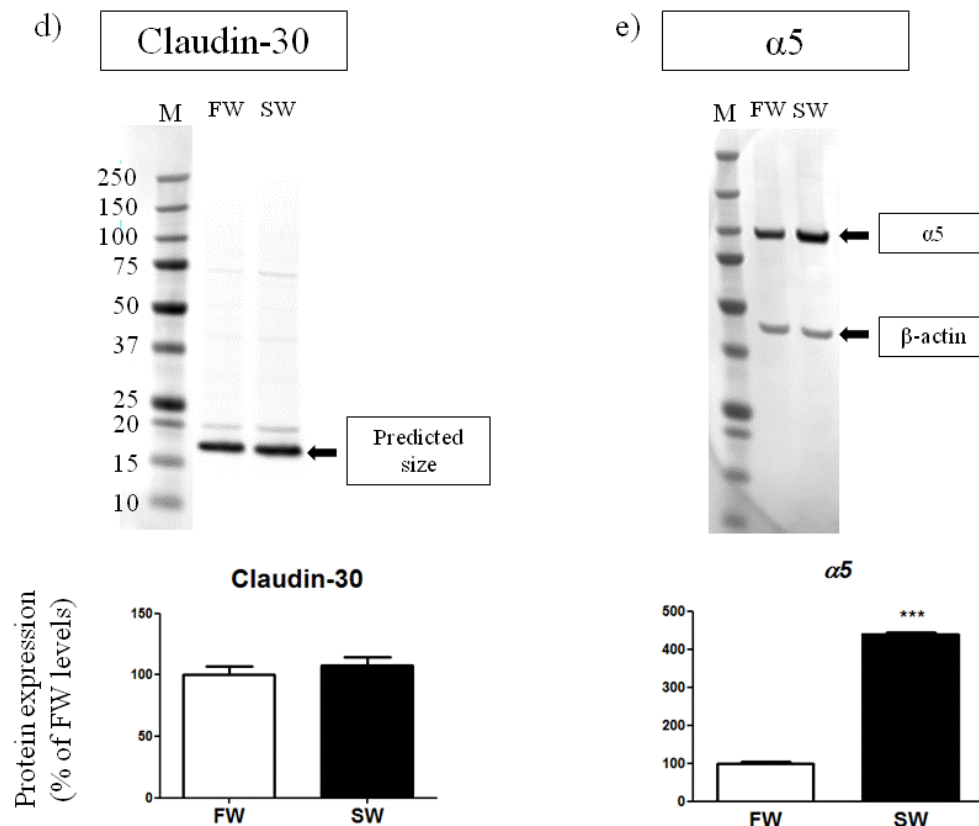


Figure 12. Protein expression of claudin-10c, -10d, -10e, claudin-30 and $\alpha 5$ in FW and SW acclimated gills of rainbow trout. Gill lysate from FW and SW acclimated rainbow trout was immunoblotted with Claudin-10c, -10d, -10e and Claudin-30. $\alpha 5$ antibodies were used as a reference. Data represent mean values \pm 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: $\alpha 1a$, which is a FW type and $\alpha 1b$, responsible for SW acclimation (Jorgensen, 2008). Relative mRNA expression of $\alpha 1a$ subunit of NKA was significantly higher in FW water when compared with SW (Fig.11e). Expression of $\alpha 1b$ 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.

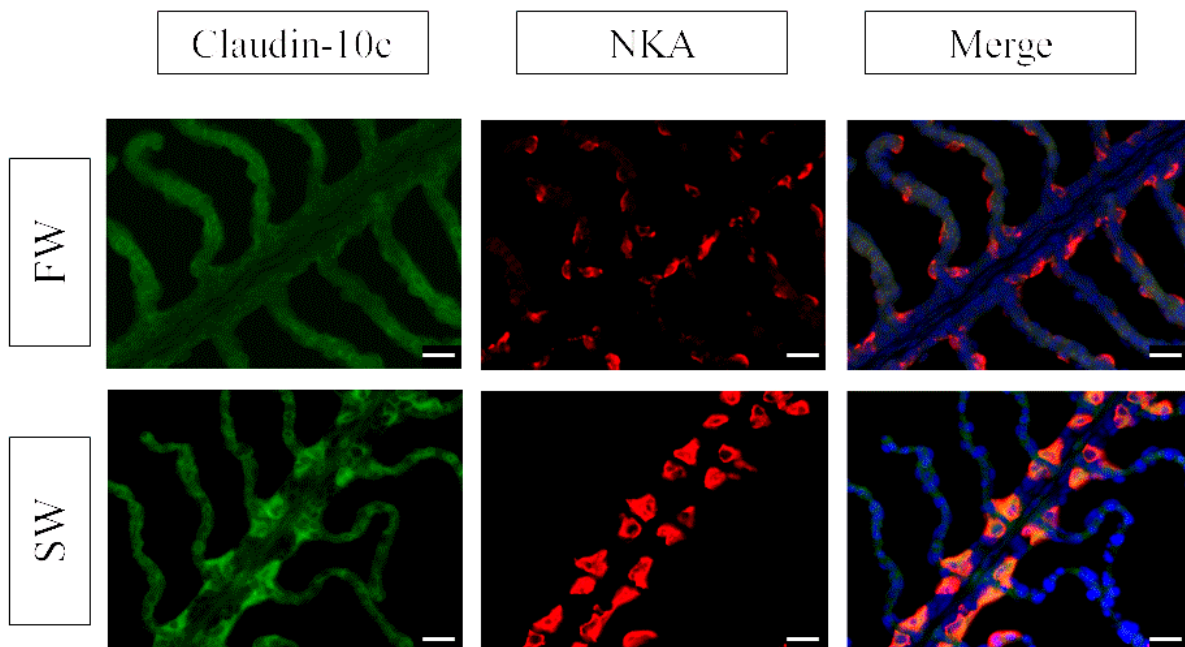
My data focus on four claudin isoforms that are encoded by four distinct genes (Appendix 2).

However, it is known that some claudin isoforms exist as spliced variants. Van Itallie, et al.

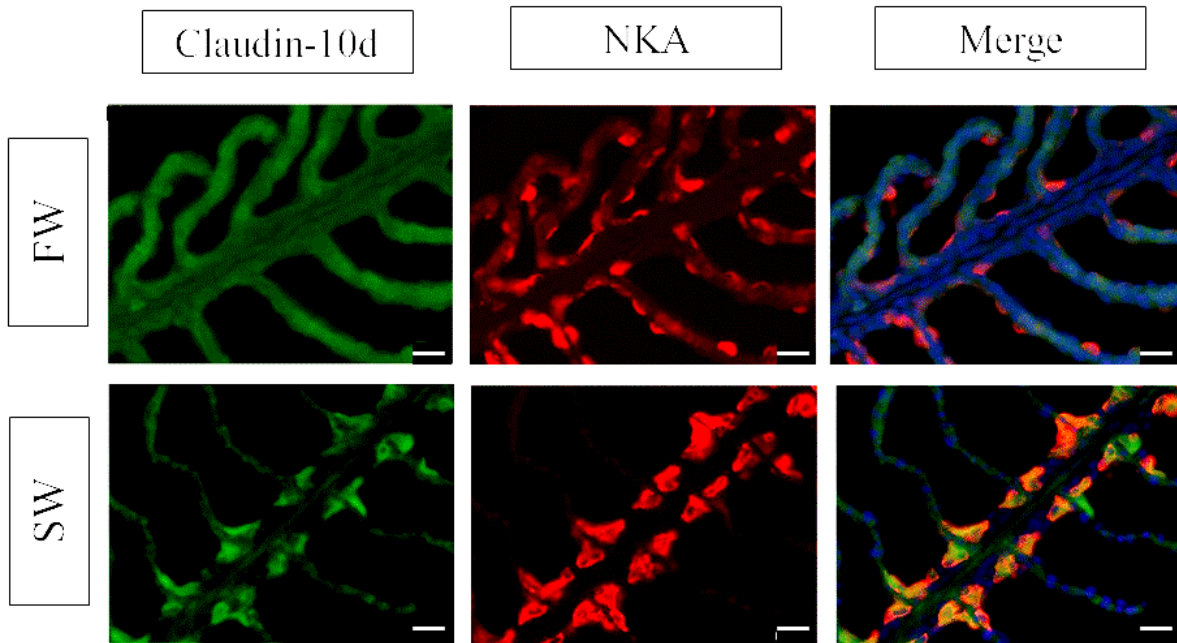
(2006) initially found that Claudin-10 in human and mouse have two spliced variants, each with

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.

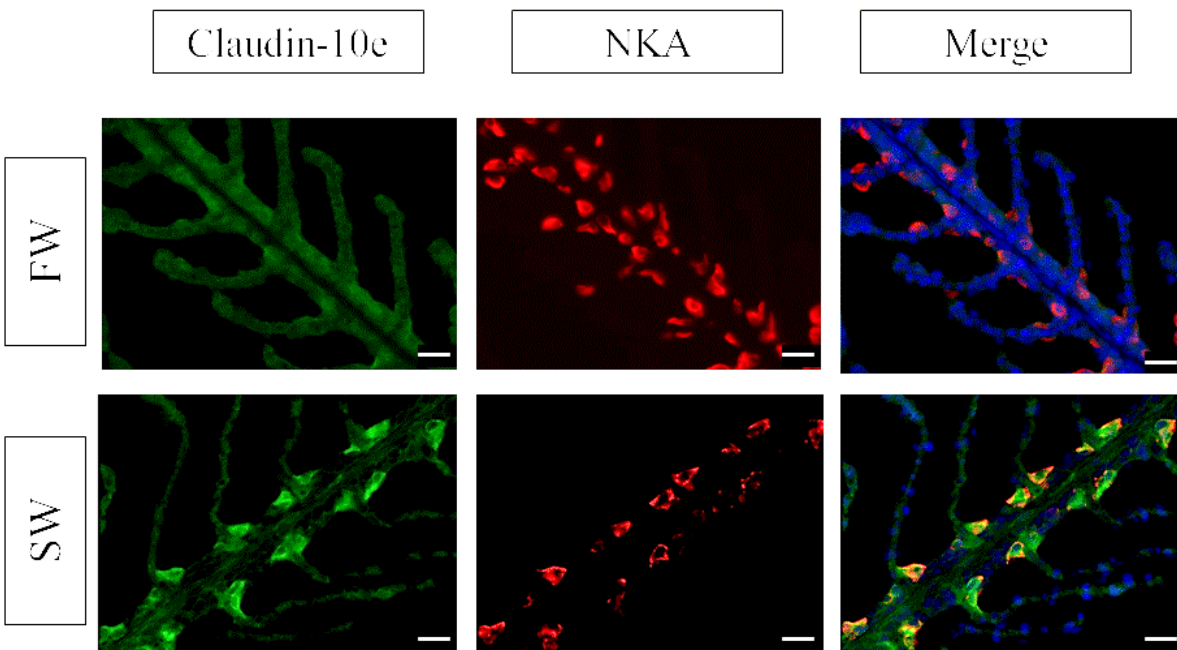
a)



b)



c)



d)

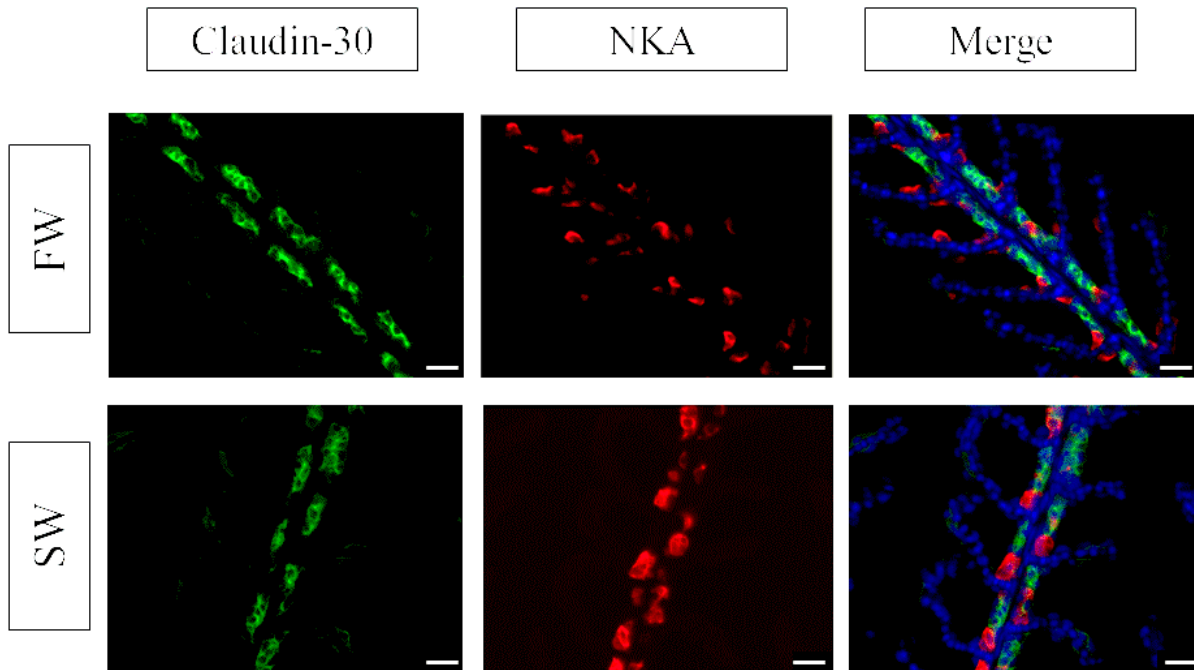


Figure 13. Immunofluorescence images of claudin-30 and claudin-10c, 10-d, -10e in the FW and SW rainbow trout gill. Gills cross sections were probed with Claudin-10c, -10d, -10e or Claudin-30 antibodies (green). $\alpha 5$ antibodies (red) were used in order to indicate position of mitochondrion-rich cells. Cells nuclei were stained with DAPI (blue). Scale bars: 20 μ m

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 $\alpha 5$, 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 $\alpha 5$

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).

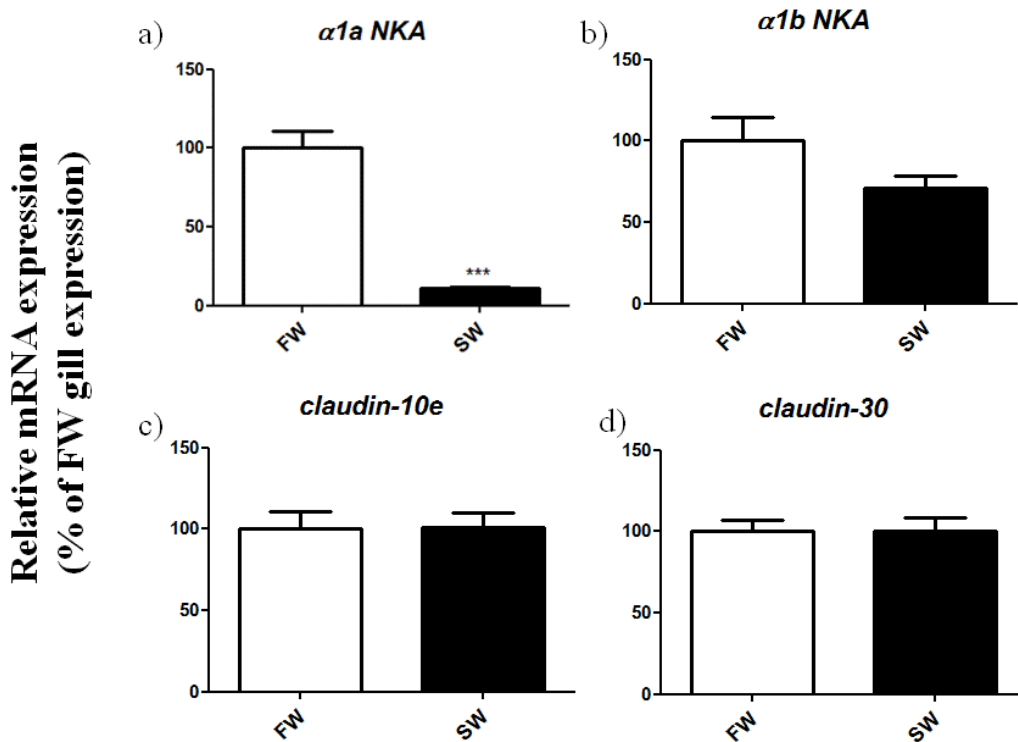


Figure 14. Relative transcript abundance in the gills of rainbow trout acclimated to 20 ppt SW for a one week. Only expression of *α1a*, a freshwater isoform of NKA, was changed after SW acclimation. Surprisingly, *claudin-10e* and *α1b* were not elevated by SW. Data represent mean values \pm SEM (N=10). 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, this 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 ability to ionoregulate is associated not only with fish size but also with activity of NKA, which plays a crucial role in ion transport.

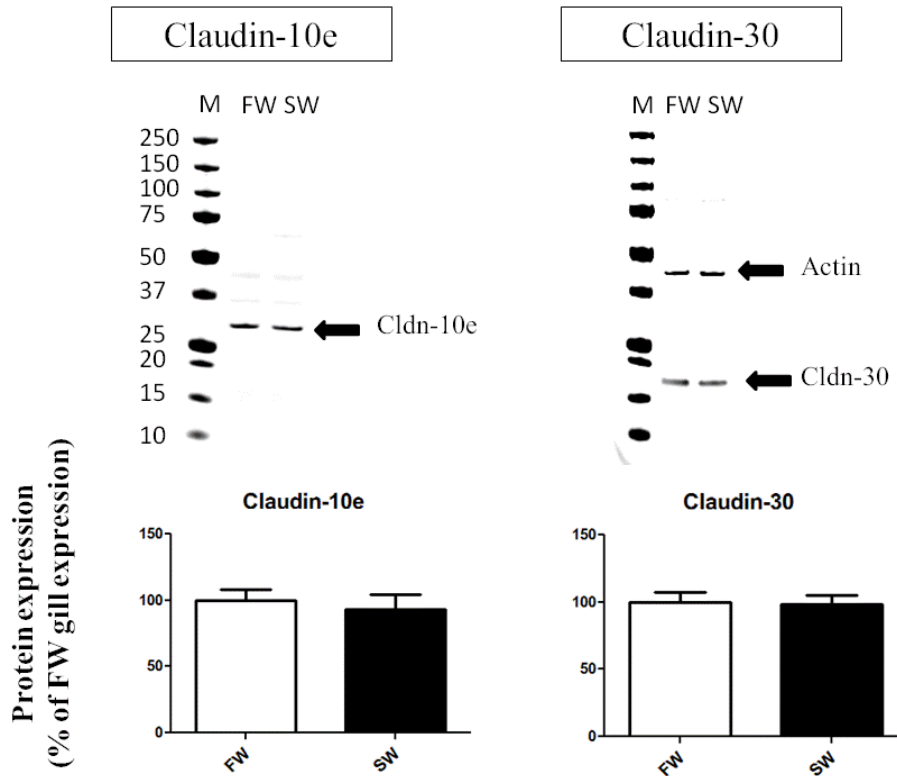


Figure 15. Claudin-10e and claudin-30 protein expression in the gills of rainbow trout acclimated to FW and 20 ppt SW for one week. Expression of Claudin-10e, supposedly SW isoforms, was not altered by SW transfer. Expression of Claudin-30 remained unchanged. Data represents mean value \pm SEM (N=5).

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 α 1a was demonstrated to be upregulated in the FW, whereas expression of α 1b was significantly induced by SW acclimation in rainbow trout gills (Richards et al., 2003). When the fish is not able to upregulate expression of α 1b 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 α 1a expression was decreased after exposure to SW but no changes were observed in regard to α 1b, 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 $\alpha 1a$ was significantly decreased in SW while level of $\alpha 1b$ 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 $\alpha 1b$ 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 *ATP $\alpha 1b$* locus (Norman et al., 2011). The same study suggests that both *ATP $\alpha 1b$* 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 *$\alpha 1b$* and *cldn-10e* in rainbow trout.

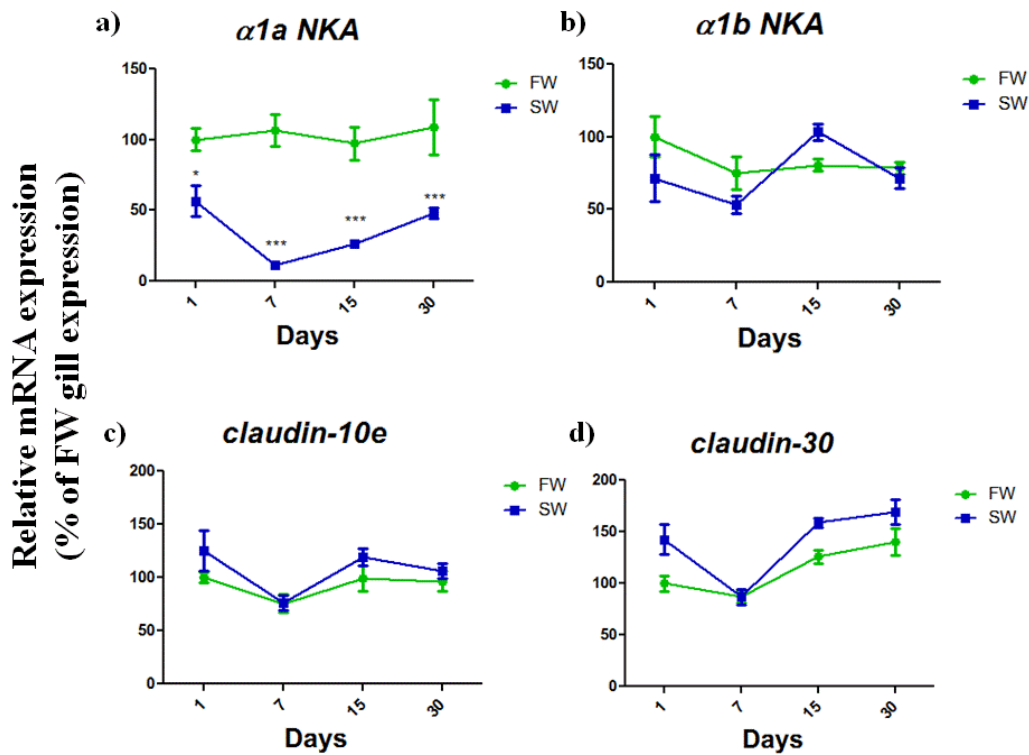


Figure 16. mRNA expression of NKA $\alpha 1a$, $\alpha 1b$, *claudin-10e* and *claudin-30* time course experiment. Only $\alpha 1a$ exhibited significant downregulation after SW-transfer. No changes in expression of *claudin-10e*, -30 and $\alpha 1b$ were observed. Data represent mean values \pm SEM (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).

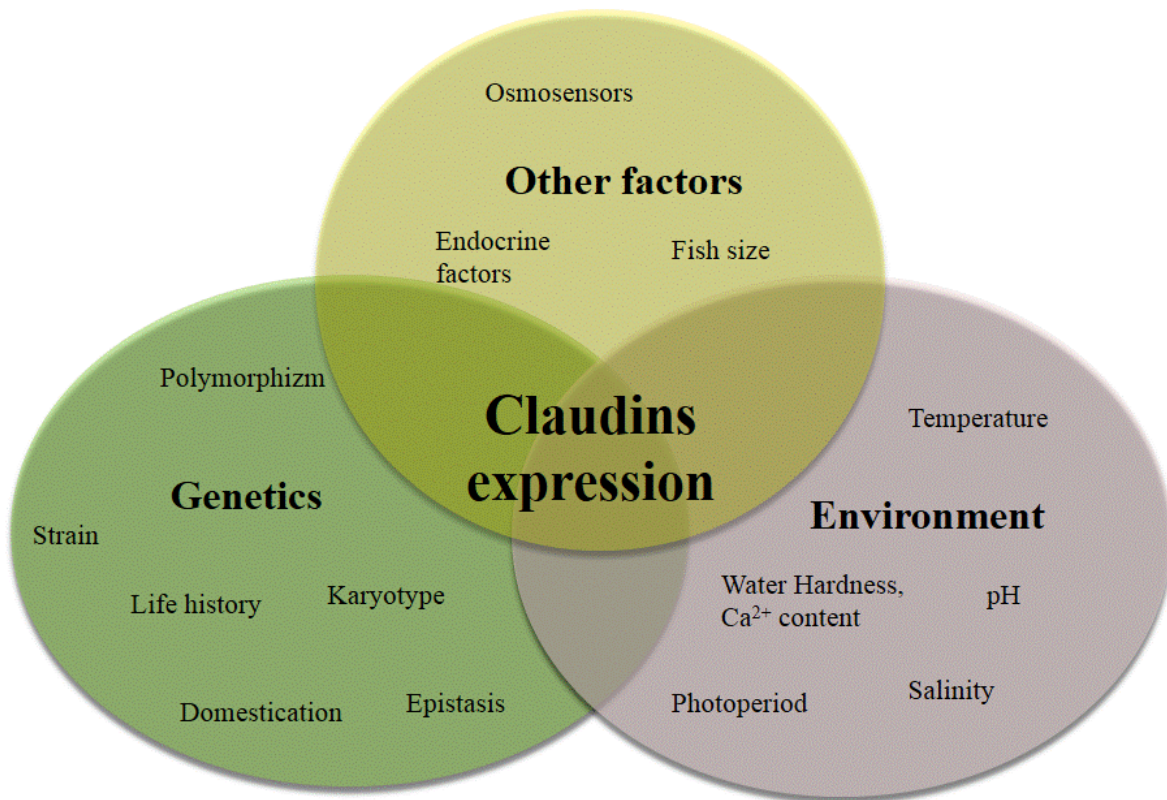


Figure 17. Influence of genetics, environment and other factors on claudin expression.

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.

Literature cited

Allen, P. J., J. J. Cech, Jr., and D. Kueltz, 2009, Mechanisms of seawater acclimation in a primitive, anadromous fish, the green sturgeon: *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, v. 179, p. 903-920.

Aono, S., and Y. Hirai, 2008, Phosphorylation of claudin-4 is required for tight junction formation in a human keratinocyte cell line, *Experimental Cell Research*, p. 3326-3339.

Bagherie-Lachidan, M., S. I. Wright, and S. P. Kelly, 2008, Claudin-3 tight junction proteins in *Tetraodon nigroviridis*: cloning, tissue-specific expression, and a role in hydromineral balance: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 294, p. R1638-R1647.

Bagherie-Lachidan, M., S. I. Wright, and S. P. Kelly, 2009, Claudin-8 and-27 tight junction proteins in puffer fish *Tetraodon nigroviridis* acclimated to freshwater and seawater: *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, v. 179, p. 419-431.

Baltzegar, D. A., B. J. Reading, and E. S. Brune, 2013, Phylogenetic revision of the claudin gene family, *Marine Genomics*, p. 17-26.

Bartels, H., and I. Potter, 1991, Structural changes in the zonular occludentes of the chloride cells of young-adult lampreys following acclimation to seawater, *Cell and tissue research*, p. 447-457.

Brenna-Hansen, S., J. Li, M. P. Kent, E. G. Boulding, S. Dominik, W. S. Davidson, and S. Lien, 2012, Chromosomal differences between European and North American Atlantic salmon discovered by linkage mapping and supported by fluorescence in situ hybridization analysis: *Bmc Genomics*, v. 13.

Bui, P., M. Bagherie-Lachidan, and S. P. Kelly, 2010, Cortisol differentially alters claudin isoforms in cultured puffer fish gill epithelia: *Molecular and Cellular Endocrinology*, v. 317, p. 120-126.

Bui, P., and S. P. Kelly, 2011, Claudins in a primary cultured puffer fish (*Tetraodon nigroviridis*) gill epithelium: *Methods in molecular biology (Clifton, N.J.)*, v. 762, p. 179-94.

Bui, P., and S. P. Kelly, 2014, Claudin-6, -10d and -10e contribute to seawater acclimation in the euryhaline puffer fish *Tetraodon nigroviridis*: *The Journal of experimental biology*, v. 217, p. 1758-67.

Bystriansk, J. S., N. T. Frick, J. G. Richards, P. M. Schulte, and J. S. Ballantyne, 2007, Failure to up-regulate gill Na⁺/K⁺-ATPase alpha-subunit isoform alpha 1b may limit seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*): *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, v. 148, p. 332-338.

Bystriansky, J. S., J. G. Richards, P. M. Schulte, and J. S. Ballantyne, 2006b, Reciprocal expression of gill Na⁺/K⁺-ATPase alpha-subunit isoforms alpha 1a and alpha 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance: *Journal of Experimental Biology*, v. 209, p. 1848-1858.

Chasiotis, H., and S. P. Kelly, 2011, Effect of cortisol on permeability and tight junction protein transcript abundance in primary cultured gill epithelia from stenohaline goldfish and euryhaline trout: *General and Comparative Endocrinology*, v. 172, p. 494-504.

Chasiotis, H., D. Kolosov, and S. P. Kelly, 2012, Permeability properties of the teleost gill epithelium under ion-poor conditions: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 302, p. R727-R739.

Colegio, O. R., C. Van Itallie, C. Rahner, and J. M. Anderson, 2003, Claudin extracellular domains determine paracellular charge selectivity and resistance but not tight junction fibril architecture, *Am J Physiol Cell Physiol*, v. 284:6, p. C1346-C1354.

Colegio, O. R., C. M. Van Itallie, H. J. McCrea, C. Rahner, and J. M. Anderson, 2002, Claudins create charge-selective channels in the paracellular pathway between epithelial cells: *American Journal of Physiology-Cell Physiology*, v. 283, p. C142-C147.

Coyne, C. B., T. M. Gambling, R. C. Boucher, J. L. Carson, and L. G. Johnson, 2003, Role of claudin interactions in airway tight junctional permeability: *American Journal of Physiology-Lung Cellular and Molecular Physiology*, v. 285, p. L1166-L1178.

D'Souza, T., R. Agarwal, and P. Morin, 2005, Phosphorylation of claudin-3 at threonine 192 by cAMP-dependent protein kinase regulates tight junction barrier function in ovarian cancer cells, *Journal of Biological Chemistry*, v. 280, p. 26233-26240.

D'Souza, T., F. E. Indig, and P. J. Morin, 2007, Phosphorylation of claudin-4 by PKC epsilon regulates tight junction barrier function in ovarian cancer cells, *Experimental Cell Research*, v. 313: 15, p. 3364-3375

Docker, M. F., and D. D. Heath, 2003, Genetic comparison between sympatric anadromous steelhead and freshwater resident rainbow trout in British Columbia, Canada: *Conservation Genetics*, v. 4, p. 227-231.

Duffy, N. M., P. Bui, M. Bagherie-Lachidan, and S. P. Kelly, 2011, Epithelial remodeling and claudin mRNA abundance in the gill and kidney of puffer fish (*Tetraodon biocellatus*) acclimated to altered environmental ion levels: *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, v. 181, p. 219-238.

Edwards, S. L., and W. S. Marshall, 2013, Principles and patterns of osmoregulation and euryhalinity in fishes: *Fish Physiology*, v. 32, p. 1-44.

Engelund, M. B., A. S. L. Yu, J. Li, S. S. Madsen, N. J. Faergeman, and C. K. Tipsmark, 2012, Functional characterization and localization of a gill-specific claudin isoform in Atlantic salmon: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 302, p. R300-R311.

Evans, D. H., P. M. Piermarini, and K. P. Choe, 2005, The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste: *Physiological Reviews*, v. 85, p. 97-177.

Flores, A.-M., and J. M. Shrimpton, 2012, Differential physiological and endocrine responses of rainbow trout, *Oncorhynchus mykiss*, transferred from fresh water to ion-poor or salt water: *General and Comparative Endocrinology*, v. 175, p. 244-250.

Furuse, M., H. Sasaki, and S. Tsukita, 1999, Manner of interaction of heterogeneous claudin species within and between tight junction strands: *Journal of Cell Biology*, v. 147, p. 891-903.

Guenzel, D., M. Stuiver, P. J. Kausalya, L. Haisch, S. M. Krug, R. Rosenthal, I. C. Meij, W. Hunziker, M. Fromm, and D. Mueller, 2009, Claudin-10 exists in six alternatively spliced isoforms that exhibit distinct localization and function: *Journal of Cell Science*, v. 122, p. 1507-1517.

Hecht, B. C., M. E. Valle, F. P. Thrower, and K. M. Nichols, 2014, Divergence in Expression of Candidate Genes for the Smoltification Process Between Juvenile Resident Rainbow and Anadromous Steelhead Trout: *Marine Biotechnology*, v. 16, p. 638-656.

Hootman, S., and C. Philpott, 1980, Accessory cells in teleost branchial epithelium, *American Journal of Physiology*, p. 199-206.

Hwang, P.-P., and T.-H. Lee, 2007, New insights into fish ion regulation and mitochondrion-rich cells: *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, v. 148, p. 479-497.

Johnston, C. E., and J. C. Cheverie, 1985, Comparative analysis of ionoregulation in rainbow trout (*Salmo gairdneri*) of different sizes following rapid and slow salinity adaptation.: *Canadian Journal of Fisheries and Aquatic Sciences*, v. 42, p. 1994-2003.

Jorgensen, P. L., 2008, Importance for absorption of Na(+) stop from freshwater of lysine, valine and serine substitutions in the alpha 1a-isoform of Na,K-ATPase in the gills of rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*): *Journal of Membrane Biology*, v. 223, p. 37-47.

Kiuchi-Saishin, Y., S. Gotoh, and M. Furuse, 2002, Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments, *Journal of the American Society of Nephrology*.

Kolosov, D., H. Chasiotis, and S. P. Kelly, 2014, Tight junction protein gene expression patterns and changes in transcript abundance during development of model fish gill epithelia: *Journal of Experimental Biology*, v. 217, p. 1667-1681.

Koval, M., 2013a, Claudin Heterogeneity and Control of Lung Tight Junctions, in D. Julius, ed., *Annual Review of Physiology, Vol 75: Annual Review of Physiology*, v. 75: Palo Alto, Annual Reviews, p. 551-567.

Koval, M., 2013b, Differential pathways of claudin oligomerization and integration into tight junctions: *Tissue barriers*, v. 1, p. e24518-e24518.

Kwong, R. W. M., Y. Kumai, and S. F. Perry, 2013, Evidence for a role of tight junctions in regulating sodium permeability in zebrafish (*Danio rerio*) acclimated to ion-poor water: *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, v. 183, p. 203-213.

Kwong, R. W. M., and S. F. Perry, 2013, The tight junction protein claudin-b regulates epithelial permeability and sodium handling in larval zebrafish, *Danio rerio*: *American journal of physiology. Regulatory, integrative and comparative physiology*, v. 304, p. R504-13.

Le Bras, Y., N. Dechamp, F. Krieg, O. Filangi, R. Guyomard, M. Boussaha, H. Bovenhuis, T. G. Pottinger, P. Prunet, P. Le Roy, and E. Quillet, 2011, Detection of QTL with effects on osmoregulation capacities in the rainbow trout (*Oncorhynchus mykiss*): *Bmc Genetics*, v. 12:46, p. 14.

Loh, Y. H., A. Christoffels, S. Brenner, W. Hunziker, and B. Venkatesh, 2004, Extensive expansion of the claudin gene family in the teleost fish, *Fugu rubripes*: *Genome Research*, v. 14, p. 1248-1257.

McCormick, S. D., 2001, Endocrine control of osmoregulation in teleost fish: *American Zoologist*, v. 41, p. 781-794.

McCormick, S. D., and R. J. Naiman, 1984, Osmoregulation in the brook trout, *Salvelinus fontinalis*. Effects of size, age and photoperiod on seawater survival and ionic regulation.: *Comparative Biochemistry and Physiology a-Physiology*, v. 79, p. 17-28.

McLaughlin, J., P. Padfield, and J. Burt, 2004, Ochratoxin A increases permeability through tight junctions by removal of specific claudin isoforms, *American Journal of Physiology - Cell Physiology*, v. 287:5, p. C1412-C1417.

Milatz, S., S. M. Krug, R. Rosenthal, D. Guenzel, D. Mueller, J.-D. Schulzke, S. Amasheh, and M. Fromm, 2010, Claudin-3 acts as a sealing component of the tight junction for ions of either charge and uncharged solutes, *Biochimica et Biophysica Acta - Biomembranes*, p. 2048-2057.

Mitic, L. L., V. M. Unger, and J. M. Anderson, 2003, Expression, solubilization, and biochemical characterization of the tight junction transmembrane protein claudin-4: *Protein Science*, v. 12, p. 218-227.

Nichols, K. M., A. F. Edo, P. A. Wheeler, and G. H. Thorgaard, 2008, The genetic basis of smoltification-related traits in *Oncorhynchus mykiss*: *Genetics*, v. 179, p. 1559-1575.

Norman, J. D., R. G. Danzmann, B. Glebe, and M. M. Ferguson, 2011, The genetic basis of salinity tolerance traits in Arctic charr (*Salvelinus alpinus*): *Bmc Genetics*, v. 12.

Norman, J. D., M. Robinson, B. Glebe, M. M. Ferguson, and R. G. Danzmann, 2012, Genomic arrangement of salinity tolerance QTLs in salmonids: A comparative analysis of Atlantic salmon (*Salmo salar*) with Arctic charr (*Salvelinus alpinus*) and rainbow trout (*Oncorhynchus mykiss*): *Bmc Genomics*, v. 13.

Payan, P., J. Girard, and N. Mayergostan, 1984, Branchial ion movements in teleosts - the roles of respiratory and chloride cells, *Fish Physiology*, Academic Press Inc., p. 39-63.

Rajasekaran, S. A., and A. K. Rajasekaran, 2009, Na,K-ATPase and epithelial tight junctions: *Frontiers in Bioscience*, v. 14, p. 2130-2148.

Richards, J. G., J. W. Semple, J. S. Bystriansky, and P. M. Schulte, 2003, Na⁺/K⁺-ATPase (alpha-isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer: *Journal of Experimental Biology*, v. 206, p. 4475-4486.

Sardet, C., M. Pisam, and J. Maetz, 1979, Surface epithelium of teleostean fish gills - cellular and junctional adaptations of chloride cell in relation to salt adaptation: *Journal of Cell Biology*, v. 80, p. 96-117.

Scott, M. A., R. S. Dhillon, P. M. Schulte, and J. G. Richards, 2015, Physiology and performance of wild and domestic strains of diploid and triploid rainbow trout (*Oncorhynchus mykiss*) in response to environmental challenges: *Canadian Journal of Fisheries and Aquatic Sciences*, v. 72, p. 125-134.

Tipsmark, C. K., P. Küllerich, T. O. Nilsen, L. O. E. Ebbesson, S. O. Stefansson, and S. S. Madsen, 2008, Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification: *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*, v. 294, p. R1563-R1574.

Van Itallie, C., C. Rahner, and J. Anderson, 2001, Regulated expression of claudin-4 decreases paracellular conductance through a selective decrease in sodium permeability, *Journal of Clinical Investigation*, p. 1319-1327.

Van Itallie, C. M., A. S. Fanning, and J. M. Anderson, 2003, Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins: *American Journal of Physiology-Renal Physiology*, v. 285, p. F1078-F1084.

Van Itallie, C. M., S. Rogan, A. Yu, L. S. Vidal, J. Holmes, and J. M. Anderson, 2006, Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities: *American Journal of Physiology-Renal Physiology*, v. 291, p. F1288-F1299.

Van Itallie, C. M., A. J. Tietgens, K. LoGrande, A. Aponte, M. Gucek, and J. M. Anderson, 2012, Phosphorylation of claudin-2 on serine 208 promotes membrane retention and reduces trafficking to lysosomes: *Journal of Cell Science*, v. 125, p. 4902-4912.

White, S. L., D. Sakhrani, R. G. Danzmann, and R. H. Devlin, 2013, Influence of developmental stage and genotype on liver mRNA levels among wild, domesticated, and hybrid rainbow trout (*Oncorhynchus mykiss*): *BMC Genomics*, v. 14, p. 1-16.

Yu, A. S. L., M. H. Cheng, S. Angelow, D. Günzel, S. A. Kanzawa, E. E. Schneeberger, M. Fromm, and R. D. Coalson, 2009, Molecular basis for cation selectivity in claudin-2 – based paracellular pores: identification of an electrostatic interaction site, *Journal of General Physiology*, p. 111-127.

Zydlewski, J., and M. P. Wilkie, 2013, Freshwater to seawater transitions in migratory fishes: *Fish Physiology*, v. 32, p. 253-326.

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.

Literature cited

- Akazawa, Y., T. Yuki, H. Yoshida, Y. Sugiyama, and S. Inoue, 2013, Activation of TRPV4 Strengthens the Tight-Junction Barrier in Human Epidermal Keratinocytes: *Skin Pharmacology and Physiology*, v. 26, p. 15-21.
- Gonzalez-Mariscal, L., A. Betanzos, P. Nava, and B. E. Jaramillo, 2003, Tight junction proteins, *Progress in Biophysics & Molecular Biology*, v. 81:1, p. 1-44.
- Gunzel, D., and A. S. L. Yu, 2013, Claudins and the modulation of tight junction permeability: *Physiological reviews*, v. 93, p. 525-69.
- Heiskala, M., P. A. Peterson, and Y. Yang, 2001, The roles of claudin superfamily proteins in paracellular transport: *Traffic*, v. 2, p. 92-98.
- Jouret, F., J. Wu, M. Hull, V. Rajendran, B. Mayr, C. Schofl, J. Geibel, and M. J. Caplan, 2013, Activation of the Ca²⁺-sensing receptor induces deposition of tight junction components to the epithelial cell plasma membrane: *Journal of Cell Science*, v. 126, p. 5132-5142.
- Loretz, C. A., 2008, Extracellular calcium-sensing receptors in fishes: *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology*, v. 149, p. 225-245.
- Nearing, J., M. Betka, S. Quinn, H. Hentschel, M. Elger, M. Baum, M. Bai, N. Chattopadhyay, E. M. Brown, S. C. Hebert, and H. W. Harris, 2002, Polyvalent cation receptor proteins (CaRs) are salinity sensors in fish: *Proceedings of the National Academy of Sciences of the United States of America*, v. 99, p. 9231-9236.
- Reiter, B., R. Kraft, D. Guenzel, S. Zeissig, J.-D. Schulzke, M. Fromm, and C. Harteneck, 2006, TRPV4-mediated regulation of epithelial permeability: *Faseb Journal*, v. 20, p. 1802-1812.
- Shen, L., C. R. Weber, D. R. Raleigh, D. Yu, and J. R. Tumer, 2011, Tight, Junction Pore and Leak Pathways: A Dynamic Duo: *Annual Review of Physiology*, v. 73, p. 283-309.
- Van Itallie, C. M., S. Rogan, A. Yu, L. S. Vidal, J. Holmes, and J. M. Anderson, 2006, Two splice variants of claudin-10 in the kidney create paracellular pores with different ion selectivities: *American Journal of Physiology-Renal Physiology*, v. 291, p. F1288-F1299.

Van Itallie, C. M., A. J. Tietgens, K. LoGrande, A. Aponte, M. Gucek, and J. M. Anderson, 2012, Phosphorylation of claudin-2 on serine 208 promotes membrane retention and reduces trafficking to lysosomes: *Journal of Cell Science*, v. 125, p. 4902-4912.

Yue, Z., J. Xie, A. S. Yu, J. Stock, J. Du, and L. Yue, 2015, Role of TRP channels in the cardiovascular system: *American Journal of Physiology-Heart and Circulatory Physiology*, v. 308, p. H157-H182.

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)
<i>claudin-10c</i> [Ssa]	AGTGGATGGATCCTGGTCTG	TCCTTCCAGAGGTTGGAGAA	BK006389
<i>claudin-10d</i> [Ssa]	ACTGGACCTGGTCTGAGGTG	TGGAGGGGTACTCTTTGCAG	BK006390
<i>claudin-10e</i> [Ssa]	ATCAAGGTGGCCTGGTACTG	GACCAGAGCACAGGGAAGTC	BK006391
<i>claudin-30</i> [Ssa]	TGATCATTGGAGGAGGGTTC	AACATAGTCCCTGGGTGCTG	BK006405
<i>Efla</i> [Omy]	AGAACCATTGAGAAGTTCGAGA AG	GCACCCAGGCATACTTGAAA G	AF498320
<i>β-actin</i> [Omy]	TCCTCGGTATGGAGTCTTGC	AGCACTGTGTTGGCGTACAG	AJ438158
<i>rplp0</i> [Ssa]	GTTCTGGAGGGTGTTCGTA	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

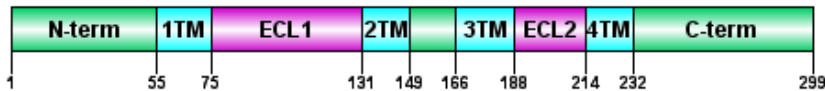
GenBank: DAA06154

>Ssa_cldn10c

```
MGQQTPIFFHQCGARAGNSSTAIFPPSCLVSLHTEAAMNYRTVVMYMEIGCFVVCVSGWIL
VCSTMPTEIWTWSEVESIVLTSSNYFSNLWKDCVSDSTGVSDCKGIPSMFGLNWDIHMCRAL
IISIILGFFGAILVLVGMKCTKIGGSEVANARVTFAGGMNYLVSGLCSMIAFSYYGNKIRAEF
QDPNFRAQKFEIGVAVYIGWGGSTLLVIGGLIYSVFAGKEACQSSSKNKHMPVYKLPDADVA
PPAKPTYRPVSTALTEGGESRESKTSRVSSETRRSGSSSKTLSSLNAYV
```

299 aa

Claudin-10c



2. Claudin-10d

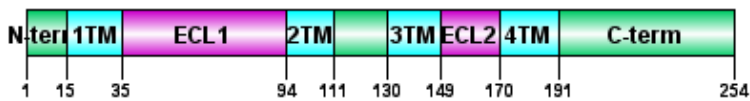
GenBank: DAA06155.1

> Ssa_cldn10d

```
MKHRTVMMYMEIGCFVSCLAGWILVSSTLAIEYWTWSEVGSVVLTTGNYFSNLWKDCVSD  
STGVSDCKEYPSMLGLPVFLHSVRALSICSVILGFFAGVLTLLIGMKCTKIGGSELANARVTFA  
GGITYLASGFAGLIVYSWWGNKVRSEFVDPNFKAQKFEIGAAVFIGWGGSELLITGGFVLSFF  
SGKEGLRSTSKKRPRRPNYSYATARTRRTYMMPNSSRVTPMPQLVQGSRGGRKIRKTRTTGTY  
SRDDFV
```

254 aa

Claudin-10d



3. Claudin-10e

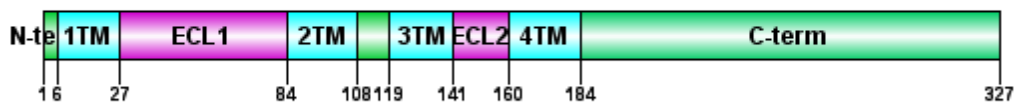
GenBank: DAA06156

> Ssa_cldn10e

```
MKIRVMQIWGFLMTVLGWIFVACTMAMEGWKVTSIGGMGGSAVIKVAWYWSNLWKACF  
TDSTSVTNCQDFPVLWSVDNHIQIVRGLLMGALSVGMLGFVLSLIGMECTFLGGKDKAKHR  
KLFTGGVCHIISGFLAASGYAVYAKYVSGEYFNPYFDGLKFDLGTPLFLGWVGSFAHMTGG  
WFYLVSVCKLLCGDKSKTIVPELPEVERDQAKSTTAQYPVSPITSKIMVSSASKISSKA  
AHSVSAISSKSGQSGRASKSERSGRSSKSVQSSKSAGGSFTSGRSSRSRSHGSDSEVS  
SGSSSTVSSLSSGSRRRERKPFIKNSYI
```

327aa

Claudin-10e



4. Claudin-30

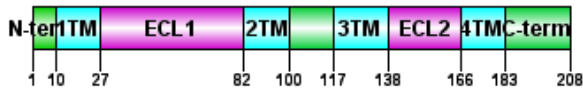
GenBank: NP_001233202.1

>Omy_cldn30

```
MASAGFQMLGTALGIIGWIGAIVVCALPQWKVTAFIGENIITAQTTWQGIWMNCVVQSTGQ  
MQCKVYDSMLALPQDLQAARALIIISIMMGLVGILLSVAGGKCTNCVEDERAKSRIGVGS  
GVVFIAGILCLIPVCWSANTIIRDFYNPMLMSSQKMELGAALYIGWGAAALMIMGGGFLC  
ANCPKEDNYPTKYSAARSTAPKDYV
```

208 aa

Claudin-30



Literature cited

Hofmann K., Stoffel W., 1993. TMbase - A database of membrane spanning proteins segments *Biol. Chem. Hoppe-Seyler* 374, 166.

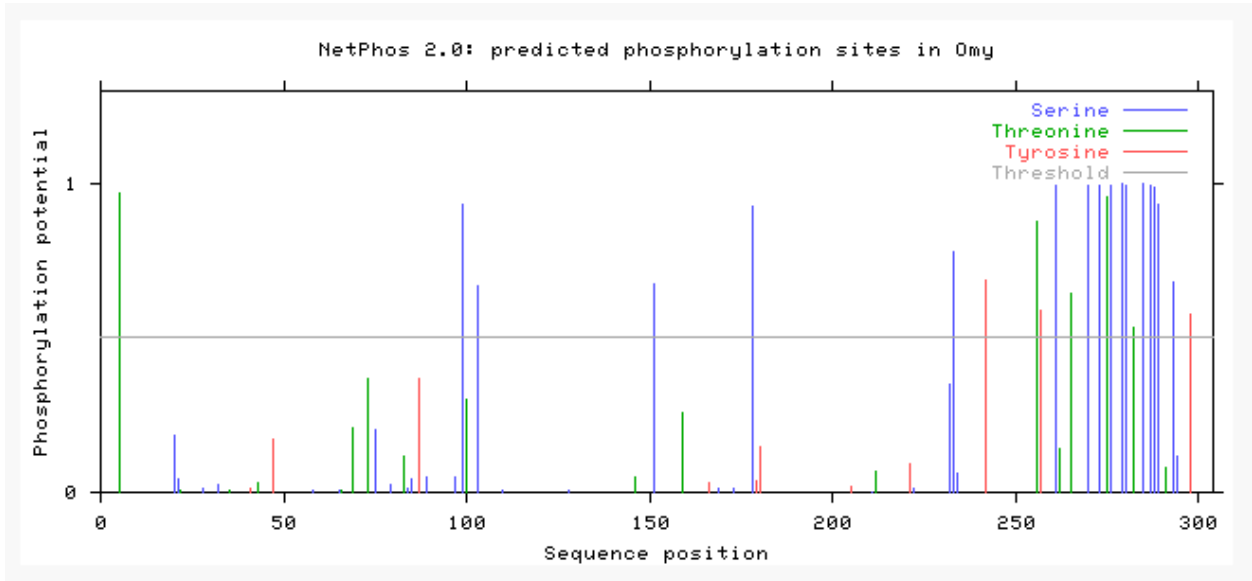
Jian Ren, Longping Wen, Xinjiao Gao, Changjiang Jin, Yu Xue and Xuebiao Yao, 2009. DOG 1.0: Illustrator of Protein Domain Structures, *Cell Research* 19:271–273.

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.

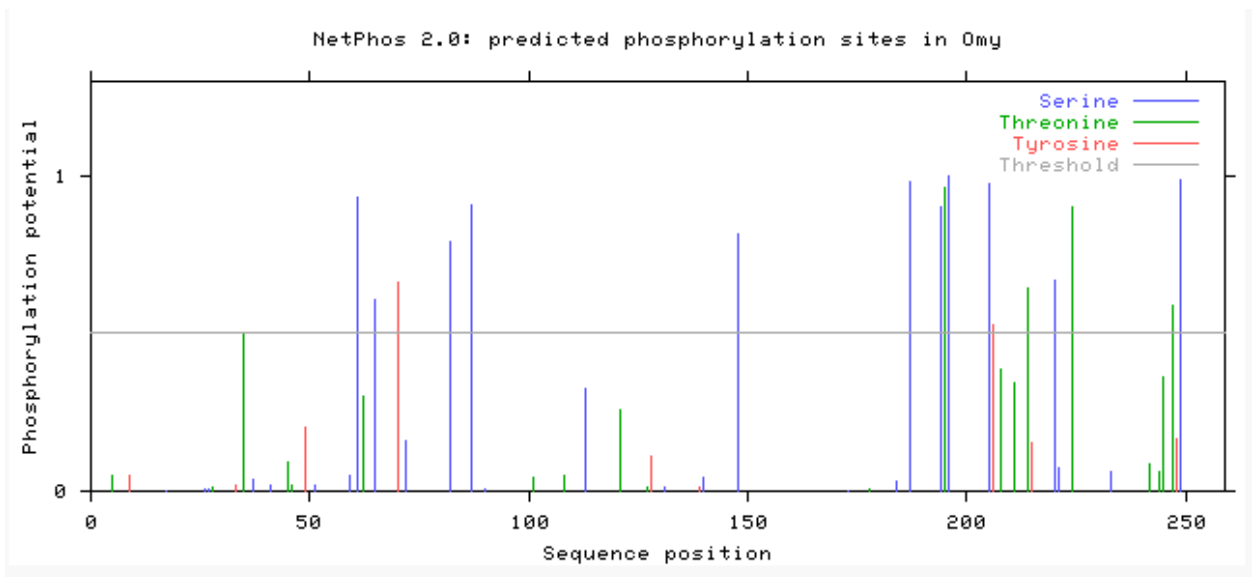
Appendix 3:

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

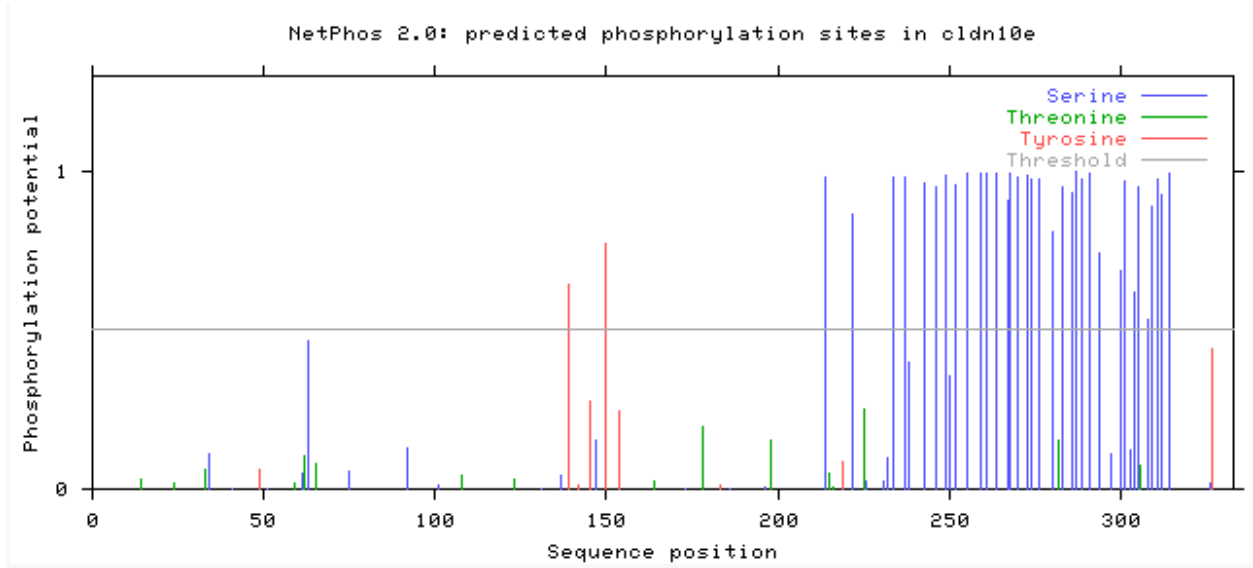
1. Claudin-10c



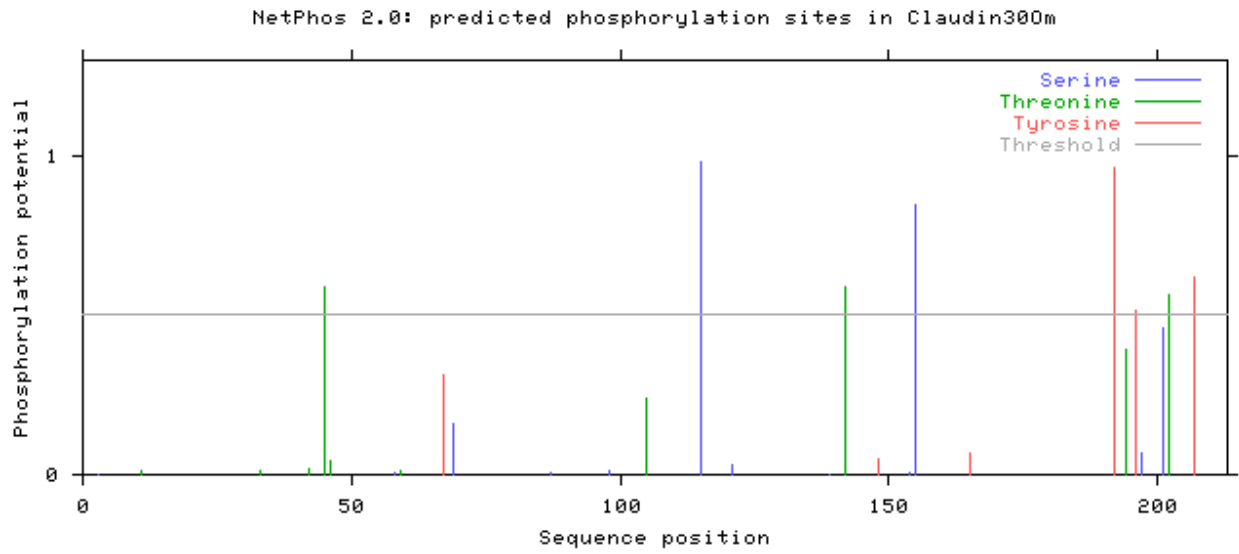
2. Claudin-10d



3. Claudin-10e



4. Claudin-30



Appendix 4:

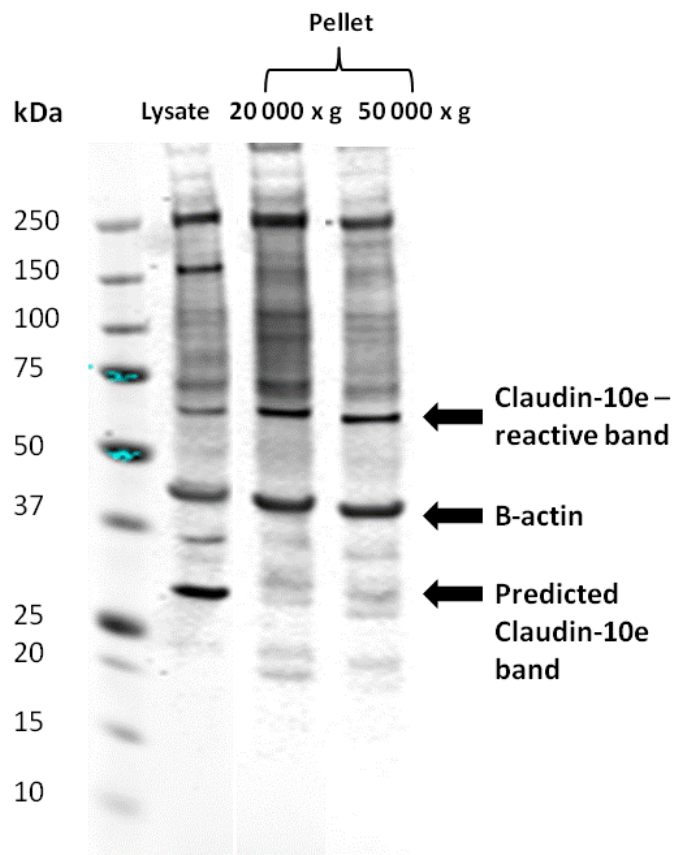


Figure A4.1 Differential centrifugation test for Claudin-10e. Lysate and pellet, obtained by lysate centrifugation at 20000 x g for 90 minutes and 50000 x g for 60 minutes, were used for detection and comparison of enrichment in Claudin-10e on western blot.

Appendix 5:

IACUC approval for research.

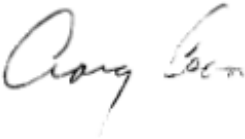


UNIVERSITY OF
ARKANSAS

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

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572

Fax: 479-575-3846 • <http://vpred.uark.edu/199>

The University of Arkansas is an equal opportunity/affirmative action institution.

Appendix 6:

Copyright permissions obtained for:

Figure 1.

Copyright: © Crête-Lafrenière et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Figure 2.

License Number	3612800688915
License date	Apr 19, 2015
Licensed content publisher	Elsevier
Licensed content publication	Elsevier Books
Licensed content title	Encyclopedia of Fish Physiology
Licensed content author	S.J. Cooke,G.T. Crossin,S.G. Hinch
Licensed content date	2011
Number of pages	8
Type of Use	reuse in a thesis/dissertation
Portion	figures/tables/illustrations
Number of figures/tables/illustrations	1
Format	both print and electronic
Are you the author of this Elsevier chapter?	No
Will you be translating?	No
Original figure numbers	Figure 1
Title of your thesis/dissertation	Functional significance of gill proteins in rainbow trout (<i>Oncorhynchus mykiss</i>) osmoregulation
Expected completion date	May 2015
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.00 USD / 0.00 GBP
Total	0.00 USD

Figure 3.

[Copy order >](#)

Confirmation Number: 11344906
Order Date: 04/22/2015

 [Print this page](#)
[Print terms & conditions](#)
[Print citation information \(What's this?\)](#)

Customer Information

Customer: Joanna Bujak
Account Number: 3000907385
Organization: Joanna Bujak
Email: jkbujak@uark.edu
Phone: +1 (479)4225626

Search order details by:

This is not an invoice

Order Details

Fish Physiology: Euryhaline Fishes : Fish Physiology Vol 32

Order detail ID: 66801086
ISBN: 978-0-12-396951-4
Publication Type: Book
Publisher: Academic Press

Permission Status:  **Granted**
Permission type: Republish or display content
Type of use: Thesis/Dissertation
Order License Id: 3614250192126

View details

Note: This item was invoiced separately through our **RightsLink service**. [More info](#) **\$ 0.00**

Figure 4.



The screenshot shows the RightsLink interface for a document. At the top left is the Copyright Clearance Center logo. The main header features the RightsLink logo in orange. Navigation buttons for Home, Create Account, and Help are visible, along with a Live Chat icon. On the left is the American Physiological Society logo. The document details are as follows:

- Title:** Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys
- Author:** David H. Evans
- Publication:** Am J Physiol-Regulatory, Integrative and Comparative Physiology
- Publisher:** The American Physiological Society
- Date:** Aug 1, 2008


Copyright © 2008, The American Physiological Society

A LOGIN button is present, with a text box stating: "If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already a RightsLink user or want to [learn more?](#)"

Permission Not Required

Permission is not required for this type of use.

Figure 5.



The screenshot shows the RightsLink interface for a document. At the top left is the Copyright Clearance Center logo. The main header features the RightsLink logo in orange. Navigation buttons for Home, Create Account, and Help are visible, along with a Live Chat icon. On the left is the American Physiological Society logo. The document details are as follows:

- Title:** The Multifunctional Fish Gill: Dominant Site of Gas Exchange, Osmoregulation, Acid-Base Regulation, and Excretion of Nitrogenous Waste
- Author:** David H. Evans ,Peter M. Piermarini ,Keith P. Choe
- Publication:** Physiological Reviews
- Publisher:** The American Physiological Society
- Date:** Jan 1, 2005

Copyright © 2005, The American Physiological Society

A LOGIN button is present, with a text box stating: "If you're a copyright.com user, you can login to RightsLink using your copyright.com credentials. Already a RightsLink user or want to [learn more?](#)"

Permission Not Required

Permission is not required for this type of use.

Figure 6.

License Number	3610810340074
License date	Apr 16, 2015
Licensed content publisher	Oxford University Press
Licensed content publication	Integrative and Comparative Biology
Licensed content title	Endocrine Control of Osmoregulation in Teleost Fish:
Licensed content author	Stephen D. McCormick
Licensed content date	08/01/2001
Volume number	41
Issue number	4
Type of Use	Thesis/Dissertation
Requestor type	Academic/Educational institute
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	1
Will you be translating?	No
Author of this OUP article	No
Order reference number	None
Title of your thesis / dissertation	Functional significance of gill proteins in rainbow trout (<i>Oncorhynchus mykiss</i>) osmoregulation
Expected completion date	May 2015
Estimated size (pages)	100
Publisher VAT ID	GB 125 5067 30
Total	0.00 USD

Figure 7.

This Agreement between Joanna K Bujak ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms of the license. For more information, please contact John Wiley and Sons and Copyright Clearance Center.

[Get the printable license.](#)

License Number	3604840124184
License date	Apr 09, 2015
Licensed Content Publisher	John Wiley and Sons
Licensed Content Publication	Clinical Genetics
Licensed Content Title	Claudins: unlocking the code to tight junction function during embryogenesis and in disease
Licensed Content Author	IR Gupta,AK Ryan
Licensed Content Date	Feb 17, 2010
Licensed Content Pages	12
Type of use	Dissertation/Thesis
Requestor type	University/Academic
Format	Print and electronic
Portion	Figure/table
Number of figures/tables	2
Original Wiley figure/table number(s)	Figure 2 and 3
Will you be translating?	No
Title of your thesis / dissertation	Functional significance of gill proteins in rainbow trout (<i>Oncorhynchus mykiss</i>) osmoregulation
Expected completion date	May 2015
Expected size (number of pages)	100
Requestor Location	Joanna K Bujak 1754 N Chestnut Ave None None FAYETTEVILLE, AR 72703 United States Attn: Joanna K Bujak
Billing Type	Invoice
Billing address	Joanna K Bujak 1754 N Chestnut Ave None None FAYETTEVILLE, AR 72703 United States Attn: Joanna K Bujak
Total	0.00 USD