Branchial Ionoregulatory Mechanisms of Sodium Regulation in Freshwater Salmonids with Conservational Implications for Arctic Grayling (*Thymallus arcticus*)

by

Salvatore Donald Blair

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in

Physiology, Cell and Developmental Biology

Department of Biological Sciences
University of Alberta

Abstract

This thesis on fish osmoregulation focuses particularly on the mechanisms utilized by freshwater salmonids to absorb Na^+ ions from dilute hypotonic environments and at the same time, their capacity to make necessary changes in order to tolerate higher saline environments.

In this thesis, I present data that indicate rainbow trout embryos/larvae reared in low Na^+ soft water maintain homeostasis by way of an EIPA-sensitive Na^+ uptake pathway. In addition rainbow trout showed significantly increased *nhe3b* expression at the embryo/larvae and juvenile life stages and overall, these data support a primary role for *nhe3b* at these life stages. Three nhe isoforms: *nhe2*, *nhe3a*, and *nhe3b* were cloned, from trout gill (*2* and *3b*) and kidney (*3a*), into pDisplay expression vectors for transfection into the AP – 1 cell line in an attempt to further characterize the pharmacological properties of these transporters. However, at this time stable transfections have not resulted in successful Nhe protein expression.

From a conservation physiology perspective I have shown that the Arctic grayling (a threatened native salmonid) demonstrates a reduced salinity tolerance and develops a novel associated interlamellar cell mass, in response to hypersaline waters, which has strong implications for hypersaline spills from hydraulic fracturing and other oil and gas operations. I presented data indicating this interlamellar cell mass and osmotic stress can be reversed if the salinity exposure lasts 48 hrs or less and recovery in freshwater is achievable. In

addition to salinity tolerance data and in order to have a comprehensive management plan for Arctic grayling, thermal tolerance data were collected indicating CT_{max} of 26.9°C and 27.8°C when acclimated to 13°C and 17°C water temperatures (see Appendix A).

This work provides evidence of three *nhe* isoform expression patterns during development in rainbow trout despite thermodynamic constraints, demonstrates reduced salinity tolerance of a threatened native salmonid in Alberta, provides the first reported instance of a salinity-induced ILCM in salmonids, and provides necessary physiological data for conservation management strategies for Arctic grayling.

Preface

This thesis is an original work by Salvatore D. Blair. This research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, and all experiments were conducted under Animal Use Protocol #00000072 as approved by the University of Alberta and the Biosciences Animal Policy and Welfare Committee under the directions provided by the Canadian Council for Animal Care in Canada. As a Ph.D. student, this work was conducted in majority under the supervision and counsel of Dr. Greg Goss, professor at the University of Alberta. Some of this work was performed as a collaborative effort with other researchers in our lab and thus the terms "we" or "our" are used throughout this thesis. Two data chapters of this thesis have been previously published in the following peer-reviewed research journals: Comparative Biochemistry and Physiology (Chapter II) and Conservation Physiology (Chapter IV). Chapter V is currently being formatted for submission to Conservation Physiology as a follow-up to the previously published work. The roles of all authors for each data chapter along with specific experimental contributions of certain co-authors are briefly described below:

Chapter II: Characterization of developmental Na^+ uptake in rainbow trout embryos supports a significant role for Nhe3b.

Published article: Boyle, D., Blair, S. D., Chamot, D., & Goss, G. G. (2016). Characterization of developmental Na^+ uptake in rainbow trout larvae supports a significant role for Nhe3b. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *201*, 30-36.

Dr. David Boyle and I share co-1st authorship for this article submission in CBP-A, as we contributed equally to this manuscript. SDB was primary researcher on this project. Experiments were designed and carried out by SDB, DB, and GG; SDB and DB performed data analysis and manuscript prep and revisions. DB was instrumental in statistical analysis and DC contributed to molecular aspects.

Chapter III: Expression and Pharmacological Profiling of Rainbow Trout nhe2, nhe3a, and nhe3b.

Salvatore Blair and Dr. Danuta Chamot carried out cloning, while SDB performed all cell culture work, expression analysis, activity assays, data analysis, and writing.

Chapter IV: Reduced salinity tolerance in the Arctic grayling (*Thymallus arcticus*) is associated with rapid development of a gill interlamellar cell mass: implications of high saline spills on native freshwater salmonids.

Published article: Blair, S. D., Matheson, D., He, Y., & Goss, G. G. (2016). Reduced salinity tolerance in the Arctic grayling (*Thymallus arcticus*) is associated with rapid development of a gill interlamellar cell mass: implications of high-saline spills on native freshwater salmonids. *Conservation Physiology*, *4*(1), cow010.

SDB and GG conceived and designed experiment. Experiment was conducted by SDB. DM assisted in taking some select microscopy images and tissue sampling, and YH assisted in qPCR analysis. SB analyzed data and prepared the manuscript with GG revisions.

Chapter V: Physiological and Morphological Investigation on Arctic grayling (*Thymallus arcticus*) Gill Filaments and Interlamellar Cell Mass with High Salinity Exposure.

SDB and GG conceived and designed experiment. Experiment was carried out by SDB. Data analysis and writing was performed by SDB.

Acknowledgements

My Ph.D. research began when I moved from North Carolina to Edmonton in August 2011 and since then my last five years have been challenging, rewarding, and priceless. Throughout this journey there have been a great number of influential, enlightening, and overall extremely supportive people whom I truly cannot thank enough. I would like to begin with thanking my research supervisor Dr. Greg Goss, known for his extensive research in fish physiology, who was the sole reason I chose Edmonton over anywhere else. I am extremely grateful for all of the opportunities he has provided, for the financial support, as well as the constant mentorship by Greg during my time here at the University of Alberta.

Although your Ph.D. thesis is an individual's own original work by definition, research is not and cannot be an independent venture and there are a number of people who have assisted me along the way. My co-authors Dr. Danuta Chamot, Dr. David Boyle, Derrick Matheson, and Dr. Yuhe He have directly contributed to the success of some of this work. These individuals offered priceless research knowledge and assistance in molecular, microscopy, and radioisotope techniques throughout my research process. Dr. Aaron Schultz, Van Ortega, Dr. Aga Dymowska, also played a vital role in my Ph.D. training which has lead to the success of this thesis. My mentors here at the U of A are extensive, but especially Dr. John Chang and Dr. Declan Ali who have always had an open door for chats, for the use of their labs, spontaneous off campus lunches, and for providing an endless supply of research knowledge. I would like to

thank past and present members of the Goss lab who have become my collaborators and friends that provided overall support during this Ph.D. Special thanks to close friends Van Ortega, Aaron Shultz, Dave Boyle, Erik Folkerts, and Josh Pemberton for fun times throughout my time here in Edmonton. I thank everyone above for all of their support both technical and social, they have contributed greatly to my success in these last five years.

There are also a lot of people who from behind the scenes were instrumental to the completion of this thesis with their molecular, microscopy, animal care, ordering, and student services. Special thanks to Troy Locke, Cheryl Nargang, Arlene Oatway, Clarence Gerla, Jesse Edgington, Ben McDonald, and Ches Mason for all of their assistance throughout my time here.

Finally, and arguably the most important people to thank and who need to be acknowledged in this thesis are my family. My beautiful wife Daneel Blair, who has witnessed all of the failures and successes associated with doing a Ph.D., has been an incredible support throughout this journey and I could not have done it without her. And to my parents who have always shown their endless love and support, have been a source of constant reassurance and encouragement, and whom I can always look to for guidance in any situation. I am also very grateful to my longtime mentor and dear friend Dr. Susan Edwards, who is the main reason I pursued a Ph.D. in the first place, following my undergraduate and M.Sc. work in her lab. Thank you to all my friends and family who have stood next to me throughout my Ph.D., without you this would not be possible.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Ab	antibody
AEP	Alberta Environment and Parks
AG	Arctic grayling
ANOVA	analysis of variance
AP-1	NHE deficient mammalian cell line
ASIC	acid-sensing ion channel
ATP	adenosine triphosphate
Au	gold
BCECF	2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein,-Acetoxymethyl Ester
bp	base pair
Ca^{2+}	calcium
CC	chloride cell
CTM	critical thermal maximum
cDNA	complementary DNA
Cl^-	chloride
DAPI	4', 6-diamidino-2-phenylinodole
DMA	dimethyl amiloride
DMSO	dimethyl sulfoxide
dpf	days post fertilization
dph	days post hatch
DNA	deoxyribonucleic acid

Ef1	elongation factor 1
EIPA	ethyl-iso-propyl-amiloride
ENaC	epithelial Na^+ channel
FW	freshwater
gDNA	genomic DNA
GDP	gross domestic product
H^+	hydrogen (proton)
H^+-ATPase	proton ATPase
HA	hemagglutin
H and E	Hematoxylin and Eosin
hr	hour
HCO^-	bicarbonate
HMDS	hesamethyldisilazane
IGF	insulin like growth factor
ILCM	interlamellar cell mass
ILT	insipient lethal temperature
K^+	potassium
LB	Lysogeny broth
Li^+	lithium
LOE	loss of equilibrium
M	molar
mM	millimolar
mOsm	milliosmols

mRNA	messenger RNA
MRC	mitochondrion-rich cell
MS 222	tricane methanesulfonate
Mya	million years ago
Na^+	sodium
Na^{22}	radioactive sodium isotope
NH_3	ammonia
NH_4^+	ammonium
Nhe	sodium hydrogen exchanger
Nka	sodium potassium ATPase
PAS	Periodic acid-Schiff
PBS	phosphate buffered saline
PC	pillar cell
PCR	polymerase chain reaction
Pd	palladium
pH_i	intracellular pH
PNA	peanut lectin agglutinin
ppt	parts per thousand
PVC	pavement cell
qPCR	quantitative PCR
R	recovery
Rh	Rhesus
rhqPCR	RNase H-Dependent qPCR

RNA	ribonucleic acid
RT	rainbow trout
RT-PCR	reverse transcription PCR
SD	standard deviation
S.E.M.	standard error of the mean
SEM	scanning electron microscopy
SITS	4-acetamido-4-isothiocyanostilbene-2,2-disulfonate
SLC	solute carrier
SW	seawater
TBS	Tris buffered saline
TEM	transmission electron microscopy
µM	micromolar
UV	ultraviolet

CHAPTER I: General Introduction

Introduction

A fish's external environment dictates the physiological behaviour necessary to maintain internal fluid homeostatic balance. Fish in both freshwater and marine environments are constantly performing physiological processes allowing them to adequately maintain internal osmotic balance against the forces of diffusion and osmosis from varying salt and ion concentration gradients (Evans et al., 2005). The ability to *take up* salts from dilute freshwaters as well as *excrete* salt loads when exposed to higher external salinities is a key physiological process for anadromous fish like those of the salmonid family (Hiroi and McCormick 2012). These specific regulatory mechanisms allow fish to tolerate environments with a wide range of salt concentrations. In this thesis I studied the regulation of sodium (Na^+) ions as a proxy for ion regulation, and examined the cellular mechanisms involved in controlling the transport of Na^+ ions to better understand osmoregulation and salinity tolerance. The Salmonidae family is an ideal group to study osmoregulation due to their common anadromous life history with extant species demonstrating both euryhaline and stenohaline characteristics (Dalziel et al., 2014). This thesis follows a micro- to macro- compilation of physiological research. Initially, an investigation of ionoregulatory mechanisms of Na^+ regulation at the cellular level was conducted and these results were applied to complete this thesis with conservation implications at the whole animal level. In this thesis, the rainbow trout (*Oncorhynchus mykiss*) was chosen to gain a

mechanistic understanding of the ability to take up Na^+ from dilute freshwater environments. Rainbow trout were also used as a euryhaline comparison model species while investigating the salinity tolerance of the Arctic grayling (*Thymallus arcticus*); a strictly freshwater salmonid that we hypothesize is at a higher risk to hypersaline produced water spills originating from the oil and gas industry.

The Salmonids

Taxonomy

The family Salmonidae commonly referred to as "salmonids" represent a diverse and extensive branch of the fish lineage. The family encompasses not only the group of fish sharing its common name (the multiple species of salmon) but also includes three sub-families: Salmoninae, Thymallinae, and Coregoninae (Figure 1.1). Using common names, these families correspond to the "true" salmonids (salmon, trout, and charr), the grayling, and the whitefish, respectively. Members of the salmonids can be found on every continent other than Antarctica, although some likely come quite close during their time in the ocean (e.g. anadromous S. America species). From the tiny and beautiful golden trout (*Oncorhynchus mykiss aguabonita*) native to the upper Sierras, the mighty powerful taimen (*Hucho taimen*) of the untouched Mongolia rivers, the native southeastern brook trout (*Salvelinus fontinalis*) of the Appalachian creeks, the Arctic grayling (*Thymallus arcticus*) of northern tundra and boreal forests, the massive chinook salmon (*Oncorhynchus tshawytscha)* of the Pacific Ocean, the

historic brown trout (*Salmo trutta*) of Europe, the sea-run steelhead (*Oncorhynchus mykiss mykiss)* of the Pacific Northwest, the deep swimming lake whitefish (*Coregonus clupeiformis*) of the Great lakes, and the widely adaptive rainbow trout (*Onchorynchus mykiss*), the salmonids inhabit nearly every water body-type imaginable and have been very successful at doing so.

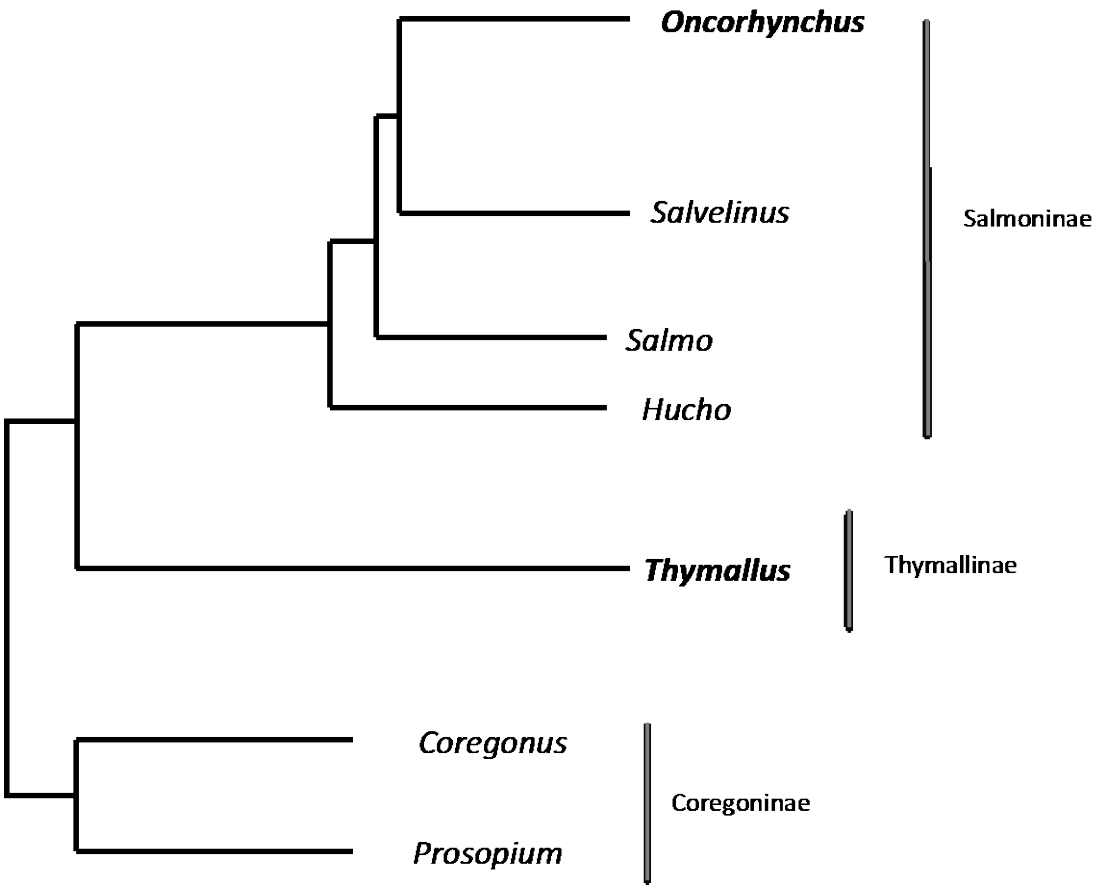


Figure 1.1. Phylogenetic relationship of the Family Salmonidae. (Adapted from Crête-Lafrenière et al 2012).

The taxonomic profile of the family Salmonidae shows a deep genetic diversity and demonstrates the ability of a species to evolve and adapt to its environment. There are currently 11 genera and 225 validated species, including 124 species in the subfamily Salmoninae, 86 Coregoninae, and 15 Thymallinae. The phylogeny of salmonids has been a topic of research for many years and recent morphological, molecular, and most recently, mitochondrial genome comparisons have demonstrated the three subfamily relationships. Crete-Lafreniere and colleagues (2012) performed a comprehensive study across 63 species of salmonids established relationships based on cytochrome B, as well as mitochondrial and nuclear genes, concluding that the Thymallinae (grayling) were the sister group to the rest of the family, diverging around 59 million years ago (mya). These data suggests that the Salmoninae (trout, salmon, charr) and the Coregoninae are more closely related to a common ancestor around 50 mya, with the Thymallinae the least related out of the three subfamilies (Crete-Lafreniere et al., 2012). A more recent study focusing on grayling phylogeny by Ma et al. (2016), utilizing complete mitochondrial genomes from four distinct species, as well as published sequences on NCBI, reconfigured the Salmonid family structure showing support for a Thymallinae-Coregoninae sister relationship stemming from a common ancestor ~46 mya. Furthermore, this study showed Salmoninae diverging separately from Thymallinae and Coregoninae, ~54 mya (Ma et al., 2016). The oldest known fossil of a salmonid, *Eosalmo driftwoodensis*, was described by Wilson (1977)

and was dated to the Eocene epoch (56-33mya). As alluded to previously, this relationship has been constantly altered and will likely continue to be refined throughout the years as more molecular data become available for all of the various salmonid species.

Behaviour

A behaviour representative of, but not exclusive to, the salmonids is the tendency towards anadromy. Anadromy, along with homing and semelparity, are the three attributes distinguishing the family (Quinn and Meyers, 2005). Homing or being able to migrate back to natal streams/rivers in order to reproduce has been strongly linked to olfaction (Dittman and Quinn, 1996) and although important to all salmonids, will not be a subject of this thesis. Likewise, semelparity, the occurrence of mortality after spawning, will also not be a focus. Anadromy, the act of migrating from saltwater to freshwater to spawn is of importance to many members of the Salmonidae, and this osmoregulatory ability will form a focus for this thesis. A large number of salmonid species (but not all) are anadromous, seemingly to be dictated by ancestral life history and genetics. Salmonid anadromy has been characterized over the years by a few unique features. Specific criteria for anadromy was provided by Rounsfell from the U.S. Fish and Wildlife Service (1958), which include: 1) extent of migrations in the sea, 2) duration of time spent in the sea, 3) maturation state while at sea, 4) spawning habits and habitats, 5) mortality post-spawn, and 6) the occurrence of freshwater strains. These criteria are further subdivided into other distinct

qualities which in turn are used to assess the degree of anadromy for a certain species or strain. The classic example of anadromy is usually represented by one of the Atlantic or Pacific salmon species and can be described as follows: 1) adult spawning occurs in freshwater streams or rivers, 2) deposited fertilized eggs then hatch and develop into larvae or alevin feeding from yolk sac, 3) larvae then become swim-up fry, resembling small fish, 4) fry become parr, characterized by dark blotches along lateral line, 5) parr undergo the process of smoltification, which allows for the switch to a seawater environment, 6) downstream swimming of smolts who migrate to the estuaries and eventually to the sea 7) growth and maturation occurs while at sea over the course of a few years varying depending on the species, 8) fertile adults then migrate from the oceans back up natal freshwater rivers to spawn, 9) death occurs in the adults following spawning. The key aspect to this anadromous behaviour and migratory behaviour is the ability to transition from freshwater to saltwater and back to freshwater during an individual's lifetime. In most cases this pattern is a one-time event, especially for the true salmon. However, some species of salmonids, such as Pacific steelhead, are iteroparous (able to spawn multiple times without death ensuing), while other salmonids remain in freshwater throughout their life cycle. Examples of this lifestyle include introduced Pacific salmon in the Great Lakes, who maintain their migratory behaviour by which the adults spawn in rivers and the juveniles then make their way downstream, where they enter into a freshwater lake instead of the marine environment of an ocean. Other examples would include landlocked salmonids, either by way of

physical barrier (i.e. a waterfall that would not allow passage upstream), or other freshwater dwelling salmonids such as members of the grayling or whitefish species, which do not have the ability to encounter marine environments. The behaviour of anadromy and the ability of salmonids to tolerate both freshwater and seawater environments is the basis on which this thesis is built and will be discussed in detail mechanistically later on in the physiology section.

Species of Interest

Rainbow trout, *Oncorhynchus mykiss*, (formerly *Salmo gairdneri*) are arguably the most widely distributed salmonid on the planet (resulting from hatchery stocking practices) and a well-known member of the subfamily Salmoninae. Its native range was confined mostly to the Pacific drainages on the northwest coast of North America, reaching south to Mexico and north to Alaska, with additional native populations found in Kamchatka and in the Peace and Athabasca river drainages in British Columbia and Alberta (Smith and Stearly, 1989; MacCrimmon 1971). There are various subspecies of the rainbow trout, ranging from the species native to Kamchatka (*O. mykiss mykiss*) to the Mexican golden trout (*O. mykiss. aguabonita*). Rainbow trout are found in naturally in cold clear rivers, feeding on a wide variety of aquatic insects, small fish, and occasional terrestrial species that fall into the water such as mice and frogs. These trout naturally spawn in the spring, although hatcheries are able to influence spawning times throughout the year by controlling light cycles and

water temperature. Rainbow trout, although genetically similar, come in two main life history forms, the landlocked or non-migratory form which inhabit inland rivers and lakes throughout its lifecycle, and the anadromous or migratory form which enters marine environments (or the Great Lakes) and is referred to as a "steelhead." Due to significant increases in hatcheries and stocking practices, and overall success of the species, the population range now extends to every continent other than Antarctica. Beginning in 1874, with the transport of eggs from the McCloud River (California), the stocking of rainbow trout has led to this fish being the most widely spread salmonid species (MacCrimmon 1971).

In the research community, the rainbow trout provides a great model species for many areas of study including toxicology and physiology. Early physiological studies on trout began around the turn of the 20th century. We find spawning and temperature preferences on many salmonid species including the rainbow trout from a study by Gurley (1902). August Krogh (the "father" of fish osmoregulation) worked on rainbow trout early on as well (referring to them by their earlier taxonomic names: *Salmo irideus* or *Salmo gardineri*) with investigations on fish respiration (Krogh and Lietch, 1919) as well as his iconic ionic and osmotic regulation study in fish (Krogh 1937). Search engine results for "rainbow trout" yield a long history of research encompassing 587,000 results. This is greater than any other searchable fish by common name, including Atlantic salmon, goldfish, or even zebrafish, which results in a close second at 534,000 results. The popularity of the rainbow trout is due to many

factors including ease of transport, rearing and maintenance, ability to tolerate salinity, and its environmental relevance given its wide population range and comparatively sensitive nature to toxicants and adverse environmental stimulus. There is now a large amount of genetic information available for the rainbow trout, including the full genome sequencing (Berthelot et al 2014) and over 684,954 DNA and RNA sequences available in the National Center for Biotechnology Information (NCBI 2016). This high availability of genetic and molecular information makes the rainbow trout a prime candidate for molecular, cellular, and physiological studies as we can now observe the molecular effects of environmental stressors that the fish encounters. Due to their anadromous lifestyle (and anadromous ancestry for the landlocked varieties), rainbow trout make an excellent model species to observe and compare ion and osmoregulation capabilities and is a species of interest for this thesis.

Arctic grayling, *Thymallus arcticus*, is a representative of the salmonid subfamily Thymallinae. Their appearance is identified by their unique sail-like dorsal fin, and like many salmonids display distinct colour patterns on their skin. As their name suggests, Arctic grayling are found throughout the freshwater regions of the Arctic drainages, including watersheds across the northern United States and Canada from Alaska to Hudson Bay and the northern reaches of Russia. The southern limit of their range has historically included some remnant populations in Michigan (now functionally extirpated) as well populations in the upper Missouri River watershed in Montana (recovering). Mitochondrial DNA evidence suggests the North American Arctic grayling is the most derived lineage

of Eurasian decent, first crossing the Bering land bridge during the Pliocene Epoch, 3 – 5 mya (Stamford and Taylor, 2004). The ancestral crossing of the land bridge from Asia, survival of glaciation periods in glacial refugia, and current strict freshwater existence, points to the likelihood that Arctic grayling in North America have not experienced a marine environment for several million years. In Alberta where this species is currently being studied, grayling require cool and low to sediment-free freshwater streams and rivers while also inhabiting some lakes. Grayling feed on mostly aquatic and terrestrial insects, occasionally eating minnows or crustaceans (Walker 2005). Being strictly freshwater and non-anadromous, the grayling do not undergo a smoltification stage. However, they are considered a migratory species and have been shown to travel large distances (Stamford and Taylor, 2004; West et al., 1992). Population data on Arctic grayling in Alberta show drastic declines from historical levels (AEP 2015). As of 2015, the current status of Alberta Arctic grayling is listed as a species of special concern and a zero possession limit has been implemented province wide in order to limit fishing pressure (AEP 2015). Although this governmental regulation is now in place, the exact reason for this decline is unknown and is likely the result of a multifaceted network of factors including but not limited to overharvest, habitat fragmentation, climate change, as well as a variety of other environmental perturbations including watershed effects brought on by the oil and gas and forestry industries (Walker 2005).

Compared to other salmonids, and especially the rainbow trout, the amount of research on Arctic grayling is minimal. General research on Arctic

grayling is quite limited with the majority of it focused on genetic distribution, population ecology, and behaviour. Very few studies have looked at the physiology in terms of ion regulation of these fish. The most significant physiological study performed by Cameron (1976), noted the resting blood ion concentrations and showed support for acid-base balance in fish was controlled via the gill. In comparison to the widely studied rainbow trout having a total of around 85,000 annotated protein sequences available on NCBI database, the Arctic grayling only has around 200. Increased research on this species on a whole scale (molecular to ecology) is necessary for conservation efforts to be most successful.

Salmonid Significance

Salmonids are an important fish to humans in terms of food, culture, ecology, and recreational fishing. From 1990 to 2010, salmonid production increased from 299,000 to 1.9 million tons per year (FAO 2016). Next to carp and tilapia, salmonids are the highest produced fish in aquaculture with Atlantic salmon production alone in 2010 at 1.4 million tons. In addition to the increase in aquaculture, wild caught salmon (combined six salmon species) numbers are also high, at around 0.9 million tons globally in 2010 (FAO 2016).

Salmon are a part of the culture in the Pacific Northwest, and especially relevant to the indigenous peoples of North America. Archeological data show evidence of sustainable harvest of salmon by the Native American people for ~7500 years (Campbell and Butler, 2010). Salmon are the main focal point for

much of the culture of the Pacific Northwest for many reasons: 1) salmon are part of the native spiritual and cultural identity and are used in religious services, 2) annual celebrations are centered around the salmon's return from the ocean representing a renewal and continuation of life, 3) salmon are the essential foundation to the well-being of the entire ecosystem including other the animals and plants which the people utilize, 4) they were and continue to be the primary food source, and 5) the annual harvest is a critical event which allows for the transfer of traditional values from one generation to the next (Columbia River Inter-tribal Fish Commission 2016). Salmon are sacred and essential to the identity of the native populations.

As seen in the textbook portrayal of bears feasting on freshly caught salmon during their spawning runs, salmonids play an integral part in the overall health and function of their ecosystems. Their unique anadromous behaviour and life cycle provide necessary nutrients to temperate rainforests. Essential nutrients including phosphorous and nitrogen are in large part derived from salmon carcasses in many river systems (Gresh et al., 2000). Salmonids are also an ideal indicator species of the overall health of the aquatic environment, as they need cold, clean water to live in and reproduce.

Today, many recreational fishermen target various species of the salmonid family. This pattern was evident even centuries ago when salmonids were highly sought after by fisherman. A 15th century book written by Dame Juliana Berners (1496) (reprinted in 1880) gives us early evidence of the importance of salmonids above other fish "As now because that the Samon is

more statelye fysshe that any man maye angle to in fresshe water: Therfore I purpose to begyn at hym", and the author continues with trout and then grayling, holding them in higher regard than the rest. It would be difficult to estimate the overall economic impact of salmonids on the recreational fishing industry, but according to a report on salmon and the economy in the Pacific Northwest, recreational caught salmon have an estimated worth valued at $200 per fish (Niemi et al., 1999). Fly-fishing in particular, which is now being adapted to a wide variety of species, was likely innovated in order to catch trout as described in one of the earliest references from the Greek author Aelian (175-235 CE), in *De Animalium Natura*. From an English translation we understand the original author describing "a river called Astraeus, and in it there are fish with speckled skins…These fish feed on a fly peculiar to the country which hovers on the river…They (fisherman) fasten red wool around a hook, and fix on to the wool two feathers…then they throw their snare and the fish…is caught by the hook and enjoys a bitter repast, a captive" (Law and Kreh, 2003).

Conservation Physiology

Conservation physiology is the research concept and approach defined by Wikelski and Cooke (2006) as "The study of physiological responses of organisms to human alteration of the environment that might cause or contribute to population declines." One goal of this thesis was to take a conservation physiology approach and address the threats to native salmonids in Alberta. Building on previous definitions as noted in Cooke et al. (2013) for

this thesis, it is necessary to look at conservation specifically as the responsibility of humans to protect, enhance, and recover any species threatened by anthropogenic forces. The research area of conservation physiology, utilizes the study of an organism's physiology (the mechanisms allowing an organisms to work: a structure and its function on a wide range of scales from biomolecules of a single cell to the organism as a whole) and apply it to a management or conservation strategy in order to benefit a specific organism or ecosystem (Cooke et al., 2013). It is on this concept that the application side of this thesis is built, utilizing the physiology of an organism to address an anthropogenic threat to its existence.

Industry Economics

The oil and gas industry is an essential socio-economic driver for both Canada and the United States, impacting the overall gross domestic product (GDP) of both countries dramatically. It was estimated that future oil sands projects in Alberta alone from 2010 to 2035, would reach a cumulative GDP amount of $2,106 billion in Canada, and stimulate $26.6 billion in GDP for the United States (Honarvar et al., 2011). In the United States, the last decade has seen a 30% total increase of natural gas production (Vengosh 2014). The economic impacts resulting from this industry are vast and currently indispensible until alternative energy initiatives become more efficient.

Hydraulic Fracturing

Hydraulic fracturing is a technique by which water is first withdrawn from ground or surface water, and then mixed with chemicals and proppants (typically sand) to create the hydraulic fracturing fluid (USEPA 2015). This fluid is then injected under high pressures into a drilled horizontal wells deep beneath the surface usually into shale rock. The pressurized fluid causes the rock formations to fracture or break allowing access to the hydrocarbon targets. Once the pressure is released the injected fluid then flows back to the opening of the well where it is pumped out, now referred to as produced water or flowback fluid. The resulting fractures are held open by the injected proppants and oil and gas can now flow out to the production well and be collected on the fracturing pad or pumped to other destinations (USEPA 2015).

Hypersaline Risk

Due to the nature of the expanding oil and gas industry, this process may result in negative impacts on the air, land, and water, yielding the parallel need for an environmental conservation effort in concert with the industry. Although oil and gas companies are taking numerous preventative measures including: contamination avoidance, water use and management, and reclamation (CAPP 2015); impacts are still common, especially involving incidental releases of hyper-saline water resulting from hydraulic fracturing. Oil and gas extraction technology (e.g. hydraulic fracturing, in situ development) requires tremendous amounts of water (pumped from lakes or rivers) and the extractive process

results in large volumes of highly saline (up to 10X seawater) and organic contaminated flowback wastewater, with up to a 600-fold increase in sodium concentrations compared to local lakes and rivers (Allen 2008). One of the clear risks of this industry is the potential for accidental release of these high saline waters through a spill occurring at a river crossing or at an on-site location. Indeed, reviewing FracFocus documents, a total of 113 spills of flowback fluid (between years 2005 and 2012) have entered flowing water in Alberta since 2005 (Goss et al., 2015). Spills of this produced water do occur regularly, with 51 incidents of saline water releases having been reported from Jan 2016 to June 2016 (AER 2016) in Alberta alone. According to the report [EPA/601/R-14/001], on the Review and State of Industry Spill Data, of the 457 spills related to hydraulic fracturing, 300 of them reached an environmental receptor (i.e. soil or water), and 32 of them (7%) reached some form of surface water (USEPA 2015). While only a small percentage of hypersaline releases may affect standing or flowing water under these circumstances, the osmotic stress on aquatic organisms would be an immediate result. The degree of damage a release may have is proportional to the volume introduced and length of time of the release. However, a sudden influx of brine water into a freshwater lake or stream would pose an immediate osmoregulatory perturbation for aquatic organisms. Internal homeostasis of osmolytes by aquatic organisms is critical for their survival and disruptions to this delicate balance can be acutely lethal. The mechanisms of osmoregulation and responses to hypersaline environments will be discussed further in the next section.

In Alberta, the resource-rich geographical areas used by the oil and gas industry directly overlap with the habitat for many threatened native freshwater salmonids including Arctic grayling, mountain whitefish (*Prosopium williamsoni*), bull trout (*Salvelinus confluentus*), westslope cutthroat trout (*Oncorhynchus clarki lewisi*), and inconnu (*Stenodus leucichthys*) (Northcote, 1995; Rieman *et al*., 1997; McPhail and Troffe, 1998; Howland *et al*., 2001; Walker, 2005; Costello, 2006; Rodtka, 2009)]. Given the limited amount of understanding of the physiology of the Arctic grayling (and indeed any of the abovementioned species), the risks imposed and the threatened status of Arctic grayling, it is imperative to examine both salinity tolerance limits and physiological responses to salinity for grayling to ensure proper conservation strategies for this important native species. One objective of this thesis was to evaluate the physiological responses of the Arctic grayling to acute higher saline exposure. It is essential to understand the potential impacts on these native species, especially given that most regulatory guidelines are routinely based on the responses of the euryhaline rainbow trout (Environment Canada, 1990; USEPA, 2002).

Fish Physiology

Ion- and Osmoregulation

A simple conclusion was made by August Krogh (1937), that "In a number of freshwater fishes a special mechanism exists by which the losses of salt through the urine and by diffusion through the skin and gills can be made good."

This statement, along with concurrent research by Smith (1929; 1930; 1931) and Keys (1931) laid the foundation for the next ~80 years of research in fish physiology concerning osmoregulation and ionoregulation. Osmoregulation by definition is the ability of an organism to maintain their internal homeostatic balance of salts and water in the blood regardless of external environment. Ion-regulation (various ions) is closely tied with osmoregulation (salts and water) and acid-base regulation (ions contributing to overall internal pH balance), differing only by the various ions being regulated i.e. Na^+, Cl^-, Ca^{2+}, NH_4^+, H^+, HCO^- etc. (Goss et al., 1992). The external media, which the fish lives in, determines mechanisms of osmoregulation that need to be employed in order for the organism to maintain internal osmotic balance. Nearly all fish and especially all higher teleost fish (bony fishes, including salmonids) maintain a blood osmotic level of roughly ~300 mOsms despite being in marine (up to 1000 mOsm) or freshwater (down to 0 mOsm) environments (Evans et al., 2005; Edwards and Marshall, 2013). Some fish have a strictly freshwater lifestyle, while others are strictly marine species, and together, these are referred to as stenohaline. Others including many of the salmonids, are referred to as euryhaline, or being able to tolerate environments with widely varying osmotic concentrations or salinities. Regardless, in either environment these fish must overcome strong osmotic gradients. In freshwater, fish are required to compensate for the loss of salts (sodium, Na^+ and chloride, Cl^-) by active salt *absorption* across the gills against the gradient presented by the hypotonic environment they inhabit, in addition to producing copious amounts of dilute urine to offset the diffusion of

water into their bodies. In contrast in hypertonic marine environments, the fish are constantly drinking and producing very small amounts of concentrated urine while salts are actively *excreted* across the gills to counteract the gain of ions and loss of water (Evans et al., 2005). Thus, in both cases osmotic and pH homeostasis is maintained. Due to extensive research over the years, a basic knowledge of ion and osmoregulation in fishes now exists. However, a complete mechanistic understanding of the relationship between pH balance and ion regulation in fish is still lacking and variation among species is prevalent. This is especially true of Na^+ transport, which is dynamically regulated in migratory fish and is implicated in mediating osmotic and acid/base disturbances. Mechanistic knowledge of ion transport begins with an understanding of the main site of regulation, the fish gill.

Fish Gill Morphology

Teleost fish gills are covered by the operculum, a flap used to protect and help ventilate or move water across the gills allowing for gas and ion exchange between the water and the internal blood. Water enters the mouth of the fish and flows across the four sets of gill arches. Each arch is composed of many laterally branching filaments containing an afferent and efferent blood vessel, which then branches individually to the many folds of the epithelial surface or lamellae. This directional movement of water through the buccal cavity across the filaments and out the caudal opening of the operculum allows for a countercurrent exchange of gasses and ions with the water (Evans et al., 2005).

Each individual filament contains many perpendicular-orientated lamellae, thereby greatly increasing the gill surface area for absorption and excretion of ions, as well as aiding oxygen and carbon dioxide transfer. The epithelium of the lamellae is very thin, at times only two cell layers thick, minimizing the distance between the blood and the external water and allowing for a close association between the vasculature system and the environment. Lamellae consist of structural pillar cells (PCs) housing the capillary compartment allowing for the flow of blood cells, and are covered by a thin layer of epithelial pavement cells (PVCs) (Laurent and Dunel, 1980). Water flows through the interlamellar space between adjacent lamellae.

Gill Plasticity

The fish gill itself has proven to be a plastic tissue, demonstrating the ability to alter its morphology or undergo remodeling under certain environmental conditions. Exposure to metals or acidic environments has been shown to result in gill epithelial hyperplasia, hypertrophy, edema, and lamellar fusion etc. (see review, Evans 1987). More recently, there has been an increase in examples of gill plasticity or alterations resulting in the space between the lamellae being filled with an interlamellar cell mass (ILCM) consisting of cells originating from the primary filament at the base of the lamellae. Tissue plasticity was clearly shown in the gills of crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) in response to hypoxia (Sollid *et al.,* 2003). In control animals, a thick ILCM exists, and this is rapidly reduced upon exposure to

hypoxic conditions. This was proposed to create greater surface area for oxygen uptake (Sollid *et al*., 2003; Sollid and Nilsson 2006; Nilsson *et al*., 2012). The goldfish gill also demonstrates similar plasticity in response to temperature. Decreases in the ILCM have also been documented when goldfish acclimated to 25°C were compared with those acclimated to 7°C (Mitrovic and Perry, 2009). Increases in ICLM in response to high environmental ammonia were also documented in crucian carp and goldfish (Sinha *et al*., 2014). Likewise, Wright and colleagues demonstrated killifish (*Kryptolebias marmoratus*) increase their ILCM in response to air exposure to prevent water loss (Ong *et al*., 2007; Turko *et al*., 2011). Wright's group also found that the ILCM of the killifish was decreased in seawater-acclimated animals compared with freshwater-acclimated fish (LeBlanc *et al*., 2010). The only documented increase in ILCM by a salmonid was observed in brook trout (*Salvelinus fontinalis*) in response to aluminum exposure in slightly acidic water (Mueller *et al*., 1991). While plasticity or gill remodeling seems to be a mechanism shared by many fish species in response to alterations or disturbances in their environment, future studies will most likely add to these examples and clarify the nature and role of ILCM in response to differing environmental conditions.

Chloride Cells or Mitochondrion Rich Cells

Chloride cells (CCs) are responsible for Cl^- secretion in marine teleosts and commonly referred to as mitochondrial rich cells (MRCs) or ionocytes as they are found in both marine and freshwater fish. Mitochondrion rich cells are,

for the most part, found at the base of the lamellae where they meet the primary filament (Perry, 1997). As their name suggests, these cells contain numerous mitochondria providing the necessary energy (ATP) needed for their primary role as ion transporting cells. Many studies have characterized the morphology and function of these cells; (see reviews: Doyle and Gorecki, 1961; Philpott, 1980; Perry, 1997; Wilson and Laurent, 2002; Marshall, 2002; Evans et al., 2005; Hwang and Lee, 2007; Dymowska et al., 2012; Hiroi and McCormick, 2012). There is extensive variation in the structure, function, and ion transporters of mitochondrial rich cells based on the species involved and the environment occupied at the time especially that of freshwater teleosts. A recent review by Dymowska et al. (2012) outlines the variation in freshwater species ionocytes models. In rainbow trout, there are two types of documented ionocytes, one beta-type that binds peanut lectin agglutinin (PNA) and termed PNA^+, and an alpha-type which is PNA^-, differing also in their specific transmembrane ion transporter localization (Goss et al., 2001). In tilapia and zebrafish there appear to be four types of ionocytes differing in their expression of certain ion transporters. As indicated earlier, this thesis will focus on the transport (uptake/absorption as well as secretion/excretion) of salts and focused on Na^+ regulation by salmonids, therefore the rainbow trout ionocyte and corresponding ion transporter proteins will be highlighted. Although some ions diffuse freely across cellular membranes, transmembrane proteins are responsible for the bulk of ion transport in and out of the cells. Sodium potassium-ATPase (Nka), Sodium hydrogen exchanger (Nhe), and recently Acid

sensing ion channels (ASIC) have been reported to play important roles in Na^+ transport (Dymowska et al., 2012; Claiborne et al., 2002; Hwang et al., 2011; Dymowska et al., 2014).

Sodium-Potassium-ATPase

Jens Skou (1957) first described the enzymatic ion transporting ability of Nka with experiments conducted on leg nerves of the shore crab, *Carcinus maenas*. Ten years later the role of Nka in adaptation to seawater was characterized in teleosts (Eptstein et al., 1967). Sodium potassium ATPase is an electrogenic membrane bound transporter that uses ATP to transfer three sodium ions *out* and two potassium ions *into* the cell against their gradients. It is important in maintaining the resting potential of the cell, in cell volume regulation and aids in overall ion transport (Schwartz et al., 1975; Jorgensen et al., 2003). It is composed of an alpha and beta subunit along with a gamma subunit or FXYD proteins (proteins named for these specific amino acids FXYD); including ten transmembrane domains of the alpha subunit. A hallmark of the Nka is its sensitivity to the pharmacological inhibitor ouabain (Blanco and Mercer, 1998; Silva et al., 1977).

The role of Nka in fish gill cells has been extensively examined ever since Epstein's original characterization. In freshwater fish, the role of sodium potassium ATPase to maintain Na^+ gradients and to transport Na^+ from the gill epithelial cells to the blood space is generally accepted. In current models, Nka is always localized to the basolateral side of the various ionocytes of freshwater

fish including tilapia (*Oreochromis*), zebrafish (*Danio rerio*), and rainbow trout, see review (Hwang et al., 2011; Dymowska et al., 2012; Hiroi and McCormick, 2012). Using an alpha subunit antibody, Ura and colleagues (1996) performed the initial protein localization of Nka to the chloride cells in the freshwater masu salmon (*Oncorhynchus masou*). Since then Nka immunoreactivity localized to MRCs has been seen in freshwater salmonids including the rainbow trout (Witters et al., 1996), brown trout (*Salmo trutta*) (Seidelin et al., 2000), Atlantic salmon (*Salmo salar*), brook trout (*Salvelinus fontinalis*), and lake trout (*Salvelinus namaycush*) (Hiroi and McCormick, 2007). In marine fish or euryhaline fish adapted to seawater, Nka mediates the branchial excretion of Na^+ by maintaining the transepithelial electrical potential across the gill epithelium, allowing for the movement of Na^+ from the blood back to the external environment through leaky junctions between mitochondrion-rich cells and accessory cells (Evans et al., 2005).

The important role of Nka in Na^+ (and Cl^-) secretion role is vital for seawater tolerance and various studies have shown the link between salinity transfer and gill Nka expression and activity in salmonids (McCormick, 1996; Richards *et al*., 2003; Bystriansky *et al*., 2006; McCormick *et al*., 2013). A significant advancement in our understanding of Nka and salinity transfer occurred with the evidence of differential expression of separate *nka* isoforms, as initially seen in the rainbow trout. Upon seawater acclimation gill expression of *nkaα1b* (now referred to as the seawater isoform) was upregulated, while *nkaα1a* (freshwater isoform) expression decreased in rainbow trout (Richards

et al., 2003). Since then, this isoform-switching has been documented in a variety of species upon salinity transfer including Atlantic salmon (McCormick et al., 2009; Bystriansky et al., 2006), Arctic char, rainbow trout (Bystriansky et al., 2006), Mozambique tilapia (Tipsmark et al., 2011), and the freshwater climbing perch *Anabas testudineus* (Ip et al., 2012). There is also evidence for differential expression patterns of the *nka* isoforms correlating with smolting and migratory stages in the salmonids (McCormick et al., 2013), in which pre-smolts demonstrate increased expression of *nkaα1a*, but *nkaα1b* is the dominant isoform being expressed during smolting (while still in FW) and upon transfer to seawater. Similar patterns were seen again in Atlantic salmon smolts (Nilsen et al., 2007). Importantly, an inability to up-regulate or increase expression of the seawater isoform *nkaα1b* has been suggested to lead to a lack of salinity tolerance. Bystriansky and colleagues demonstrated a reduced salinity tolerance of landlocked Arctic char associated with an inability to up-regulate *nkaα1b* (Bystriansky et al., 2007). This is a critical consideration for present and future salinity tolerance studies and potentially more informative than results from enzymatic assays which do not allow for discrimination between various isoforms of osmoregulatory genes. Genetically these isoforms are present in almost all of the salmonids however their ability to be upregulated and function is the current topic of discussion (Dalziel et al., 2014). It is important to note (when making genetic comparisons across salmonid lineages with a species such as the well-studied rainbow trout) many hatchery rainbow trout had ancestors that likely experienced marine environments less than 150 years ago [assuming

a great number of hatchery rainbow trout lines retain a combination of genetics from anadromous and resident rainbow (MacCrimmon, 1971)].

Upon exposure to differing environmental conditions, maintaining internal blood osmolality and ion levels demonstrates a regulating ability and suggests an overall tolerance in fish. During salinity exposures, freshwater or euryhaline fish demonstrate initial increases in plasma ion levels (Na^+ and Cl^-) and osmolality, indicative of osmotic stress. Successful acclimation is demonstrated when those blood ion levels have returned back to or near control values. For example, rainbow trout acclimated to 40% SW experienced elevated osmolality, Na^+, and Cl^- levels following transfer and recovered after 5 days post-transfer (Richards et al., 2003). Sturgeon (*Acipenser transmontanus*) exposed to 16 ppt compared to those held in 0 ppt, experienced 72 hour (hr) increases in plasma osmolality (400 to 250 mOsm, respectively), in Na^+ (190 to 140 mEq/L, respectively), and in Cl^- (165 to 100 mEq/L, respectively) but recovered to control levels by 120 hrs (Amiri *et al.,* 2009). This pattern has been shown repeatedly; however, the time course of recovery is dependent on both the salt concentration and the fish species examined. Salinity tolerance tests used to examine different species can vary in the salinity concentration used with 32 – 35 ppt representing full strength seawater, 16 – 17ppt, 50% seawater, 0 – 1 ppt, freshwater. These tests usually involve direct acute transfer from freshwater to these higher salinities and then the fish are monitored for 24 – 96 hrs (but can vary depending on the study and species examined).

Sodium Hydrogen Exchanger

Mechanisms allowing for the transfer of Na^+ for hydrogen ions (protons, H^+) have been found universally across various phyla including bacteria, plants, and animals. Members of the SLC9A gene family dominate this role in higher vertebrates. In humans and mammals, at least 9 functional genes are present that code for the various NHEs (NHE1- NHE8) (Orlowski and Grinstein 2004; Slepkov et al., 2007). In general, NHE1-NHE5 are plasma membrane cell surface proteins while NHE6-8 are organelle-localized transporters. All NHEs are organized in a similar fashion consisting of a ~450 amino acid N-terminus made up of 11-12 transmembrane domains, with varying length intracellular C-terminal domain of ~125-440 amino acids which are isoform specific. While theorized models for NHE structure are currently utilized, the exact structure has not been solved via x-ray crystallography for any member of the SLC9 family.

In fish, recent ion-regulatory investigations theorize the Nhe as a primary mechanism for Na^+ and H^+ transport at the gill (see reviews by Wright and Wood, 2009; Takei et al., 2014). Current ionoregulatory models of trout MRCs suggest a metabolon mechanism incorporating a Na^+ transport protein (Nhe) coupled to a rhesus glycoprotein and V-type H^+-ATPase (Dymowska et al., 2012; Wright and Wood, 2009). This ion transporter organization would mediate the simultaneous efflux of H^+ and ammonia (NH_3), resulting in favourable electrogenic gradients and boundary layer acidification to facilitate Na^+ uptake (Wright and Wood, 2009). Initial protein expression studies confirmed the presence of Nhe in the gills of rainbow trout (Edwards et al., 1999). Three Nhe

isoforms involved in osmoregulation have now been identified in salmonids: Nhe2 (Slc9a2), Nhe3 [Slc9a3 (referred to hereafter as Nhe3a)] (Ivanis et al., 2008) and the more recently described Nhe3b (GenBank ID: NM_001160482.1).

The expression of Nhe in freshwater fish and has been demonstrated in numerous studies (Edwards et al., 1999; Ivanis et al., 2008; Hirata et al., 2003; Edwards et al., 2005; Inokuchi et al., 2009). Full characterization of Nhe3 including gill localization was performed in the Osorezan dace, which lives in an acidic freshwater environment (Hirata et al., 2003). Freshwater adapted *Fundulus heteroclitus* demonstrate gill protein expression of both Nhe2 and Nhe3, and exposure to hypercapnia resulted in increased expression of gill Nhe2 under freshwater conditions (Edwards et al., 2005). Expression of Nhe3 was localized to the MR cells of the tilapia, and gill expression of *nhe3* was upregulated upon acclimation to artificial freshwater containing lower Na^+ and Cl^- concentrations (Inokuchi et al., 2009). Zebrafish (*Danio rerio)* are perhaps the most researched freshwater fish when it comes to molecular and genetic studies, and to date, 8 nhe isoforms have been cloned including *nhe1*, *nhe2*, *nhe3a*, *nhe3b*, *nhe5*, *nhe6*, *nhe7*, and *nhe8* (Yan et al., 2007). This comprehensive nhe expression study demonstrated gill expression of all *nhe* isoforms with the exception of *nhe3a*, which was strongly expressed in the kidney. Furthermore, while the 7 isoforms were expressed in the gill under control freshwater conditions, the authors importantly demonstrate down regulation of *nhe3b* under acidic conditions and upregulation under low Na^+ conditions (Yan et al., 2007). Kumai and Perry (2011) however provide conflicting evidence that

under low pH conditions morpholino knockdown of zebrafish *nhe3b* results in significantly decreased Na^+ uptake. These differences however, may be the result of one being a functional assay (Na^+ uptake) and the other only a relative change in expression.

Among salmonids, Ivanis et al. (2008) performed initial cloning and localization of *nhe2* and *nhe3* in rainbow trout, with apical expression of Nhe2/3 found on MR cells of the gill. However, no differentiation between Nhe3a and Nhe3b was achieved with the indiscriminate antibodies used in this study. Nhe3b immunoreactivity has been detected on the apical side of MR cells in rainbow trout and co-localized on cells possessing basolateral Nka and sodium potassium chloride co-transporter (Nkcc) (Hiroi and McCormick 2012). Additionally, feeding as well as exposure to high environmental ammonia resulted in increases in rainbow trout gill *nhe2* mRNA expression (Zimmer et al., 2010). Expression of Nhe in salmonids is supported by other studies including the detection of Nhe2 immunoreactivity in freshwater Coho salmon, where it was localized to elongated accessory cells on the gill filaments with minimal co-localization with that of Nka (Wilson et al., 2002). Clarification of the expression pattern of the various isoforms of Nhe and their role in Na^+ uptake in the model organism rainbow trout was one of the goals of this thesis.

In addition to uptake of Na^+, the other role of Nhe is in acid-base regulation by providing a direct path of H^+ excretion at the gill epithelium. This regulation would be necessary for fish during internal metabolic acidosis events following intense activity (exercise in the form of chasing prey, evading

predators, upstream migrations, or angling pressures). Acid-base and ion regulation at the gill are closely linked in both freshwater (FW) and seawater (SW) species, resulting from the excretion of acidic and basic equivalents and the absorption of Na^+ and Cl^- (Perry and Gilmore, 2006). In marine environments favourable gradients (high external Na^+) would allow for this Na^+/H^+ exchange to occur more easily. This exchange is supported by studies indicating the increase of *nhe3* mRNA expression following hypercapnia in the SW mummichog (*Fundulus heteroclitus*) (Wall et al., 2001), increases in mRNA of *nhe2* in the longhorned sculpin (*Myoxocephalus octodecemspinosus*) gill following HCl infusion associated with increased H^+ extrusion (Hair et al., 2002; Claiborne et al., 1997; Claiborne et al., 2002), and increases in Nhe2 protein abundance following HCl injection in the spiny dogfish (*Squalus acanthias*). In freshwater environments, thermodynamic constraints have led to questioning the function of Nhe despite numerous physiological studies stated earlier showing presence and increased expression under various freshwater conditions. Theoretically, even at low environmental Na^+, following intense exercise the Nhe could function given the increase of H^+ in the epithelial cells, as a result of the action of cytosolic carbonic anhydrase which catalyses the reaction of CO_2 and water to form protons (H^+) and bicarbonate ions (HCO_3^-). We know that a metabolic and respiratory acidosis occurs in rainbow trout following exhaustive exercise (Milligan and Wood, 1986). Arterial blood pH underwent sudden acidification dropping from 7.8 to 7.25 following exercise, followed by progressive recovery by 8 hrs with slight alkalosis over-compensation at 12 hrs, but returned to pre-

exercise levels by 24 hrs associated with acid excretion to the environment (Milligan and Wood, 1986). The exact mechanism of H^+ excretion is still up for discussion as evidence for H^+-ATPase has gained much support, however that route would likely be linked with the presence of an associated Na^+ channel such as ENaC (epithelial sodium channel), which has not been found to exist in teleost fish. The recently proposed ASIC4b has been shown to be expressed in rainbow trout and zebrafish at low pH and low Na^+ environments and has been suggested to be the missing sodium channel (Dymowska et al., 2014). The question still remains of the complete role of Nhe, as the presence of this transporter in freshwater salmonids is clear despite thermodynamic constraints.

Pharmacological Inhibitors

The use of pharmacological inhibitors or drugs that block the action of an ionoregulatory protein is a popular method used to demonstrate the presence or function of a specific ion channel or transporter in many physiological studies (Eigler et al., 1967; Kirshner, 1973; Kleyman and Cragoe, 1989). Commonly the inhibitory drugs utilized are and have been chosen based on their interaction in mammalian studies (in accordance with medical application) and have been applied with overall theoretical assumptions for species crossover. In fish, pharmacological agents are usually administered to the surrounding water environment, by which they will in theory come in contact with the gills and the transporters situated in the gill epithelium. Pharmacological agents have been frequently employed in studies attempting to resolve the mechanistic debate

surrounding the mode of Na^+ acquisition by various freshwater fish (Kirshner, 1973; Wright and Wood, 1985; Avella and Bornancin, 1989).

The application of pharmacological inhibitors in fish physiology studies was likely prompted by studies revealing the use of amiloride (MK 870; N-amidino-3,5-diamino-6-chloropyrazinecarboxamide), a diuretic compound, in inhibiting active Na^+ transport across the frog skin (Eigler et al., 1967). Amiloride was used in a wide range of animal species to investigate Na^+ transport (Benos, 1982). Kirshner (1973) first utilized amiloride in a fish physiological study, and demonstrated a significant decrease of net Na^+ movement across the trout gill. Again amiloride, as well as 4-acetamido-4-isothiocyanostilbene-2,2-disulfonate (SITS), an anion exchange inhibitor, was demonstrated to inhibit Na^+ and Cl^- transport, respectively, in the rainbow trout (Perry and Randall, 1981). The addition of amiloride to the external media resulting in inhibition of Na^+ influx suggests the exchange is on the apical side of the branchial epithelium of trout (Wright and Wood, 1985; Avella and Bornancin, 1989). Due to the non-specificity of amiloride, the support for Nhe as the main path of Na^+ uptake was challenged with contrasting pharmacological studies demonstrating Na^+ uptake inhibition by bafilomycin (a H^+-ATPase inhibitor), in turn suggesting the presence of an apical Na^+ channel working in conjunction with the H^+-ATPase (Fenwick et al 1999; Lin and Randall, 1991; Lin and Randall, 1995; Reid et al., 2003). However, evidence for Nhe was further supported with immunological and further pharmacological support using more specific amiloride analogues including DMA (5-N, N-dimethyl amiloride), MIA (5-methyl-N-isopropyl

amiloride), HMA (5-Nethyl-N-isopropyl amiloride), or EIPA (5-N-ethyl-N-isopropyl amiloride) which all caused significant inhibition of Na^+ uptake in the goldfish at 100μM concentrations or less (Preest et al., 2005). These inhibitors were also chosen based on assumptions from studies in the medical field on ion transport in mammalian cell preps and effects in the presence of amiloride analogues (Kleyman and Cragoe, 1988). Until we have complete characterization of pharmacological inhibitor effects in whole animal studies, isolated fish cells, and isolated proteins, our interpretation of the drug effects seen in fish physiological studies is limited by the assumptions from the mammalian literature. One goal of this thesis was to provide further fish-specific ion-transporter (i.e. Nhe) drug inhibitor interactions, by expressing these transporters in a NHE-deficient cell system, allowing for direct pharmacological characterization of these transporters. Nhe activity would be indicated by measuring intracellular pH (pH_i) calibrated to fluorescence of the pH-sensitive dye BCECF, which was previously taken up by the cell. This method of calculating NHE activity has been utilized in numerous studies (Grinstein et al., 1993; Murtazina et al., 2001; Parks et al., 2010).

AP-1 Cell Line

Upon researching the role of human NHE in the growth-arrest state of mammalian cells, a technique was performed that produced cells which were deficient of the NHE. Utilizing a wild-type Chinese Hamster ovary cell line (WT-5), an acid suicide method was developed which produced a mutant cell line

deficient of NHE activity (AP-1) (Rotin and Grinstein, 1989). This method is described in Pouyssegur et al., (1984) and also in Rotin and Grinstein (1989) as follows: Cells are first loaded with LiCl (lithium chloride) via 2 hr incubation in 130mM LiCl, causing the intracellular Li concentration to reach 80-90mM and pHi 7.1. Next, two chemical gradients of opposite direction were created: an inward directed H^+ gradient and an outward directed Li^+ gradient by replacing external medium with Na^+- and Li^+ -free choline chloride saline solution, buffered at pH 5.5. This resulted in a detrimental H^+ uptake (which could be inhibited by the amiloride analogue, DMA). After 60-min exposure to the choline acid saline, cell viability dropped dramatically, 100μM DMA efficiently prevented cell death. Cell pH dropped from 7.1 to 4.8, and was associated with the rapid H^+ uptake and significant efflux of Li^+. This cycle was performed twice and the surviving clones were given a third 60-min suicide test, and resistant clones were picked and passed over 4 months of passage in the absence of selective pressure. Subsequently, 90% of the clones resistant to H^+ uptake were found to be defective in NHE activity. Also, in a HCO_3^- buffered medium Cl^-/HCO^- exchange can efficiently overcome a NHE anti-port defect. Loading with NH_4^+ kills 100% of the NHE-deficient cells with no effect on the wild-type cell.

The establishment of this NHE-deficient cell line allows for the transient or stable expression of NHEs into the cell line, which can now be characterized for transport kinetics and or pharmacological interactions. Extensive studies utilizing this system to do exactly that have been performed to investigate the activity of various NHEs including the rat NHE2 (Yu et al., 1993), rat NHE1 and

nhe3 (Orlowski, 1993), human NHE1 (Murtazina et al., 2001), human NHE1, -2, and -3 (Kandasamy et al., 1995) and shark Nhe2 and -3 (Guffey et al., 2015). Given all of the teleost physiological studies utilizing pharmacology against the Nhe, there is a lack of functional characterization of any teleost Nhe utilizing a similar expression system.

Fish Embryo Physiology

Salmonid embryo hatching times vary greatly depending on the species and incubation temperature, however from fertilization, rainbow trout embryos if held at constant temperature around 10°C hatch at ~34 days (Velsen, 1987). Historically the majority of fish physiological studies concerning osmoregulation or ionoregulation were conducted on juvenile or adult fish. However, there is an increasing number of fish embryo or larval studies emerging looking at ion transport and respiration at this initial stage of life (Eddy and Talbot, 1985; Rombough, 2007; Zimmer et al., 2014). Just as adult freshwater fish face the challenges of living in a hypotonic environment where they must acquire ions from the environment and rid themselves of excess water brought on by osmotic pressures due to their increased surface area-to-volume ratio fish embryos and larvae arguably face even greater osmotic challenges. Unlike adult fish, embryos do not have the ability to seek out favourable environments if an environmental perturbation does exist, although the chorion and perivitellin fluid act as protective buffering layers for the embryo (Eddy and Talbot, 1985). As stated earlier, the gill is the main site for ion regulation, osmoregulation and respiration

in adult fish; however, because this tissue is greatly underdeveloped in embryonic and larval fish, research suggests the skin or yolk sac membrane to be the initial location for these physiological processes (Wells and Pinder, 1996; Rombough, 1999; Rombough, 2007; Fu et al., 2010; Zimmer et al., 2015). Indeed similar cells to those of the gill epithelial MR cells have been localized to the yolk sac of embryonic fish (Ayson et al., 1994; Nakada et al., 2007; Esaki et al., 2009; Kaneko et al., 2002). The ability of embryonic fish to osmoregulate has been discussed for a number of years (see review by Versamos et al., 2005). Shen and Leatherland (1978) suggested that rainbow trout embryos possessed some capacity to osmoregulate, demonstrating differences in eggs held in distilled water compared to those in 13 or 16ppt salinity. Additionally, significant differences in Na^+ content were established between the different treatments, suggesting embryonic Na^+ regulation (Shen and Leatherland, 1977). Barrett et al. (2001) demonstrated that Na^+ uptake in whole rainbow trout embryos was maintained at stable rates from fertilization up until 10 days before hatch at which point there was a significant increase in Na^+ uptake rate.

Much like adult freshwater fish, the exact mechanisms involved in osmoregulation at these early life stages, especially those responsible for the uptake of salts from the surrounding environment have not yet been fully determined. In larval tilapia, Nka was immunolocalized to MR cells on the yolk sac membrane suggesting the MR cells role in early osmoregulation (Hwang et al., 1999). In larval zebrafish in acidic freshwater, Kumai and Perry (2011) demonstrate an EIPA-sensitive Na^+ uptake mechanism (localization of Nhe3b)

which is linked to ammonia transport via Rh protein, as well as a Na^+ uptake component facilitated by H^+-ATPase, suggesting the existence of multiple mechanisms. Sodium uptake was also decreased upon knockdown of *nhe3b* in zebrafish larvae in low Na^+ water (Shih et al., 2012). It has been suggested in rainbow trout embryos that there is a direct coupling of Na^+ uptake and ammonia excretion over early development with the documented increase of expression of *nhe2* and *Rhcg1*, and that this shifts from the skin to the gills over time (Zimmer et al., 2014). The role of the various Nhes in embryonic Na^+ uptake during rainbow trout development was an aspect investigated in this thesis.

Thesis Projects and Aims

Fish constantly perform physiological processes allowing them to live in aquatic environments with varying salt and water gradients. Maintaining internal homeostasis against the forces of diffusion and osmosis that may perturb the delicate balance of pH or salt to water ratios in their blood is a function of the gill. Gills are the main routes of ion and gas exchange between fish and their environment, and mitochondria rich cells lining branchial epithelium are equipped with specialized proteins acting as transporters or channels for ions to pass across the water/blood barrier. Sodium is a vital ion for maintaining both acid-base balance and internal osmotic homeostasis and is closely regulated by in fish, especially species such as those in the salmonid family that commonly migrate between freshwater and marine environments.

The goal of my research was to investigate the various mechanisms of the gill or branchial region, which allow members of the salmonid family to efficiently exchange Na^+ ions with the environment. Failure to do so under specific conditions will cause imbalances to the fish's blood homeostasis. The aims of my thesis were as follows:

1) Examine the developmental expression of Nhe isoforms and their role of sodium uptake in trout embryos
2) Clone trout *nhe2*, *nhe3a*, and *nhe3b* and conduct a pharmacological profile following transfection into nhe deficient AP-1 cells
3) Investigate the salinity tolerance and identify ion transporters in Arctic grayling (*Thymallus arcticus*)
4) Examine possible recovery from salinity exposure of Arctic grayling and perform a comprehensive morphology analysis of the resulting ILCM

CHAPTER II: Characterization of developmental Na+ uptake in rainbow trout embryos supports a significant role for Nhe3b

A version of this chapter has been published previously, in which I share co-1st author with Dr. David Boyle for the following journal article.

Boyle, D., Blair, S. D., Chamot, D., & Goss, G. G. (2016). Characterization of developmental Na+ uptake in rainbow trout larvae supports a significant role for Nhe3b. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *201*, 30-36.

Introduction

In freshwaters, fish face a challenge to overcome unfavourable ion gradients to replenish ions, including Na^+, lost by diffusion. In adult fishes, ion uptake is primarily achieved at the gill, a multifunctional epithelium with additional coupled roles in gaseous exchange, acid-base balance and nitrogenous waste excretion (Evans et al., 2005). In larvae, these physiological processes are first performed cutaneously, with the role of the gill progressively increasing during development (Fu et al., 2010; Rombough, 2007; Zimmer et al., 2014a). Recently, it has been demonstrated that ion uptake shifts earliest to the developing gill with gaseous exchange following later, and this has been interpreted as the primacy of ion acquisition over respiration in driving the evolution of gill development in fish (Fu et al., 2010). Nevertheless, the molecular identities and cellular organizations of the proteins involved in ion uptake and especially their expression under variable environmental conditions have yet to be fully elucidated and are the subject of continuing debate.

Early models of acid-base balance and ion-regulation in fish postulated Na^+ uptake to occur in exchange for acidic equivalents (H^+) at the gill (e.g. Krogh, 1937, 1938). This has since been empirically demonstrated [*e.g.*, in rainbow trout (*Oncorhynchus mykiss*); Goss and Wood, 1991]. Sodium uptake at the gill has also been suggested to be coupled to ammonia (total NH_3/NH_4^+) excretion (Liew et al., 2013; Wright and Wood, 1985; Zimmer et al., 2010). Although less studied, Na^+ uptake is also required for embryonic fish, Na^+ balance in chorionated embryos is acid-sensitive (Eddy et al., 1990) and the timing of the

ontogenetic shift of Na^+ uptake and NH_4^+ excretion to the gill in embryonic trout is highly correlated (Zimmer et al., 2014). The mitochondrion-rich ionocytes in the gill (MR cells; Perry, 1997) also appear early in fish development, being present on the yolk-sac membrane and prior to the development of gills (Nakada et al., 2007; Esaki et al., 2009).

Recently proposed models of Na^+ transport in adult rainbow trout gill MR cells, have a sodium transport protein potentially coupled to rhesus glycoproteins and V-type H^+-ATPases in a metabolon (Dymowska et al., 2012; Wright and Wood, 2009). Together, these act in concert to mediate NH_3 and H^+ extrusion and maintain favourable electrogenic gradients and boundary-layer acidification to facilitate Na^+ uptake. The electroneutral sodium/proton exchange proteins (Nhe) in the solute carrier (Slc) family of membrane transport proteins are currently considered primary candidates for this role (see reviews by Wright and Wood, 2009; Takei et al., 2014). First documented in rainbow trout by Lin and Randall (1991) and with localization at the branchial epithelial membrane confirmed later (Edwards et al., 1999), three isoforms have now been identified: Nhe2 (Slc9a2), Nhe3 [Slc9a3 (referred to hereafter as Nhe3a)] (Ivanis et al., 2008) and the more recently described Nhe3b (GenBank ID: NM_001160482.1). However the function of Nhe transporters in low Na^+ waters has been questioned due to thermodynamic constraints of combined low pH and low [Na^+] (Parks et al., 2008). Indeed, it has been demonstrated that acid sensing ion channels (Asic) may substitute for the function of a Nhe protein in juvenile rainbow trout under unfavourable conditions (Dymowska et al., 2014); Asics are

voltage-insensitive Na^+ channels gated by extracellular H^+ and are expressed in the gill of juvenile trout.

The goal of this study was to investigate Na^+ uptake and the role of Nhe in rainbow trout embryos and larvae reared from fertilization in soft synthetic freshwater containing 0.1 mM Na^+ at pH 6. This [Na^+] and pH are below the point of transport equilibrium of Nhe (Parks et al., 2008). We utilized unidirectional whole embryo/larvae $^{22}Na^+$ fluxes and dose-increments of pharmacological inhibitors of Na^+ uptake to determine the role of Nhe. Expression of *nhe* isoforms (*nhe2*, *nhe3a* and *nhe3b*) was also analyzed in whole embryos/larvae. A second group of embryos/larvae reared in hard water (2.2 mM Na^+, pH 8, higher [Ca^{2+}]) was also included in the experimental design. Importantly, fluxes with hard water reared embryos/larvae were performed in soft water, only. The rationale behind this approach was that if rearing embryos/larvae in soft and hard waters leads to differential expression of *nhe* isoforms then embryos/larvae would display different Na^+ uptake profiles when fluxed under the same water conditions. These data could therefore add further weight to the roles of specific Na^+ uptake pathways in soft water. To complement these data and to address a data gap in the trout literature tissue specific expression analyses of *nhe* isoforms were also performed in juvenile rainbow trout. This is the first study to differentiate developmental expression patterns of isoforms of *nhe* in rainbow trout during early development and the data gathered suggest a predominant role of Nhe3b in Na^+ acquisition in low [Na^+] soft water.

Materials and Methods

Experimental animals and environmental waters

All experiments were conducted under Animal Use Protocol #00000072 as approved by the University of Alberta and the Biosciences Animal Policy and Welfare Committee under the directions provided by the Canadian Council for Animal Care in Canada. Rainbow trout embryos (n = 500) were collected on the day of fertilization from Raven Brood Trout Station, Raven, Alberta and transported to the University of Alberta. Embryos were equally divided between Heath chambers in two continuously aerated and UV-treated dechlorinated recirculating water systems containing 130 L of either soft [low Na^+ (0.1 mM, nominal concentration)] or hard [high Na^+ (2.2 mM, nominal concentration)] synthetic freshwaters maintained at 10°C. The chemical compositions of the synthetic freshwaters used correspond to those previously used in trout ionoregulatory research (Allin and Wilson, 2000; Fu et al., 2010) and standardized waters recommended for use by the U.S. Environmental Protection Agency (USEPA, 2002). Measured [Na^+] were (means ± S.D., n = 26): 0.105 ± 0.006 and 2.267 ± 0.201 mM for soft and hard waters, respectively. Soft and hard waters were pH 5.9 ± 0.2 and pH 8.1 ± 0.1, respectively. Concentrations of other elements in freshwaters were: Ca^{2+} 0.015 mM; Mg^{2+} 0.035 mM; K^+ 0.035 mM, in soft water and Ca^{2+} 0.9 mM; Mg^{2+} 1.0 mM; K^+ 0.1 mM, in hard water.

Immediately after the final flux (see section 2.3), when complete reabsorption of the yolk sac (~22 d post hatch, dph) had occurred, excess fry were transferred to 120 L flow-through tanks supplied with aerated 10°C re-

circulating dechlorinated Edmonton City tapwater (Na^+ 0.46 mM; Ca^{2+} 0.99 mM; Mg^{2+} 0.65 mM; K^+ 0.02 mM) and maintained as stock fish. Later, and in light of gathered data of developmental expression of *nhe* isoforms in whole embryos/larvae (see section 2.4 and results), it was apparent that these data could be better interpreted by complementing them with analyses of expression of *nhe* isoforms in specific tissues of juvenile trout. These expression analyses also addressed a data gap in juvenile rainbow trout. Therefore, at 6 months, a sub-sample (same cohort) of the juvenile trout (2.1 ± 0.3 g, n = 6) were re-acclimated for 7 d in aerated tanks containing 20 L of either soft or hard synthetic freshwater (chemistry as indicated above). Following the acclimation period, fish were euthanized in pH buffered MS222, and tissues excised and snap frozen in liquid N_2 for reverse transcriptase-PCR analysis (*nhe* isoform).

Measurement of embryo sodium concentration

At time points selected to span development from pre-hatch (chorionated embryos) to immediately post-hatch and then the period up until the absorption of the yolk sac, embryos/larvae (n = 3) were randomly selected and removed from Heath trays, and individually snap frozen in liquid N_2. Embryos were subsequently dried to constant weight at 80°C, transferred to metal-free polyethylene tubes, digested in 250 µL trace analysis grade concentrated nitric acid and diluted to a known volume with ultrapure water for measurement of $[Na^+]$ via atomic absorption spectrophotometry (Model 3300, Perkin Elmer, CT, USA). Embryo $[Na^+]$ was calculated as µmol Na^+ mg^{-1} dry weight.

Unidirectional $^{22}Na^{+}$ fluxes

Unidirectional $^{22}Na^{+}$ (in the form of $^{22}NaCl$, Perkin Elmer) fluxes in whole embryos/ larvae reared were conducted in soft water, only (water chemistry as described in section 2.1). The broad-spectrum Na^{+} channel blocker, amiloride and the purported Nhe specific inhibitor 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) were used to examine the involvement of Nhe in Na^{+} uptake in embryos/larvae (Kleyman and Cragoe, 1989). Embryos/ larvae (n = 6 per dose/treatment) were transferred in system water from the Heath chambers to individual 12 × 75 mm borosilicate tubes and placed in a constant temperature water bath at 10°C. At t = -30 min, the system water was removed, and 1 mL of soft water (both treatment groups) premixed (vortexed) with pharmacological agents (0, 0.1, 1, 10, 100 µM amiloride or EIPA in 0.1% dimethyl sulfoxide (DMSO vehicle; all reagents from Sigma) was added. Previous studies have indicated that 0.1% DMSO does not interfere with ion uptake in trout embryos (Boyle et al., 2015) and comparison of Na^{+} uptake rates in hard and soft water reared embryos/larvae exposed to 0.1% DMSO with controls (i.e. no DMSO), indicated no significant difference (Student's t-test, $p > 0.05$). Accordingly, flux measurements performed without DMSO are not shown in figures and were not included in further statistical analyses. After the pre-incubation period (t = 0), the solutions were removed and immediately replaced with 1 mL of soft water premixed (vortexed) with both the pharmacological agent (in 0.1% DMSO) and 0.0125 µCi $^{22}Na^{+}$ mL^{-1}, as appropriate. After t = 5 min, a 100 □L sample was withdrawn from each tube and placed in a borosilicate tube for later counting to

calculate the specific activity of $^{22}Na^{+}$ in the water. At the completion of the experimental flux period (t = 60 min), the flux media was removed with a Pasteur pipette and replaced with 1 g L^{-1} MS222 prepared in soft water and buffered to pH 6.0. Euthanized embryos/ larvae were then washed 3 times in ~4 mL ice-cold 2.9 g L^{-1} NaCl to displace residual unbound $^{22}Na^{+}$. A sub-sample (1 mL) of the final wash solution was also removed to borosilicate tubes for counting to measure carry-over of residual externally bound $^{22}Na^{+}$. The activities of $^{22}Na^{+}$ in all trout embryos/larvae and water samples were counted with a Cobra Quantum gamma counter (model E5003, Packard Instrument Company Inc., Downers Grove, Illinois). Total [Na^{+}] in low [Na^{+}] water was measured using atomic absorption spectrometry (Model 3300, Perkin Elmer, CT, USA) and compared to appropriate Na^{+} standards (as NaCl). Unidirectional Na^{+} influx was calculated as nmol Na^{+} $embryo^{-1}$ h^{-1} (Fu et al., 2010).

RNA extraction, cDNA synthesis and PCR

Whole embryos for transcript analysis were collected on the same day and from the same tray as those used for $^{22}Na^{+}$ uptake fluxes. Transcript tissue analysis (gill, kidney, brain, intestine, liver) was also conducted on acclimated juvenile trout at ~6months post-fertilization. All collected samples were immediately frozen in liquid N_2 and stored at -80°C for later analysis. Total RNA was extracted from tissues and embryos using Trizol (Invitrogen) and combined with further RNA purification and genomic DNA (gDNA) removal using the RNeasy mini plus kit (Qiagen) as previously described (Boyle et al., 2015).

Briefly, tissues (approximately 20 mg) or individual (n = 1) whole trout embryos were homogenized in 1 or 1.5 mL Trizol, respectively, using a motor-driven hand homogenizer (Gerresheimer Kimble Kontes LLC, Düsseldorf, Germany). After centrifugation (12000 × g, 10 min, 4°C) to remove tissue debris, the supernatant was transferred to a second tube with 0.2 or 0.3 mL chloroform and then shaken by hand (15 s). Tubes were then incubated at room temperature for 3 min and then centrifuged again (12000 × g, 10 min, 4°C). The upper phase was transferred to a gDNA removal column and further RNA purification continued according to RNeasy kit manufacturer's instructions (Qiagen). Total RNA was eluted in ultrapure nuclease-free H_2O and the concentration and presence of impurities assessed through spectrophotometry (NanoDrop, ND-1000, Thermo Scientific, Wilmington, USA). Synthesis of cDNA was carried out with SuperScript III reverse transcriptase (Invitrogen) with random primers and 1.5 μg (tissues) or 5 μg (embryos) RNA template according to the manufacturer's instructions. cDNA was diluted 1 in 25 with ultrapure nuclease-free H_2O for use in quantitative PCR (qPCR) or Reverse Transcription PCR (RT-PCR).

The gene-specific oligonucleotide primers for *nhe2*, *nhe3a* and *nhe3b* and *elongation factor 1α* (*ef1α*, house-keeping gene) for qPCR and RT-PCR were designed using Primer3 software (*v.* 0.4.0) and are listed in Table 2.1. Expression of *ef1α* has previously been shown to be stable during embryo/larval development and has comparable expression between tissues in juvenile rainbow trout (Boyle et al., 2015).

Quantitative PCR was carried out using a light cycling PCR machine (ABI Prism 7500 sequence detection system) with cycling conditions as follows: 2 min denaturation at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min, and using SYBR green PCR mastermix (produced in-house, Molecular Biology Services Unit, University of Alberta) in a final reaction volume of 10 µL containing 300 nM primers. All samples were analyzed in triplicate with appropriate no-template controls included on each plate and dissociation analysis performed in every well to verify primer specificity. The relative copy number of mRNA transcripts was calculated according to a Ct-based relative quantification with efficiency of reaction correction and normalizing to *ef1α* (Pfaffl, 2001). Efficiencies of the PCR reactions were calculated using a cDNA dilution curve and were 105-110%.

Reverse transcription PCR was performed on whole larvae (11 dph, only) and tissues (gill, kidney, brain, intestine, and liver) of juvenile trout. Conditions used in the PCR were as follows: 3 min denaturation at 95°C; and 35 cycles of 95°C for 30 s, and 30 s at 60°C (*nhe2, nhe3a, nhe3b*), or 57°C (*ef1a*), and 72°C for 1 min; and followed by a final elongation at 72°C for 5 min. The resulting PCR products were visualized by electrophoresis on a 2% agarose gel stained with ethidium bromide and imaged using AlphaImager 2200 (ProteinSimple, California). The identities of all PCR products were confirmed by sequencing.

Data handling and statistical analyses

All data are presented and reported as means ± standard error (S.E.M.). Statistical analyses were performed using GraphPad Prism (GraphPad Software, Inc. *v.* 6) or IBM SPSS Statistics 21 (SPSS Inc., *v.* 21). All data were tested for normality (Shapiro-Wilk test) and if not normally distributed were $\log_{10}$ or arcsine transformed as appropriate. Statistically significant differences between groups were detected using ANCOVA (Na^+ concentrations in embryos), Two-Way ANOVA with Holm-Sidak's multiple comparisons test *a posteriori*, One-Way ANOVA with Dunnett's test *a posteriori* and Student's *t*-test. A p value of ≤ 0.05 was considered significant.

Results

Whole embryo [Na^+]

There was a small difference (of 1 d) in the time of hatch between embryos reared in soft and hard water. Accordingly, sampling was staggered by 1 d in all post-hatch analyses and all data are presented normalized to date of hatch. There was a clear and significant increase in [Na^+] in whole embryos/larvae as development progressed and especially post-hatch that was evident in both treatment groups (Fig. 2.1). However, there were no significant differences in [Na^+] in soft *versus* hard water (using ANCOVA to normalize for difference in time of hatch, $p > 0.05$). For example, [Na^+] at 20 dph when yolk sacs had receded was: 59.4 ± 8.0 and 67.5 ± 2.4 μmol Na^+ mg^{-1} in larvae reared in soft and hard water, respectively.

Developmental profile of Na^+ acquisition

When assessed in soft water, unidirectional Na^+ uptake rates in embryos/larvae reared in both soft and hard water progressively increased during development ($p < 0.001$, Fig. 2.2). Starting at 0 dph, significant differences in Na^+ uptake were seen between soft and hard water treatments with larvae acquiring Na^+ at 7.6 ± 0.9 and 4.7 ± 0.6 nmol larva^{-1} h^{-1}, respectively. By 20 dph, Na^+ uptake had increased to 29.5 ± 3.7 and 20.8 ± 3.0 nmol larva^{-1} h^{-1} in soft and hard water treatments, respectively (Fig. 2.2).

Pharmacological profile of Na^+ acquisition

To profile proteins and pathways of Na^+ uptake in soft water, unidirectional $^{22}Na^+$ flux experiments were also performed with increasing doses of amiloride and EIPA at 0 and 20 dph (Figs. 2.3, and 2.4, respectively). The data clearly demonstrate that both pharmacological agents, and in both soft and hard water reared larvae, inhibited Na^+ uptake. At 0 dph, 10 μM EIPA caused an approximate 50% inhibition in Na^+ uptake, while 100 μM EIPA resulted in an approximate maximal 85% inhibition (Fig. 2.3A). Amiloride also caused similar patterns of inhibition with a maximum 85-90% inhibition of Na^+ uptake observed. This pattern of pharmacological inhibition was also apparent in larvae fluxed at 20 dph (Fig. 2.4A).

There were clear and statistically significant differences in absolute measurements of Na^+ uptake rates in larvae reared in soft and hard water throughout the experiment, including during exposures to amiloride and EIPA;

however, when uptake rates were expressed as % inhibition of their respective controls (soft or hard water), there were no significant differences observed in the effects of the drugs between the two treatment groups (Figs. 2.3B, 2.4B).

mRNA Expression of nhe isoforms

Expression of all three *nhe* isoforms was not consistently detected in whole embryos/ larvae during development. While *nhe2* was detectable in embryos/larvae with RT-PCR (at least at 11 dph, Fig. 2.5) it was not consistently quantifiable with qPCR (data not shown). Expression of *nhe3a* was not detected with either technique (e.g. RT-PCR, Fig. 2.5). In contrast, expression of *nhe3b* was consistently detected throughout development and was observed to increase significantly post-hatch (Figs. 2.5 and 2.6A). Moreover, and at most time-points analyzed, expression was significantly greater in soft water reared embryos compared to hard water reared embryos. There was also a strong positive correlation between unidirectional Na^+ uptake and *nhe3b* expression across all time and treatment groups for which data were available (Fig. 2.6B). In juvenile rainbow trout acclimated to soft and hard water, *nhe2, nhe3a and nhe3b* exhibited a clear tissue specific expression pattern (Fig. 2.5B). Expression of *nhe2* and *nhe3b* was detected in the gills, only, while *nhe3a* was localized to the kidney (Figure 2.5B). Furthermore, expression of *nhe3b* was found to be about 75-fold higher than *nhe2* in the gills of juvenile rainbow trout under both water conditions (Figure 2.7).

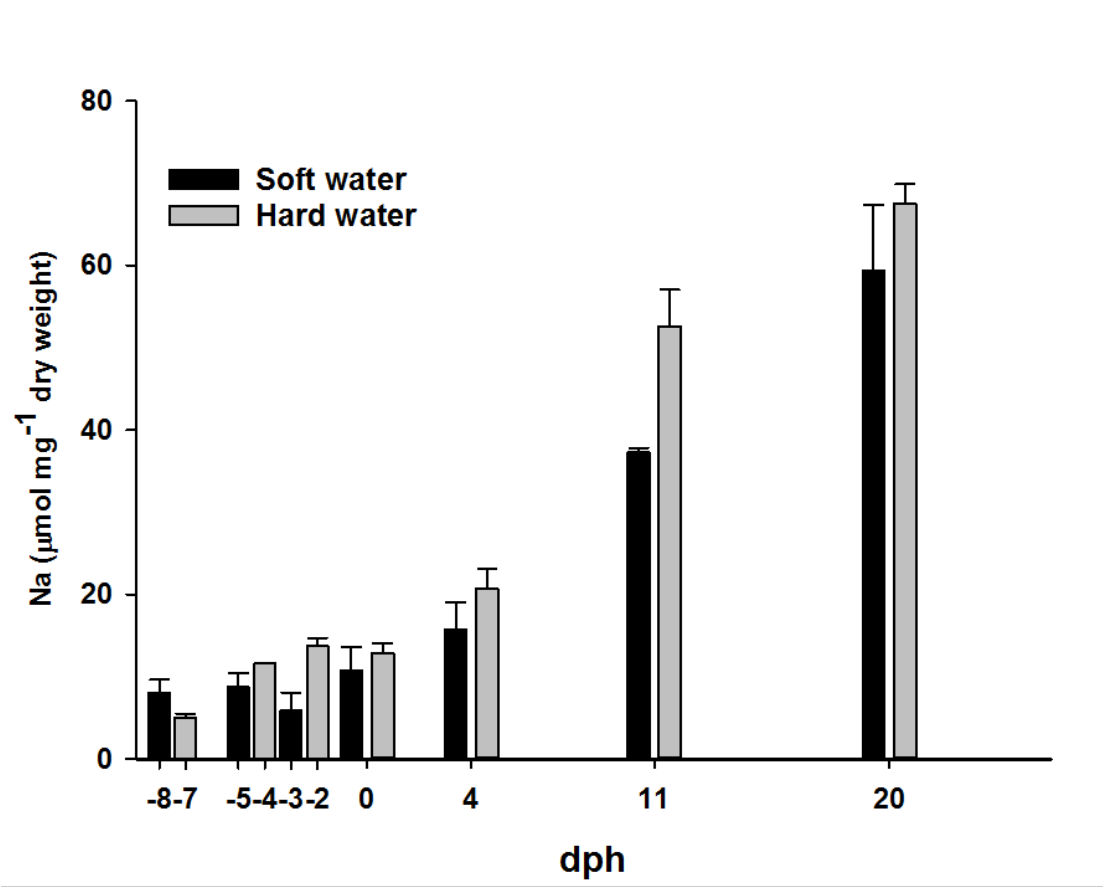


Figure 2.1. Sodium (Na^+) concentrations (µmol mg^{-1} dry weight) in embryos/larvae reared in soft (0.1 mM Na^+) and hard (2.2 mM Na^+) waters. Time is expressed as day post hatch (dph) and the non-alignment of pre-hatch (i.e. < 0 dph) data points reflect the 1 d difference in hatch between treatments. Data are means ± S.E.M., $n = 3$. There was no significant difference between treatment groups (ANCOVA, $p > 0.05$).

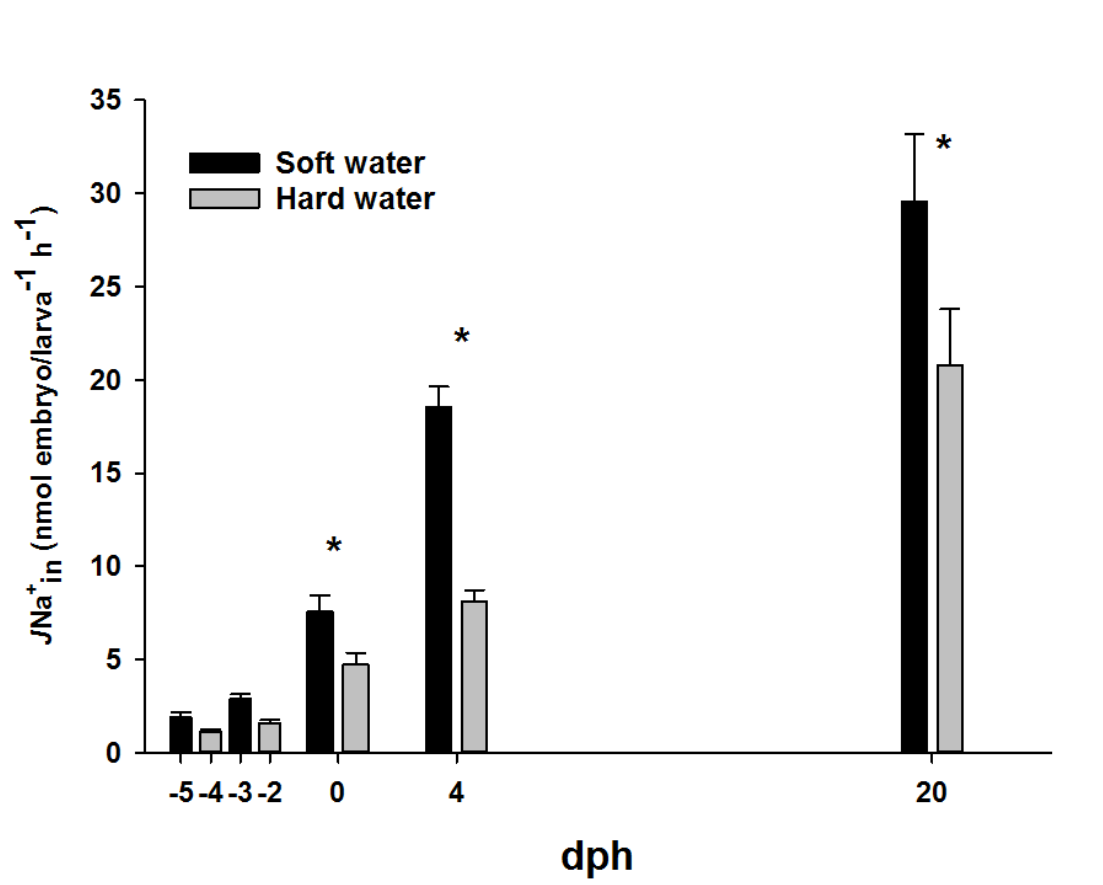


Figure 2.2. Unidirectional Na^+ flux (JNa^+_{in} (nmol embryo^{-1} h^{-1})) in trout embryos/larvae reared in soft (0.1 mM Na^+) and hard (2.2 mM Na^+) waters. Embryos were fluxed in soft water, only. Data are means ± S.E.M., $n = 6$. JNa^+_{in} increased significantly over time in both treatment groups. * denotes significant difference between treatments within time-points (Two-Way ANOVA, $p < 0.001$).

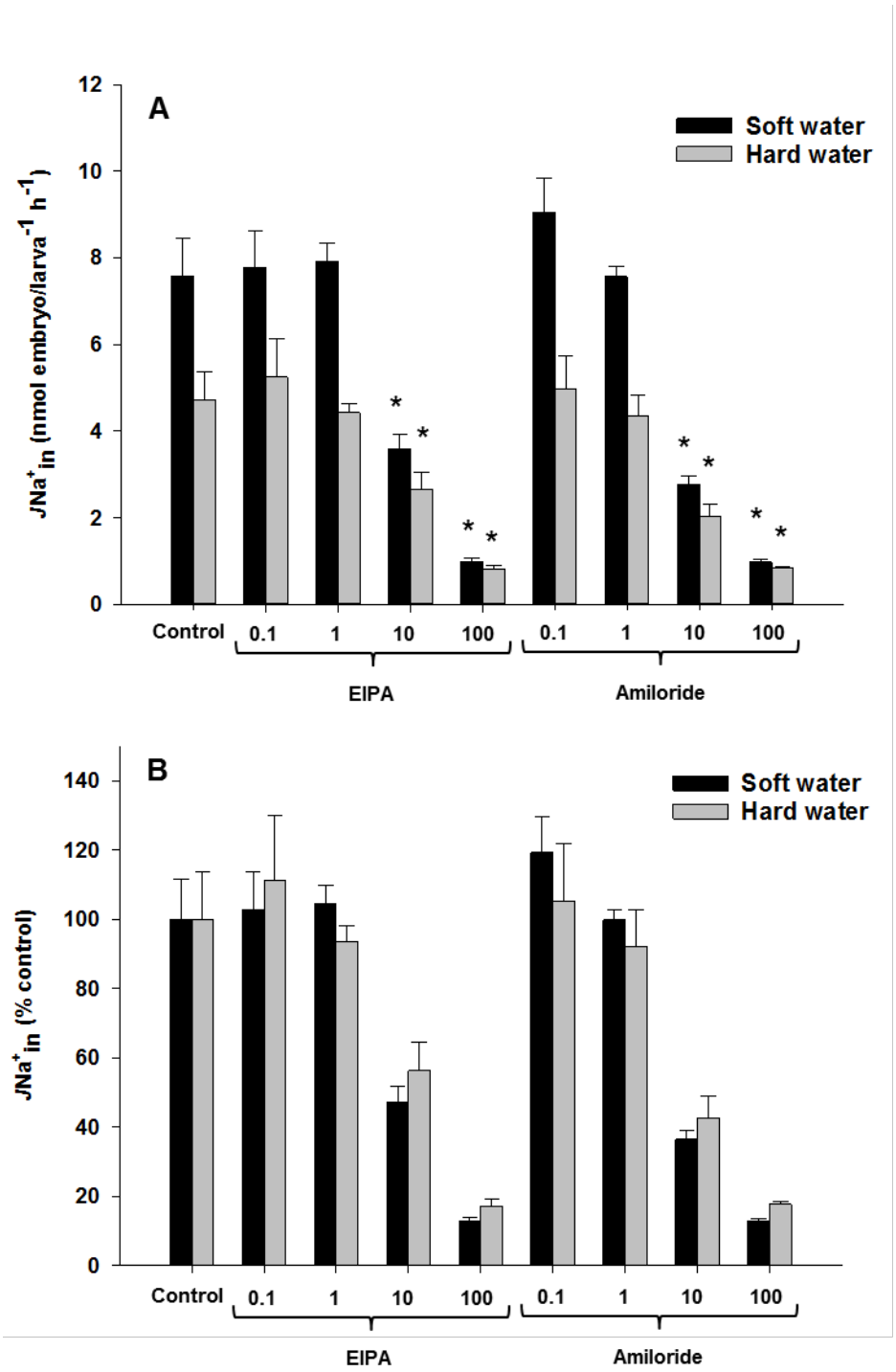

A
Soft water
Hard water
JNa^+_{in} (nmol embryo/larva^{-1} h^{-1})
12
10
8
6
4
2
0
Control
0.1
1
10
100
0.1
1
10
100
EIPA
Amiloride
B
Soft water
Hard water
JNa^+_{in} (% control)
140
120
100
80
60
40
20
0
Control
0.1
1
10
100
0.1
1
10
100
EIPA
Amiloride

Figure 2.3. (previous page) Profile of unidirectional Na^+ flux [$J_{in}Na^+$ (nmol embryo^{-1} h^{-1})] in soft water (0.1 mM Na^+) in trout larvae immediately post-hatch that were reared from fertilization in soft (0.1 mM Na^+) and hard (2.2 mM Na^+) waters. Larvae were fluxed in 0.1 – 100 μM EIPA and amiloride prepared in 0.1% DMSO. Controls were fluxed in 0.1% DMSO. Panel A: * denotes significant differences compared to the within treatment control (Two-Way ANOVA, $p < 0.05$). Panel B: when inhibition was expressed as % respective controls, there were no significant differences in the effects of pharmacological agents between treatment groups ($p > 0.05$). Data are means ± S.E., $n = 6$.

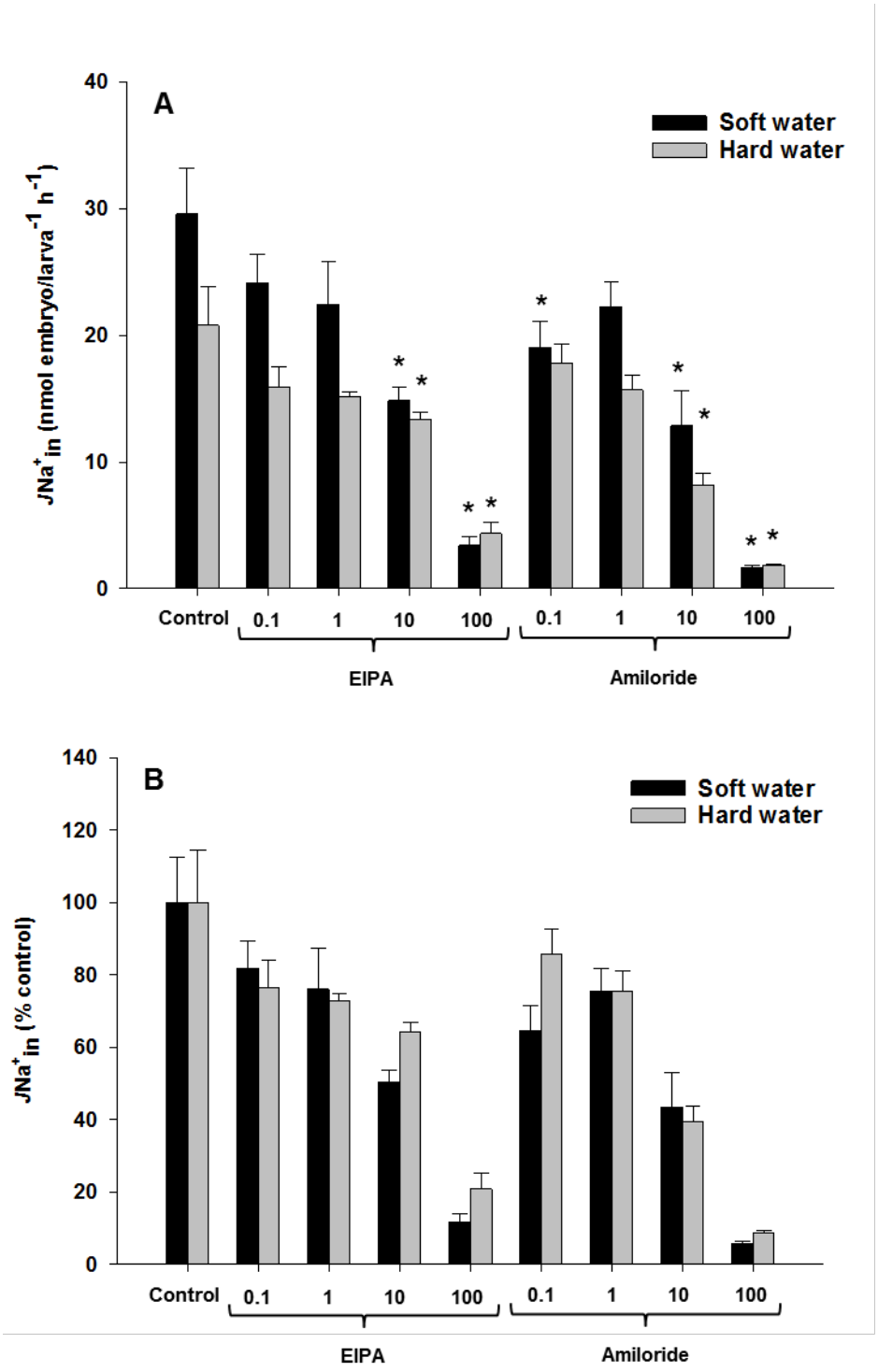

A
Soft water
Hard water
JNa^+_{in} (nmol embryo/larva^{-1} h^{-1})
40
30
20
10
0
Control
0.1
1
10
100
0.1
1
10
100
EIPA
Amiloride
B
Soft water
Hard water
JNa^+_{in} (% control)
140
120
100
80
60
40
20
0
Control
0.1
1
10
100
0.1
1
10
100
EIPA
Amiloride

Figure 2.4. (previous page) Profile of unidirectional Na^+ flux [JNa^+_{in} (nmol embryo^{-1} h^{-1})] in soft water (0.1 mM Na^+) in trout larvae at 20 days post hatch that were reared from fertilization in soft (0.1 mM Na^+) and hard (2.2 mM Na^+) waters. Larvae were fluxed in 0.1 – 100 μM EIPA and amiloride prepared in 0.1% DMSO. Controls were fluxed in 0.1% DMSO. Panel A: * denotes significant differences compared to the within treatment control (Two-Way ANOVA, $p < 0.05$). Panel B: when inhibition was expressed as % respective controls, there were no significant differences in the effects of pharmacological agents between treatment groups ($p > 0.05$). Data are means ± S.E.M., $n = 6$.

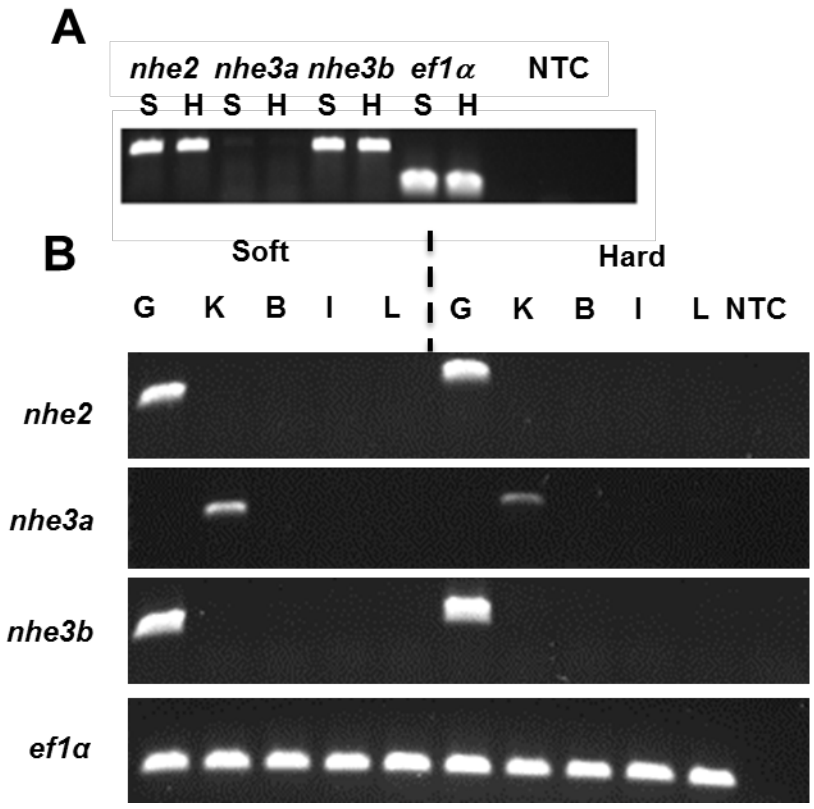
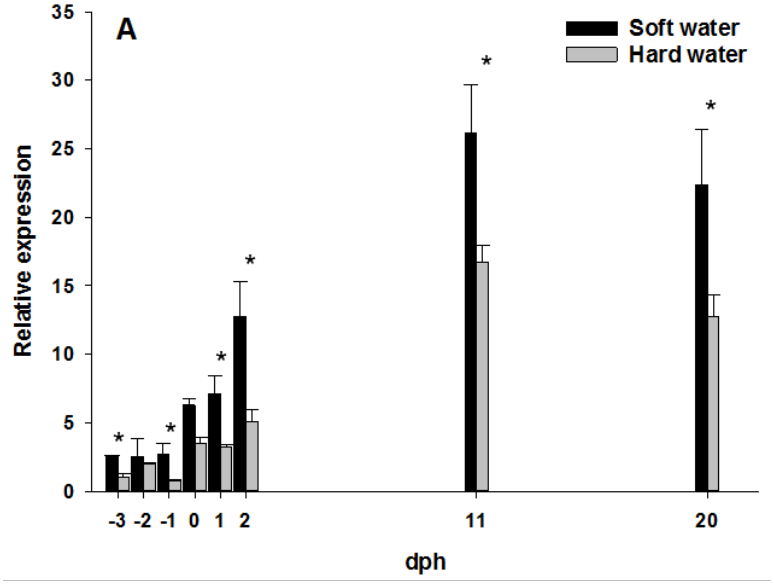


Figure 2.5. Representative RT-PCR gel images showing expression of *nhe* isoforms (*nhe2, nhe3a, nhe3b*) and the housekeeping gene *ef1α* in whole larvae at 11 dph (Panel A) and in tissues of juvenile rainbow trout (Panel B) acclimated to soft (0.1 mM Na^+) and hard (2.2 mM Na^+) waters for 7 days. S/H indicates soft/hard water; NTC = no template control; gill (G), kidney (K), brain (B), intestine (I), and liver (L).


A
Soft water
Hard water
Relative expression
35
30
25
20
15
10
5
0
-3 -2 -1 0 1 2
11
20
dph
*

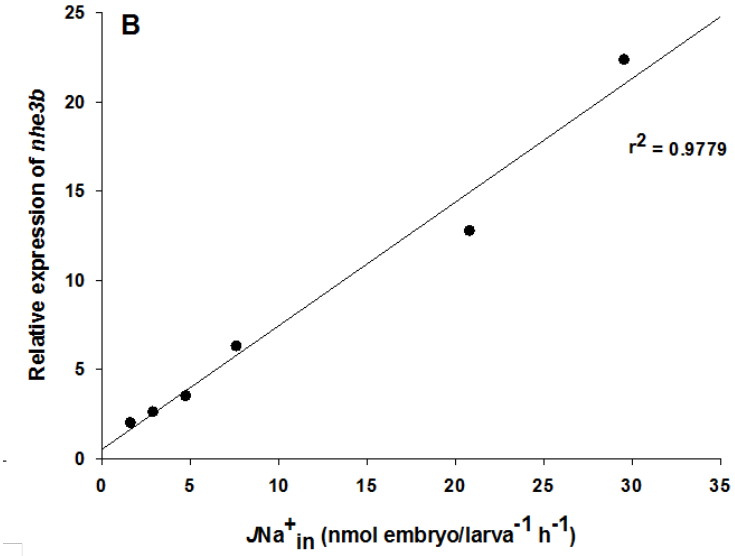

B
Relative expression of nhe3b
25
20
15
10
5
0
0 5 10 15 20 25 30 35
r2 = 0.9779
JNa+in (nmol embryo/larva-1 h-1)

Figure 2.6. (previous page) Expression of *nhe3b* in whole trout embryos/larvae reared in soft (0.1 mM Na^+) and hard (2.2 mM Na^+) water. Expression is shown after normalization to *ef1α* and relative to embryos/larvae reared in hard water at -3 days post hatch (dph). Panel A: * denotes significant differences between treatments (Two-way ANOVA, $p < 0.05$). Data are means ± S.E.M., $n = 3$. Panel B: correlation between mean unidirectional Na^+ flux [$J_{in}Na^+$ (nmol embryo/larvae^{-1} h^{-1})] in soft water and mean relative expressions of *nhe3b* (data duplicated from Fig. 6 Panel A and Fig. 2). Data points are taken from both treatments and all time-points when both measurements were taken.

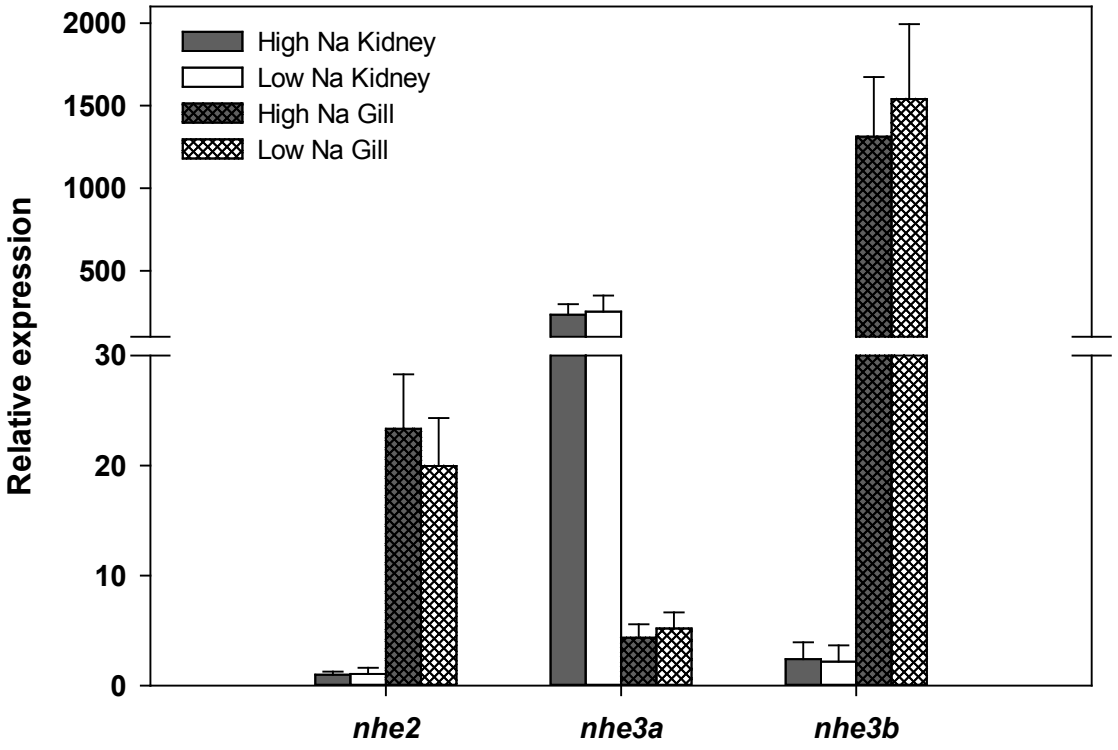


Figure 2.7. Expression of *nhe2*, *nhe3a* and *nhe3b* in kidney and gill of juvenile trout acclimated to soft or hard synthetic waters. Expression of all genes are shown relative to expression of *nhe2* from kidney of trout acclimated to hard water. Data are means ± S.E.M.. There were no significant differences in expression between treatment groups (Student's *t*-tests, $p > 0.05$).

Table 2.1. Gene specific primers used.

Gene	qPCR 5'-3'	RT-PCR 5'-3'	Accession # & GenInfo Identifier
nhe2	*F* – GCCCTCTAGCTCTGTTGTGG *R* - ATCCGGGAATCACTGGAGGA	*F* - ATTGGGCTGATTGTAGGGGC *R* - TCGTTGAACAGGCACTCTCC	Accession: NM_001130994.1 GI: 196049372
nhe3(a)	*F* - AGTCGGCCAAAATGGGTGTC *R* - GCCTTCACCCCTGACCTTTT	*F* - ACCAAGGCCGATGTGGATTT *R* - CACCCCTGACCTTTTGTGGT	Accession: NM_001130995.1 GI: 167534366
nhe3b	*F* - GCATCCAGCTTGAAGATGCC *R* - GCTGGGCCTACTAATGGCAA	*F* -TGCATGAGCAGGACCTGAAG *R* - TCTCATGGGGAATGAACCGC	Accession: NM_001160482.1 GI: 210062149
ef1α	*F* - CTGTTGCCTTTGTGCCCATC *R* - CATCCCTTGAACCAGCCCAT	*F* - CTGTTGCCTTTGTGCCCATC *R* - CATCCCTTGAACCAGCCCAT	Accession: NM_001124339.1 GI: 20269865

Discussion

The molecular identities of proteins involved in apical Na^+ uptake in MR cells in freshwater fish are much debated (see reviews by Dymowska et al., 2012; Parks et al., 2008; Wood and Wright, 2009). Data presented in the current study provide evidence for a principal role of Nhe3b in Na^+ acquisition in rainbow trout larvae in soft water with 0.1 mM Na^+. A role for the other Nhe isoforms was less apparent. Pharmacological profiling of Na^+ uptake in larvae reared from fertilization in soft water with EIPA indicated a dominant contribution of Nhe to Na^+ uptake immediately post-hatch. Further progressive increases in rates of Na^+ uptake throughout development were also commensurate with elevations in expression of *nhe3b* while increases in expression of *nhe2* and *nhe3a* could not be demonstrated. The conclusion of an important role of Nhe3b in Na^+ uptake in low Na^+ waters is given further confidence by observations made in embryos/larvae reared in hard water with a higher [Na^+]. In these embryos/larvae expression of *nhe3b* was lower. When transferred to soft water Na^+ uptake was also lower than in embryos/larvae reared from fertilization in soft water. In fact there was a strong correlation between the rate of Na^+ uptake and expression of *nhe3b* when data from multiple time-points and including embryos/larvae sampled from both treatment groups were incorporated into a single analysis. However, a role for other Nhe isoforms in Na^+ uptake cannot be completely discounted: gene expression analyses were performed on whole embryos/larvae rather than measuring expression in discrete tissues or cell types in embryos.

Increases in whole larva [Na^+] were consistently observed over the time course of post-hatch sampling points (Fig. 2.1). This has been suggested to be due to the development of the circulatory system in salmonid larvae and the growing importance of Na^+ in interstitial fluid (Barrett et al., 2001). These increases, and their magnitudes, were also apparent in both hard- and soft-water reared rainbow trout. This observation is consistent with data from other teleost fish, e.g. zebrafish, where internal ion concentrations (including Na^+) were unaffected by the ion concentrations of the rearing media [35 and 1480 μM Na^+ (Boisen et al., 2003)]. Pre-hatch embryo [Na^+] was also similar between treatment groups, although a direct comparison between embryos was complicated by a 1 d difference in time of hatch. The rate of rainbow trout development is closely correlated with temperature (From and Rasmussen, 1991) and very small differences in water temperature (< 0.5°C) between systems might account for the 1 d difference in time of hatch. Nevertheless, and following normalization of all data to day of hatch, the lack of a significant difference between profiles of whole embryo/larva [Na^+] during development, despite a 22-fold difference in water [Na^+] between treatments, indicates that rainbow trout embryos/larvae may adjust their Na^+ transport physiology to maintain Na^+ homeostasis.

Measurements of Na^+ uptake rates were performed in soft water, only. Therefore, no inference can be drawn from these data as to mechanisms of Na^+ acquisition and Na^+ homeostasis in hard water; however, comparative flux data from embryos/larvae reared in soft and hard water can provide better insight

into mechanisms of Na^+ homeostasis in trout embryos/larvae under conditions of low [Na^+]. Soft water reared embryos/larvae exhibited higher rates of Na^+ uptake at all time-points tested, and especially post-hatch, indicating a difference in physiology between the two groups (Fig. 2.2). However, inhibition of Na^+ uptake by EIPA (and also amiloride) reached near maximal levels (approximately 90%) at the highest doses in both groups of larvae indicating the principal role of Nhe, despite theoretical thermodynamic constraints to Nhe function at 0.1 mM Na^+ (Parks et al. 2008; Dymowska et al., 2012). These data are given extra support since they were observed both immediately post-hatch (Fig. 2.3A) and in larvae at 20 dph (Fig. 2.4A). Previous studies have indicated similarities in morphologies of ionocytes in gill and on yolk sac of embryos/ larvae and the data in the present study suggest close similarities in physiology also. Furthermore, when flux data were expressed as a percentage of the respective control (soft or hard water reared larvae) the increments of inhibition of Na^+ uptake observed with increasing doses of EIPA were not different between groups (Figs. 2.3B and 2.4B). These data strongly suggest that differences in mechanisms of Na^+ homeostasis in soft water between groups were mediated at the level of uptake i.e. through activity of Nhe rather than by differences in Na^+ efflux. If efflux was lower in soft water reared embryos the profile of EIPA inhibition would be different compared to hard water reared embryos, and this was not observed.

An EIPA-sensitive component of Na^+ uptake has been previously demonstrated in goldfish (Preest et al., 2005) and in larval zebrafish (Esaki et al.,

2009). Under conditions of low Na^+ (0.03 mM) and low pH (pH 6.0) a DAPI-sensitive Asic mediated Na^+ uptake mechanism has also been reported in juvenile rainbow trout (Dymowska et al., 2014). In the present study, a role of Asic cannot be discounted. Furthermore, Nhe function at lower water [Na^+] could still occur in embryos by a combination of either a lower intracellular pH (pH_i < 7.0) or a lower intracellular [Na^+] (< 0.5 mM) than used in our previous calculations of the thermodynamic limitation of Nhe function (Parks et al., 2008).

Recently, Brix and Grosell (2012) demonstrated the involvement of a higher affinity Na^+ transport pathway in a freshwater subspecies of coastal pupfish (*Cyprinodon variegatus hubbsi*) acclimated to 0.1 mM Na^+ compared to 1 mM Na^+. Interestingly, they also noted that both transport pathways were EIPA sensitive leading them to speculate that the Nhe isoforms, Nhe2 and Nhe3, in *C. v. hubbsi* may have different Na^+ transport affinities [as reported for mammalian NHE2 and NHE3 (Orlowski, 1993; Yu et al., 1993)] and be differentially utilized at 0.1 and 1 mM Na^+. In the present study, *nhe3b* expression in whole embryos/larvae from both treatment groups increased throughout development but was significantly greater in larvae in soft water. The level of expression of *nhe3b* was also highly correlated with Na^+ uptake rates in soft water across all time-points and both treatment groups (Fig. 2.6). Although *nhe2* was detectable with RT-PCR (at 11 dph, Fig. 2.5), expression was lower than *nhe3b*, and was not consistently quantifiable with qPCR. Expression of *nhe3a* was poorly detected in whole embryos/larvae with both techniques and observations in juvenile rainbow trout tissues [also reported in zebrafish (Yan et al., 2007)] indicate that

Nhe3a is a kidney specific isoform (Fig. 2.5). While gene expression analyses lack the functional significance of protein quantitation, these data, together with data demonstrating EIPA sensitivity of Na^+ uptake, provide evidence for a specific and principal role of Nhe3b in Na^+ uptake in soft water.

Data showing a role of Nhe3b in Na^+ uptake are in agreement with other studies. Expression of Nhe3b has been localized to the yolk-sac membrane of tilapia larvae and the gills of rainbow trout (Hiroi and McCormick, 2012). At low [Na^+] *nhe3* (presumably the orthologue of trout *nhe3b*) was demonstrated to have increased in expression in gills of Mozambique tilapia [*Oreochromis mossambicus* (Inokuchi et al., 2009)], Japanese ricefish [*Oryzia latipes* (Wu et al., 2010)] and in Japanese eel (*Anguilla japonica*), expression of Nhe3b at both protein and mRNA levels were reportedly increased upon transfer to distilled water [ultra-low Na^+ (Seo et al., 2013)]. A functional role of Nhe2 has also yet to be elucidated despite gene expression at the gill, albeit comparatively lower than *nhe3b*, being documented in the present study (Fig. 2.5) and in the data of others. For example, Craig et al. (2007) reported increases in *nhe2* expression in the gills of zebrafish following 6 days acclimation to soft water. Increased gill expression of *nhe2* has also been reported in response to hypercapnia (Ivanis et al., 2008) and on exposure to high environmental ammonia (Zimmer et al., 2010) although the latter response has not been consistently observed (Nawata et al., 2007; also in skin of trout Zimmer et al., 2014b). Clearly, the regulation of expression of *nhe* isoforms at the gill is complex and requires further investigation.

In conclusion, pharmacological and gene expression profiling of Na^+ uptake in trout larvae reared in a low Na^+ environment support a primary role of Nhe3b. The roles of Nhe proteins and other transporters in Na^+ uptake in low Na^+ (and low pH) environments are the subject of an on-going discourse regarding their roles and activities under hypothetical thermodynamic constraints. The data presented herein indicate Nhe proteins likely can function in embryos/larvae under these conditions, and upregulation of Nhe3b may underpin the increased Na^+ uptake rates required to increase Na^+ acquisition post-hatch.

CHAPTER III: Expression and Pharmacological Profiling of Rainbow Trout nhe2, nhe3a, and nhe3b

Introduction

Current models for ion regulation and acid-base balance in freshwater fish gills have mainly been based on both immunohistochemistry and *in vivo* pharmacological experiments (see Reviews Hwang et al., 2011; Dymowska et al., 2012; Hiroi and McCormick, 2012). Many of the *in vivo* experiments incorporate the use of radioisotopes and pharmacological drugs aimed at blocking certain ion transporters or channels in order to inhibit function (Kirshner, 1973; Wright and Wood, 1985; Avella and Bornancin 1989). Unfortunately to date, the vast majority of interpretations from the *in vivo* experiments utilizing inhibitory drugs have been based on the known pharmacological profiles and interactions of mammalian ion transporters. This extrapolation is especially true of fish physiological studies on the mechanisms involved in Na^+ uptake from the environment (Dymowska et al., 2014). However, these profiles have not been confirmed for fish transporters and additionally, multiple Na^+ transport pathways exist in gill ionocytes making results from pharmacological inhibition studies difficult to accurately interpret (See reviews Dymowska et al., 2012; Claiborne et al., 2002; Hwang et al., 2011).

The NHE is a major ion transporter involved in ion regulation and acid base balance and is found throughout the animal kingdom. Its many isoforms have been well characterized in mammalian literature (Donowitz et al., 2013). Although apically located on the brush border membranes of the intestine and kidney and involved in some Na^+ absorption at these areas, the physiological role of human NHE2 is not well understood (Hoogerwerf et al., 1996; Donowitz

et al., 2013). Studies reveal that in mammals NHE2 accounts for less than half of the NHE activity and at times no Na^+ absorption at all where it is located. On the other hand, NHE3 seems to be the major player in mammalian intestinal and renal Na^+ absorption (Hoogerwerf et al., 1996). In mammals NHE pharmacology has been characterized in various studies. Counillon et al. (1993) demonstrated k_i values (concentration of drug that results in half the maximum inhibition) for amiloride on NHE1, NHE2, and NHE3 as 3 μM, 3 μM, and 100 μM, respectively. Overall, NHE3 was the most resistant to amiloride inhibition. This pattern was the same for the other amiloride derivatives used in the study including DMA, MPA, and HOE694, with NHE1 being most sensitive, followed by NHE2, and NHE3 demonstrating highest resistance (Counillon et al., 1993).

Many whole animal fish studies incorporate the use of amiloride and its derivatives to indicate the presence or absence and function of transporters including the study in Chapter two. The drugs and concentrations used are based on those previously demonstrated on mammalian models. The only fish pharmacological characterization studies involving Na^+ transport include one recent study from our laboratory which featured the cloning and characterization of Pacific dogfish shark (*Squalus suckleyi*) Nhe2 and Nhe3 following expression in the AP-1 NHE-deficient cell line (Guffey et al., 2015) and a previous study on zebrafish (*Danio rerio*) Nhe3b expressed in *Xenopus* oocytes (Ito et al., 2014).

As previously stated, NHE transporters are found within almost every mammalian cell and across much of the animal kingdom. AP-1 cells and other

cell lines, which have been genetically engineered and selected to be deficient of endogenous NHEs, provide an efficient system to utilize for characterization of NHEs. Because they are void of endogenous NHE, researchers are confident that the majority of sodium hydrogen exchange activity occurring can be attributed the specific NHE isoform with which they have transfected the cells. This is an advantage these cells possess over the highly utilized *Xenopus* expression system, which contains an amiloride-sensitive endogenous NHE necessary for cell volume regulation (Towle et al., 1991; Goss et al., 2001). AP-1 cells have been widely used for mammalian NHE characterization (Orlowski, 1993; Wang et al., 1998; Murtazina et al., 2001) and shark Nhe (Guffey et al., 2015), and have been chosen for this study as the best expression system for the various trout nhe isoforms.

We know that rainbow trout contain at least 3 isoforms of Nhe, Nhe2, Nhe3a, and Nhe3b. From previous research by Ivanis et al. (2008) and confirmed by my data in Chapter two, we know that trout *nhe2* and *nhe3b* are highly expressed in the gills, while *nhe3a* is found mostly in the kidney. We have made conclusions on the presence of these isoforms based on mRNA data as well as pharmacological inhibition. However, as stated previously we are limited in our conclusions from the pharmacological data because a full pharmacological profile of Nhe inhibitors on teleost Nhe isoforms does not yet exist. The goal of this study was to clone all three isoforms from rainbow trout and express each of them separately in the AP-1 mammalian cell line deficient of NHE isoforms in

order to characterize their pharmacological inhibition in the presence of common Na^+ transport inhibiting drugs.

Methods

Animals

Rainbow trout were raised from embryos generously donated from Allison Creek Brood Trout Hatchery, Coleman, Alberta. Embryos were maintained in Heath trays with aerated 10°C flowing dechlorinated Edmonton city tap water until hatch and then maintained in facility water until use. All animal use was approved under University of Alberta AUP00001126. All trout were euthanized in buffered MS222 prior to tissue sampling. Filaments from right and left gill arches along with kidney tissue were excised and immediately frozen in liquid nitrogen and stored at -80°C for downstream RNA isolation.

RNA Isolation, cDNA Synthesis, Cloning

Rainbow trout frozen gill and kidney tissues (50-100 mg) were used for TRIzol Reagent (Ambion, Life Technologies, Carlsbad, CA) RNA extraction according to the manufacture's protocol. The resulting RNA pellet was resuspended in 50 µL of nuclease-free water and stored at 4°C to ensure full RNA solubility. RNA was checked for quality and purity via spectrophotometry (NanoDrop, ND-1000; Thermo Fisher Scientific, DE, USA) and underwent formaldehyde RNA gel electrophoresis to further check RNA integrity. 10 µg of RNA was treated for DNA contamination with Recombinant DNase I (Ambion)

and 2 µL of DNase-treated RNA was used in the subsequent 1st strand complimentary DNA (cDNA) synthesis using SuperScript III Reverse Transcriptase (Invitorgen) with a mix of random and oligo(dT) primers as per manufacturer's protocol.

Cloning strategies differed for each of the 3 trout *nhe* isoforms with the goal of insertion into the pDisplay plasmid vector mammalian expression system (generously donated by Dr. James Stafford, University of Alberta). Due to initial complications with amplifying the gene using restriction sites incorporated directly into initial primers for insertion into pDisplay, prior cloning into pBluescript plasmid was required. Full length gene primers were designed for *nhe2* and *nhe3b* (Table 3.1) and amplification from gill cDNA with Phusion High-Fidelity polymerase (NEB, Ipswich, MA) RT-PCR resulted in a ~2.6 and ~2.8 bp amplicon, respectively. PCR products were cleaned up with QIAquick PCR Purification Kit (Qiagen) and inserted into pBluescript SK cut with EcoRV restriction enzyme and ligation was completed using T4 DNA Ligase (NEB). TOP10 chemically Competent *E. coli* or DH5α cells (ThermoFisher Scientific) were transformed with ligated plasmids containing either trout nhe2 or nhe3b inserts. Colonies were grown overnight on LB Ampicillin (100 µg/mL) plates and underwent blue-white screening. Positive colonies were picked and cultured in 5mL of LB-Amp media at 37°C overnight. Colony PCR was performed on selected bacterial colonies from each of the plates containing individual nhe isoform transformed bacteria. Plasmids were then isolated from bacterial cultures using GeneJET Plasmid Miniprep Kit (ThermoFisher Scientific).

Restriction digest of resulting plasmid DNA along with sequencing was performed to confirm presence of specific nhe in the pBluescript vector. New primers containing Sma1 and Sal1 restriction sites (Table 3.1) were designed against trout *nhe2* and *nhe3b* and PCR amplification was run using the resulting plasmid DNA as a template. Following PCR clean up, the resulting PCR products were then ligated into pDisplay Vector (Invitrogen), which were previously digested with Sma1 and Sal1 restriction enzymes. The procedure for transforming *E. coli* bacterial competent cells and isolating plasmid DNA was repeated with this ligation product. The resulting plasmid DNA (pDisplay containing the nhe inserts) was used for transfection of the nhe-deficient mammalian AP-1 cell line.

The cloning procedure for trout nhe3a was less complex. Specific primers containing Sma1 and Sal1 restriction sites (Table 3.1) were designed and direct amplification from trout kidney cDNA resulted in a ~2.2 bp fragment. Following PCR cleanup as above, it was ligated into Sma1 and Sal1 digested pDisplay. Identity of the product was confirmed by sequencing and alignment using Clustal Omega software.

Cell Culture

The NHE-deficient cell line AP – 1 was generously donated by Dr. Larry Fliegel. Department of Biochemistry, University of Alberta. Cells were thawed from frozen stocks by immersing them in 37°C water bath until a small piece of ice is left. Cells were then added to a tube containing pre-warmed MEMα (Gibco)

supplemented with (10% Fetal Bovine Serum, 25 mM HEPES, 2% penicillin-streptomycin solution, pH 7.4). Cells were spun down (5 min at 300 x g), then re-suspended in fresh media and transferred to a cell culture plate. Once cells were confluent (covering 90-100% of the surface of the plate) cells underwent passage by removal of old media, followed by washing cell layer with a PBS solution, which was then removed, followed by 1 mL of 0.05% trypsin-EDTA being added to plate. Cells were then incubated at 37°C for 5 min to allow for cell detachment from the plate. Fresh media (2 mL) was added to the plate to deactivate trypsin and cells were harvested. 100 µL of cell suspension were added to new plate containing 10 mL fresh MEM-α media and cells grew to confluence and passed every 3-4 days. Once cells underwent 20 passage cycles, cells were re-frozen and newly thawed cells from previous time points were thawed and used. AP-1 cells used for transfection were used only in the range of 3-15 passages.

Transfection

Procedures for transfection of AP-1 cells with trout nhe2, nhe3a, and nhe3b were performed according to the Lipofectamine® 2000 Transfection Reagent manufacturer's protocol. Cells were grown to 70-90% confluence in a 24-well plate. In brief, 1 µg of plasmid DNA at a concentration of ~500 ng/µL was added to 50 µL serum free media, while 5 µL of Lipofectamine reagent was simultaneously added to 50 µL of serum-free media. These solutions were then mixed and allowed to incubate for 5 min at room temperature (21°C) in order

for DNA-lipid complexes to form. Fifty μL of the resulting DNA-lipid complex solution was added to each well (in duplicate) yielding a final concentration of 500 ng of DNA and 2.5 μL of Lipofectamine per individual well of cells. Following the transfection procedure, cells were incubated at 37°C for 24-48 hrs, and then passed into normal culture media (MEM-α) containing 800 μg/mL geneticin (G418), selecting against negatively expressing cells. Cells were allowed to grow under these conditions at 37°C to confluence with fresh media changes every 2 days. After 3 weeks, cells underwent a selection technique exposing them to an acute acid load (Wang et al 1998; Franchi et al 1986) to ensure they expressed nhe. Culture media was washed away with PBS and cells were then incubated in isotonic NH_4Cl buffer (50 mM NH_4Cl, 70 mM choline chloride, 5 mM KCl, 1 mM $MgCl_2$, 2 mM CaCl, 5 mM glucose, pH 7.4) for 60 min at 37°C in nominally CO_2 free atmosphere. Cells then rapidly washed (x2) with isotonic saline solution (120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM CaCl, 5 mM glucose, 20 mM HEPES, pH 7.4) to remove extracellular NH_4^+. This technique causes the initial buildup of NH_4^+ in the cell which then dissociates into NH_3 and H^+, resulting in strong intercellular acid loading and drops in pH_i, which must be alleviated by the function of NHE, or the cells will die. Cells that survived the acid suicide technique were selected for and allowed to continue to grow in normal culture media (MEM-α) containing geneticin (G418).

Nhe Activity Assays

Two different methods were utilized in order to measure the activity of trout nhe transfected AP-1 cells. The first method involved intracellular pHi imaging. Following early attempts at transfection, cells were grown on pretreated (1 M HCl acid-washed, 0.1% poly-L-lysine-coated, and rinsed with double-distilled H_2O and 70% ethanol) 15-mm round glass coverslips (catalog no. CS-15R, Warner Instruments). Excess media was removed and cells were briefly rinsed in Na^+-containing buffer (142.5 mM NaCl, 5.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 4.0 mM KCl, 15 mM HEPES, and 2.5 mM $NaHCO_3$, pH 7.4). The cells were then incubated in 200 µL of Na^+-containing buffer, which contained 2 µL of 5 mM pH-sensitive BCECF-AM (50 µg in 16 µL of DMSO and 20% pluronic acid), for 30 min at 37°C. Coverslips were placed into a 70 µL imaging chamber (model RC-20H, Warner Instruments) used for the perfusion experiments. The chamber was fixed to an inverted fluorescence microscope (Nikon Eclipse TE300), and the cells were subjected to differential interference contrast microscopy and fluorescent imaging. The microscope was fitted with a xenon arc lamp (Lambda DG-4, Sutter Instruments, Novato, CA), which allow for BCECF-AM excitation at 495 and 440 nm. Images were digitally captured every 1.7 s during perfusion experiments at an emission wavelength of 535 nm on a mono 12-bit charge-couple device camera (Retiga EXi, QImaging, Burnaby, BC, Canada). Ratios of fluorescence emission intensity following excitation at 495 nm versus excitation at 440 nm (detected at 535 nm) were digitally compiled using Northern Eclipse software (Mississauga, ON). Solutions were manually perfused at a rate of 0.5

mL/min (fluid replacement time of 5 sec) across the cell chamber by gravity feed from an apparatus containing 60mL syringes blocks controlled by pinch valves (model VE-6, Warner Instruments) and VC-6 valve controllers (Warner Instruments). The solutions were constantly being removed from the opposite end of the chamber via vacuum suction. Cells were monitored at resting state while being perfused with a Na^+ -free buffer (142.5 mM N-methyl-D-glucamine-Cl, 2.5 mM $C_5H_{14}NO{\cdot}HCO_3$, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 4 mM KCl, and 15 HEPES, pH 7.4). A 3-min pulse of Na^+ free buffer containing 10mM NH_4Cl was perfused over the cells, and then returned to Na^+-free buffer, which resulted in intracellular acidification. Cells were then allowed to recover in the presence of Na^+ (indicated by pH_i alkalization) in order to monitor the resulting Nhe activity. For pharmacological inhibition of Nhe, similar perfusion protocol steps were used, with the addition either 100 μM EIPA or DAPI in the presence of Na^+ to monitor any inhibition of recovery. Following the end of the perfusion, cells underwent a pH_i calibration by which four high K^+ solutions adjusted to (pH 6.6, 7.2, 7.8, and 8.4) containing the ionophore nigericin (5μM) in order to equilibrate intercellular and extracellular pH. The resulting pH-dependent ratios of the emission intensity (detected at 535 nm) of 490 versus 440 nm were calculated at each time point and used to form a regression line for each individual cell; then extrapolated across the entire raw data set for calibration

purposes. The data (ΔpH_i/time) was collected and compared between cells under control and in the presence of the pharmacological inhibitor.

The second method of characterizing Nhe activity utilizes a similar approach, this time with fluorescence measurements on the cell population as a whole as opposed to individual cells, and has been demonstrated multiple times by the Fliegel lab (Wang et al 1998; Murtazina et al 2001; Slepkov et al 2005; Ding et al 2006; Li et al 2011). In brief, transfected AP-1 cells were grown on rectangular coverslips (Thomas Red Label Micro Cover Glass Squares), which can be inserted into a manufactured holder specific to the PTI Deltascan spectrofluormeter. Cells are grown on converslips to 90% confluence and then incubated for 20 min in 400 µL serum-free media (MEM-α) containing 1.9 µg/mL BCECF-AM. Coverslips are immersed in cuvettes containing the required solution and then cuvettes are transferred to the flurometer for measurement of activity. Cells are subjected to Na^+-containing buffer (135 mM NaCl, 1.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 5.0 mM KCl, 10 mM HEPES, and 5.5 mM glucose, pH 7.3) for 3 min. Fifty µL of a 2.5 M stock NH_4Cl solution was added for a final concentration of 50 mM NH_4Cl to the bathing solution for an additional 3 min. Cells were then exposed to a Na^+-free buffer (135 mM N-methyl-D-glucamine-Cl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM glucose, and 10 mM HEPES, pH 7.3), which resulted in internal acidification. Cells were then allowed to recover in normal Na^+-containing solution where rising pHi would indicate positive Nhe activity. Calibration is then performed with cells exposed to three pH adjusted (8.0, 7.0, and 6.0) high K^+ buffers containing nigericin as above.

AP-1 Cell Nhe Expression

Following transfections and continued successful growth, samples of each transfected cell line, as well as non-transfected cells, were harvested and spun (2 min, 12,000 x g) to pellet cells. Cell pellets were then frozen in liquid nitrogen and stored at -80°C for downstream RNA isolation and cDNA synthesis. RNA isolation was performed using the NucleoSpin RNA kit (Macherey-Nagel, Germany) as per the manufacturer's protocol, with the addition of the initial homogenization buffer directly to the frozen cell pellet to avoid any degradation. Total isolated RNA was checked for purity and concentration via spectrophotometry (NanoDrop, ND-1000, Thermo Scientific, Wilmington, USA). SuperScript III reverse transcriptase (Invitrogen) was used to perform the cDNA synthesis reaction with a mix of oligo(DT) and random primers on 1.5 μg RNA template as per manufacturer's protocol. RT-PCR was performed using isoform specific primers to confirm presence/absence of Nhe expression in the cDNA templates (Table 2.2).

Following transfections, in addition to gene expression analysis, protein expression against the HA-tag incorporated in to pDisplay was also performed with Western Blot using HA-tag Antibody [HRP], (polyclonal antibody generated in goat; GenScript) generously donated by James Stafford. A positive control protein sample from Larry Fliegel's lab was run in addition to protein homogenates isolated from AP-1 cells transfected with the three trout nhe

isoforms. In brief, cell pellets were homogenized with a motorized hand homogenizer (Gerresheimer Kimble Kontes LLC, Düsseldorf, Germany) in cell lysis buffer (150 mM NaCl, 50 mM Tris, Complete EDTA-free Protease Inhibitor, PhosStop, 1% Triton X, pH 8.0). Homogenates were spun at (10min, 14,000 x g) and resulting supernatants were diluted in equal volumes of reducing buffer (Laemmili Sample buffer, 2-Bercaptoethanol, BioRad), and boiled at 95°C for 10 min prior to protein separation via electrophoresis. Twenty-five µL of protein homogenate from each sample including control were separated via SDS-Page gel on gel and transferred to a nitrocellulose membrane (BioRad). Ponceau S (Sigma) staining confirmed successful transfer and visualization of relative amounts of protein in each sample. Blots were then incubated in 5% skim milk in TBS with 0.1% Tween for 20 min, followed by overnight incubation in HA-HRP conjugated antibody in TBST (1:2000). Following three washes in TBST and a final wash in TBS, immunoreactive bands were visualized using a Super Signal chemiluminecent substrate kit (Thermo Scientific).

Results

Cloning and Sequencing

PCR amplification resulted in full-length gene fragments for trout *nhe2*, *nhe3a*, and *nhe3b* were cloned from gill (*2, 3b*) and kidney (*3a*) (Figure 3.1). These genes were then successfully ligated in vectors and restriction enzyme digested to confirmed the presence of the insert. Gene specificity was also confirmed with sequencing and alignment to previously reported sequences for

trout *nhe* isoforms (Figure 3.2-3.4). Alignments were made over the open reading frame from the starting methionine (ATG) to the last nucleotide before the stop codon (TAG), which was excluded in the cloning process in order to have transcription take place through the polyadenylation site in contained in the pDisplay plasmid. Sequenced trout gill *nhe2* had a 99.28% identity to available trout *nhe2* sequence, which included 17 nucleotide differences between the sequences (Ivanis et al 2008). Sequenced trout kidney *nhe3a* shared a 99.82% identity to trout *nhe3* sequence (Ivanis et al 2008). The sequenced trout gill *nhe3b* was 100% identical to the available trout *nhe3b* sequence (Hiroi and Yasumasu unpublished).

Transfection

AP-1 cells transfected with either trout *nhe2*, *nhe3a*, or *nhe3b* were maintained in MEMα medium supplemented with 800μg/mL geneticin. The cells maintained slow initial growth for the first 2 weeks in the presence of geneticin, however after 2 passages, growth rates returned to close to that of non-transfected AP-1 cells. The successful growth in the presence of geneticin prompted the use of the acid suicide technique as the second selecting agent. A significant die-off of cells occurred following the acid selection, however surviving cells continued to grow in G418 media over the next few days. Sustained growth rates and normal cell morphology indicated cells were ready for Nhe assay activity testing.

Intracellular pHi Imaging

Initial testing performed on cells one-day post transfection indicated little to no activity. Cell morphology post-transfection was also altered, and cells appeared to be still in the recovery phase and not spread out (Figure 3.5A-C). During the perfusion experiments cell activity was normal during the initial NH_4Cl-induced alkalization and subsequent acidification. However, the Na^+-induced alkalization which should occur following the addition Na^+-containing buffer was absent from all cell traces, including the untransfected AP-1 cells which was expected, and also from the trout *nhe2* transfected cells, as indicated in the representative cell traces (Figure 3.5D,E). Not a single cell showed any form of Nhe activity during these initial tests.

Spectrofluormeter Nhe Assay

After a second round of transfection a successfully growing group of cells was assayed for activity. Similar to the previous imaging technique, this method also produced negative results for all of the transfected AP-1 cells. Again cells responded well to the initial buffers and followed expected patterns of acidification following NH_4Cl injection. However, the expected rise in pHi attributed to the presence of Na^+ activating the expressed Nhe was minimal to absent for any of the populations of cells tested (Figure 3.6).

Expression of Nhe

In order to confirm expression of Nhe in the AP-1 cells, RT-PCR was performed on cDNA synthesized from isolated RNA. Using short primers specific to the various trout isoforms, gel electrophoresis revealed corresponding bands in the original pDisplay DNA containing the gene inserts, however no bands were present in the transfected AP-1 cell cDNA samples, indicating that these cells were indeed not expressing the transfected Nhes (Figure 3.7).

Western blot analysis demonstrated the absence of HA immunoreactivity in any of the transfected cell protein samples analyzed with the exception of the positive control sample donated by the Fliegel lab. The lack of immuoreactive bands for any of the samples indicates a lack of expression of the HA tag (Figure 3.8).

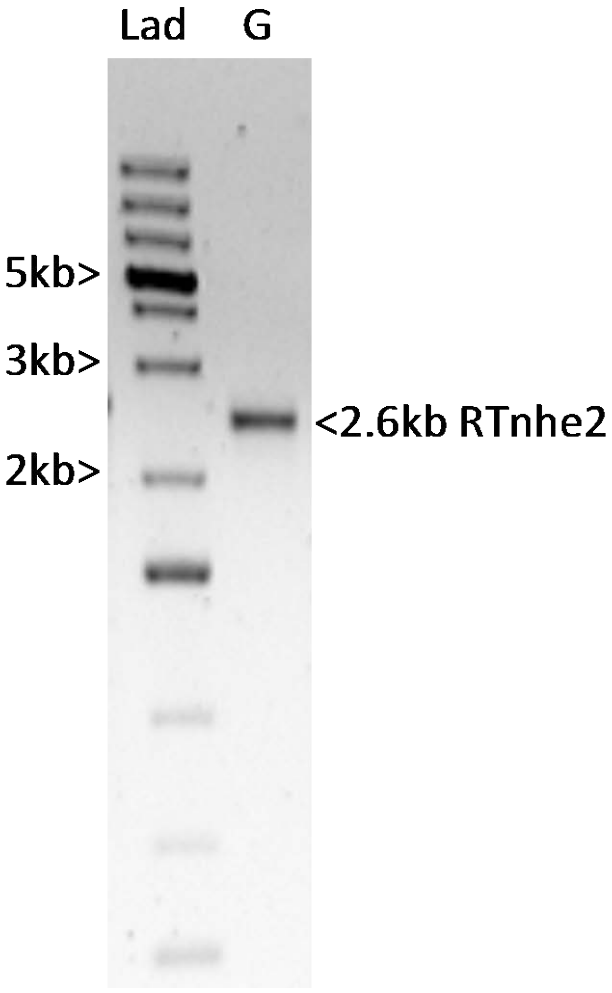


Figure 3.1. Agarose gel electrophoresis of RT-PCR for *nhe2* gene expression. A single 2600 bp sized band was amplified in the gills (G) of rainbow trout indicative of presence of *nhe2*.

5’_ATGCGTACTATGGGAGGATTTAGCCAGATGAAACTGGCAGTTAACTTTTTAACCATCGGATTGGTTATC
CTTAGTCTACCATGTGGATCATACACGGAGATTCCACCCAAACCCCCTGGTTCCGTGACCGTCCTACCCCCT
GTCAAGCCAGCGGGGGGTCCACAAGCGTTTCCCGACGCGGAGAACTCGAATCTCCCGGTCTTCACAATGG
ATTATGCCCGTATACAGGTGCCGTTTGAGGTCACGCTTTGGGTGCTGCTCGCGTCATTCGCCAAGATTGGT
TTCCATGTGTACCACAAGATTACCTTCTGGGTCCCAGAGTCATGTCTCCTGATCAGTATTGGGCTGATTGTA
GGGGCCATCATGCACTCTGTCCACGAGGAGCCCCCTGCTGTGCTCAGCTACAATGTCTTCTTCCTCTTCATG
CTGCCTCCCXATCGTACTGGAGTCTGGATACTTTATGCCCXACGCGACCGTTCTTCGAGAACGTGGGAACX
GGTGCTTTGGTTTGCGGTGGTGGGCACCCTGTGGAACAGCATTGGGATAGGGATGTCTCTGTATGCGGTG
TGTCAGATCGAGGCGTTTGAGGTGCAGGACATTAACCTTCAGGAGAACATGCTGTTTGCCGCCATCATCTC
AGCTGTGGACCCAGTGGCCGTGCTCAGCGTGTTCGAGGATGTCTCTGTCAACGAGCAGCTCTACATCGTG
GTGTTTGGAGAGTGCCTGTTCAACGATGCTGTCACTGTGGTGCTGTACAACATGTTTATCTTCGTAGCAGAT
ATGCCAGTGGTGGAGCCGGTCGATGTATTTCTGGGCGTGGCTAGGTTCTTCGTGGTGGGCCTGGGGGGTA
TGGGCTTTGGTGTCATCTTTGGCTTCGTAGCCTCCTTCACCACACGTTTCACCTCTAACGTCAGGGAGATAG
AACCCCTCTTTATCTTCATGTACAGCTATCTGGCCTACCTGGTAGCAGAGCTGTTCTCCATCTCCAGTATTAT
GGCCATTGTGACCTGTGCCCTGACCATGAAGTACTATGTGGAGGAGAATGTCTCCCAGCGGTCCTGCACCA
CTATCCGCCATGTGGTCAAGATGTTGGGCACCATCTCAGAAACCCTCATCTTCTTCTTCCTGGGTGTGGTCA
CCATAACAACAGAACACGAGTGGAACTGGGGCTACATCATGTTCACGCTGCTGTTGGCCTTAGTGTGGAG
AGGCCTGGGTGTCCTGGTGCTGACCCAGATCATTAACCCTTTCCGAACCATCCCCTTCAACTTCAAGGACCA
GTTTATTCTGGGTTATGGTGGCCTGCGAGGGGCAATCTCCTTTGCCCTGGTCTTCACCCTGCCAGACAGCAT
CGGCCGCAAGAAACTCTTTGTCACCACCACCATCTGTATGATCATGTTCACAGTCTTCATCCAGGGAATCAG
CATACGGCCCCTGATCGAGTTCATCAACGTCCGCAGAACCAACCGCAACCTGGAGACCATAAACGTAGAG
ATCCACTCCAGGCTCATAGAGCACACCATCGCTGGGGTAGAGGACCTCTGTGGCCAGTGGAGCCACTACT
ACTGGAAAGACAAGTTTCTGAAGTTTAATAACCGGATCCTGCGTAAGATCCTGATCAGGGATAACCGGGC
AGAGTCTAGCATTGTGGCTCTGTACAAGAAGCTGGAGCTGCAGAACGCAATGGAGATACTGGACACAGTG
TCCGGGGACATCAACGCTGGTTCCTCCATTGTCTCTCTACACGAGGAGAAGAAGAGCTCTGGTAAACCCAA
GAGGAAGTTTGTGGCTGCAGACCTGATGAACATGCATGACATTCTGTCCAAGAACATGTACAAGATCAGA
CAACGGACCACGGCGTACACCACCAAGCACTCCCTGCCCAATGACAGCCGGACAAAAGAGATCCTGATCC
GACGGCACACCAGCATCCGACGCAGCCTCCGACCCGGCAGCTTCAACGGACCGAATGAGGCCAAATCCCA
GAAGTACTTTTCTCTGCCTCCAGGGAAGAGTCTGGACTCAAAGTTCCCTCCTCAGAGACTGAGGCATACAG
GTAATGACGATGATGAGACCATGTCGGAGATGGATTACCCATCAGCGCGCTCCAGGTTTAACCAGCCCAG
TCGCTCCTCATCCCGAGCAATGCTGCCCCTACGCAGGCTGAACACACTGACAGAGGTGCCCTCTAGCTCTG
TTGTGGACCTGGTGGATGAGAGGAGTGGAGGAAGAGGTGCCTGGGGGCGTGGCATGTCCCGACTAAACT
CCTCCTCCAGTGATTCCCGGATACCCGCCCCTCGCCGTCACCACGACCCCTCCAACGGCCCTGATAATGGCT
CCTCAGGTGACCATGGCAGCGGTGACAACGGGCCAGAGGGGGAGCAGCAGCCCTTGTCTCCGACCCCAG
CCTGGGCTGCAGAGCCTGGTGACCGTGACGACAGCGGAACGCAGAAACCCCTGCTCAGGCCACAGTGGA
AGACTAGGAAACCCGTCGACGAACAAAAACTCATC _3’

Figure 3.2. Cloned sequence of trout *nhe2* from gill.

5’_ATGGCATGCAGAACTTGCCTCTGGCTGCTAGGGACAACATTTTTTACTTGCACAGTCCGCCTGGTTGTTG
AGGGTACGGAGGTGGGAGCAACCCAGGGGTCGCAACCGGAGCCAGGGGTGGTGGGATCAAACCACAGC
CAGGCTGGAGGGGGCCATGGGTCCAGCATCTCAGGGATACAAATCGTGACCTTTAAGTGGCACCATGTGG
AAGCCCCCTACCTCATCGCCTTATGGATACTTGTGGCTGGGGTATCCAAGATGGTGGTCGAGCTGAACCAC
AGCCTGACCAGTGTGATCCCGGAGAGCGCCCTCCTCATCCTCATCGGTTGGATCCTGGGCGGCATCATCTA
CGGGGCAGACAAGATGCAGACCTTCACCCTGACACCTACCGTCTTCTTCTTCTACCTGCTGCCCCAAGTCAT
CCTGGATGCGGGCTACTTCATGCCCAACAAGCTGTTCTTCAGCAACATGGGCGCCATCCTGGTTTACGCTGT
GATTGGCACCTGCTGGAACGCTGCCACTGTGGGTCTCGGCCTCTGGGGGTGCTGGTTGGGCGGGGCAATG
GGTGATATCGACATTGGTCTCCTTCAGTTCCTGTTGTTTGGGAGTCTAATTGCTGCTGTGGACCCCGTGGCT
GTAATTGCCGTGTTTGAGGAGGTCCATGTCAACGAGGTCCTGTTCATCATGGTGTTTGGGGAGTCCCTCCT
CAACGATGGTGTCACAGTGGTGCTGTTCAATGTGTTTGATGCATTCGTGTCGCTGGGAGGGGCAGAAATC
GATGCTGTAGAGATCATCAAAGGCATAATCTCGTTCTTCGTGGTGGCATTCGGAGGCTCCCTGGTTGGTAT
TATCTTTGCTCTGCTGATCTCGCTCCTGACCAGATGCACAAAGAACATCCAGATCATCGAGCCAGGCTTCAT
CTTCATCCTGGGCTACCTCTCCTACCTGACAGCTGAGATGCTCTCGCTATCCGCTATCCTGTCGATCGTGTTT
TGTGGCATCTGCTGTCAGAAGTATATCAATGCCAACATGGATGAGAGGTCGGTCACCACAGTACGATACGT
CATGAAGGTGTTCGCCAATGGCTCAGAGACCATCATCTTTGTCTTCCTCGGCATCTCGGCCATAGATCCAGC
TGTATGGGTTTGGAACACAGGCTTCATCCTCCTCACACTCCTCTTCATCTTTGTGTTCAGGTTCATAGGGGTC
TTTCTCCTTACCTGGATTTTGAACAAGTACCGACTGGTTCCCTTGGAGTTCATTGATCAGGTGGTGATGAGC
TACGGTGGCCTGCGAGGGGCAGTTGCCTACGGCCTTGTGGCCATGCTGGATGAGAATAAGTTCAAAGAGA
AGAATTTGATGGTCAGCACCACCCTCATAGTGGTGTACTTCACTGTCATGCTGCAGGGAATAACCATGAAA
CCACTGGTCACCTGGCTGAAAGTGAAGAGAGCAGCCGTGACAGAACTCACACTAGCTGAAAAAATACAGA
ATAGGACCTTTGACCACATGCTTGTCGCCATAGAGGACATTTCTGGACAAATAGGACATAACTACATGAGA
GACAAGTGGCATAACTTTGAGGAGAGGTGGTTGTGCTGGTTCTTGATGAAGCCCTCTGCCAGGAAATCCC
GTGACTACATATTCAACATCTTCCACCAGCTCAACCTCAAGGATGCCATGAGCTATGTAAAGGAGGGAGAA
CGCAGAGGCTCATTGGCGTTCGTTCGGAATGAGACCAAGGCCGATGTGGATTTCAATAAGAAGTTTCGTGC
CAGTTTCTCTGAGATTATGCCTGACATCATGGCAGATAATATGGGACCGGATCATGTTCCACTCTCCTCTAT
CTTACAGGACATTGTGCCCTCTGTCTGCCTAGTCATGCATGAGCAGGACCTGACAATGAGGGAGTCAGAGG
ACTTTAATGCACACCATCTACTGCAGCAGCACTTGTACAGAGGCAGGAAACAGCACAGACACAGGTACAGT
CGGAGCCACCTCGAAACCAACAGGGATGAGAATGAGGTGCAAGAGATCTTCCAGAGAACAATGAGAAGC
CGTCTAGAGTCATTCAAGTCGGCCAAAATGGGTGTCGCCCCTGCCAAGAAGATCCAAACCAAGCATCAAAA
GAAGGATAAACCACAAAAGGTCAGGGGTGAAGGCACTTTTTCCCCCTTCCTCCGTGTCCGACCTCATAGCC
ATCCTCTTATAGGCAATGTAATGTGTATGTTGTGTAATTTCATGTTATGTCATTTT_3’

Figure 3.3. Cloned sequence of trout *nhe3a* from kidney.

5’_ATGCCAGCTCTGTGGCGCTCCACTTTCGTCCTGGTGATGGTACTGCTTGCCATTAGTAGGCCCAGCCTG
GCATCTGAGGCCACAGGAAATGGAACAGAACATACTATCACCACCCTCCCCATCGTTATCTGGAAGTGGGA
GGAAATTCATTTCCCATACCTGGTGGCACTCTGGGTCCTGGTCAGCTGGCTCTGCAAACTCGTATTCCAGCT
GAACCCGTGCCTAACCAGAGTGATCCCGGAGAGCGGCCTCCTCATTTGCATCGGTTGGGTCCTGGGCGGC
ATCATCTATGGAGCAGACAAGGTGCAGACCTTCAGGCTGCAGCCCTTCACCTTCTTCTTCTACCTGCTGCCC
CAGATCGTCCTAGACGCAGGCTACTCCATGCCCAACAAGCTGTTCTTCGGCAACTTGGGCGCCATCCTGGTT
TACGCTGTTATTGGCACCTGCTGGAACGCTGCCACTTTGGGACTCGCCCTTTGGGGGTGCTGGTTGGGCGG
AGCCATGGGTAATATCGACACTGGTCTCCTGCAGTTGTTGTTGTTTGGGAGTCTGATTGCTGCTGTGGATCC
CGTGGCTGTGATTGCCGTGTTTGAGGAGGTCCATGTCAACGAGGTCCTGTACATCCTGGTGTTTGGGGAGT
CCCTTCTCAACGACGGCGTCACAGTGGTGCTGTTCAATGTGTTTGATGCATTTGTGTCGCTTGGAGGGGCA
CAAATCGACGCTGTAGAGATCATCAAAGGCATAATCTCATTCTTCGTGGTGGCGGTTGGAGGCTCCCTGGT
TGGTGTCGTCTTTGCTCTGCTGATCTCGCTCCTAACCAGATGCACCAAGAACATCCAGATTATCGAGCCAGG
CTTCATCTTCGTTCTGGGCTACCTCTCTTACCTCACAGCCGAGATGCTCTCCCTATCCGCTATCCTGTCGTGTA
CATTCTGTGGGGTCTGCTGTCAGAAGTATATCAATGCCAACATGGATGAGCGGTCGGTCAGCACGGTACG
ATCCACAATGAAGGTGTTCGCAAATGGCTCAGAGACCATCATCTTTGTCTTCCTCGGCATCTCGGCCATAGA
TCCGTCAATATGGGTGTGGAACACAGCCTTTATCCTCCTCACACTCCTCTTCATCTTTGTGTTCAGGTTCATA
GGGGTCTTTTTACTGACCTGGATTCTGAACCAGTACCGACTGGTTCCCTTGGACTTTATTGATCAACTAGTG
ATGGGCTACGGTGGCCTACGTGGGGCCGTAGCCTATGGCCTTGTGCTCCTGCTGGACGAGAATAAGTACA
AGGAGAAGAAACTGATGATCAGCACCACCCTCATAGTGGTGTACTTCACTGTCATGTTGCAGGGAATAACC
ATGAAACCACTGGTGACCTGGCTGAAAGTGAAGAGAGCAGCAGTGTCAGAACGCACACTAGCAGAAAAA
ATACAGAATAGGACCTTTGACCACATGCTTGTCGCCATAGAGGACATTTCTGGACAAATAGGAGATAACTA
CATGAGAGACAAGTGGAATAACTTTGAGGAGAAGTGGTTGTACTGGCTCTTGATGACACCCATTGCCAGG
AAGTCTTGTGACCCCATATTCAACATTTTCCACAAGCTCAACATCAAGGATGCCACAAGCTATGTGAAAGAG
GGAGAACGCAGAGGCTCATTGGCGTTCGTACGTAATGAAAACAAGGCCGATGTGGATTTCAATAAGAAGT
TTGGTGCCGATTTTTCAGAGATGATGCCTTACATCATGACAGAAAATATGGGACCAGATCATGTTCCAGTCT
CCTCCATCTTACGGGACAGTGTGCCCTCTGTCTGCCTGGACATGCATGAGCAGGACCTGAAGATGAGGGA
GTCAGAGGACTTTGATGCACACCATCTACTGCAGCAGCACTTGTACAAAGGCAGGAAACAGCACAGACAC
AGGTACAGTCGGAAGCAACTTGAAGTCAACAAAGACGAGCATGAGGTGCAAGAGATCTTCCAGAGAACCA
TGAGGAGCCGTCTAGAGTCATTCAAGTCAGCCAAAATGGGTGTCGCCCCACCCAAGAAGATAAGCAAGCA
TTTAAAGAAAGACCAGCCACAAAAGATGCCAAATGGAAAATCCACAGATAACAGTAAAAGCCATGCTTAT
GGGGATGAAGATTTTGAGTTTTCTGAAGGAGACAGTGCCTCTGGTTACGAGGCTGCTGGCGGTTCATTCCC
CATGAGAGCCTATAGAGGAGCTGGAATTGTCAACCCAGCTTTCATGGCGGAGATGGACACCATGGACCCC
ATGCCACCAATGCAGCAGATTCCTTCATGGCTGGCTGAAACCGAGGTGGACAGCAGCAACATGGTCCCTTC
TCAGAGGGCTCAGCTGAGACTGCCCTGGACACCGAGCAACCTCCGCCGCCTGGCTCCGCTGTGCCTCAGTA
CCCATTCCAATGACTCCTTCCTGCTGGCTGACTCCCCTGCCACACAGGAGGGCATCTCTGAGCTCCCACCAC
CACCTCCCCCACAAGACGGACAGGGTCAGGGAACCCACAAG _3’

Figure 3.4. Cloned sequence of trout *nhe3b* from gill.

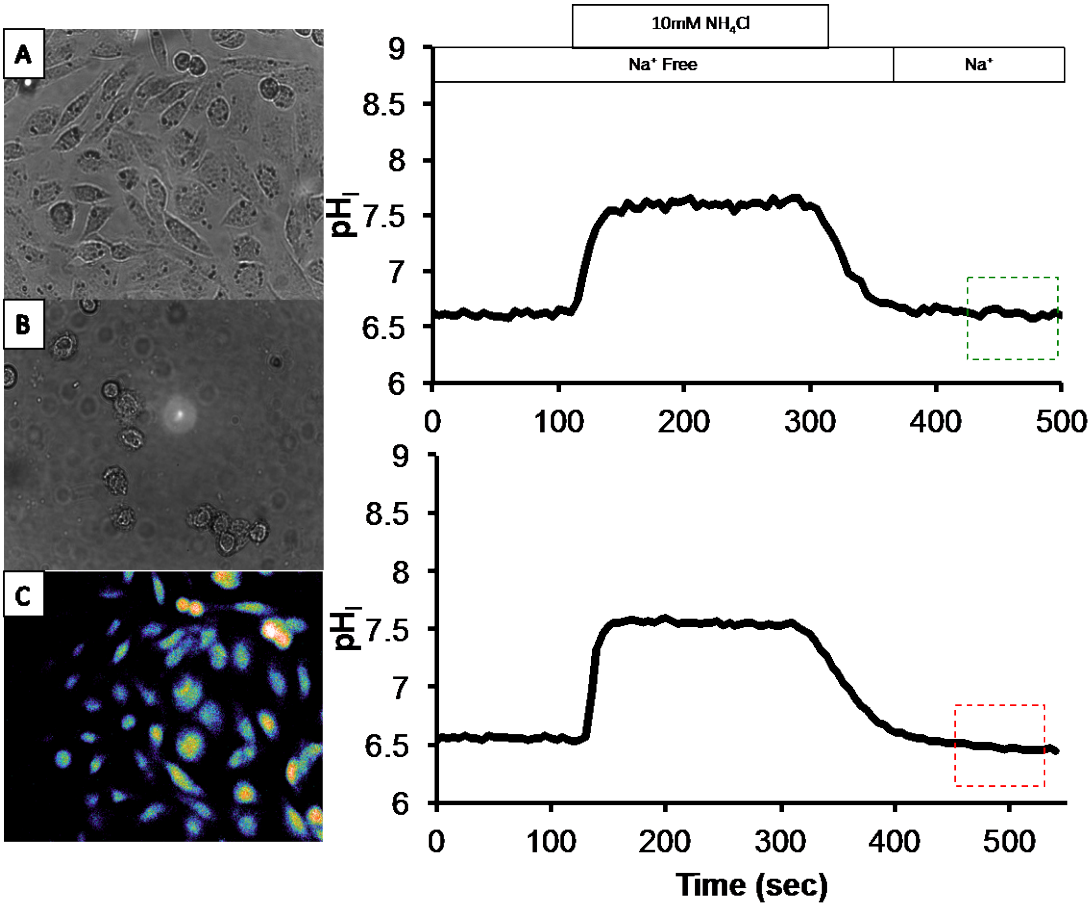

A
B
C
10mM NH4Cl
Na+ Free
Na+
pHi
9
8.5
8
7.5
7
6.5
6
0
100
200
300
400
500
Time (sec)

Figure 3.5. (Previous page) Light microscopy and intracellular pHi imaging. A) DIC image of untransfected AP-1 cells. B) DIC image of transfected AP-1 cells with trout *nhe2* (note cell morphology alterations). C) Image of fluorescence during intercellular pH imaging experiment on untransfected AP-1 cells. D,E) Representative AP-1 cell traces of intercellular pH (pH_i) in relation to external environmental Na^+- or Na^+-free buffer conditions (145 mM) on untransfected (D) and trout *nhe2* transfected (E) cells . Ammonia pre-pulse technique results in cellular acidification, and subsequent Na^+ addition should trigger an immediate cellular alkalization driven by the functioning Nhe if expressed. Green box indicates no alkalization due to absence of Nhe in untransfected AP-1 cells. Red boxes show absence of Na^+ alkalization in "transfected" cells.

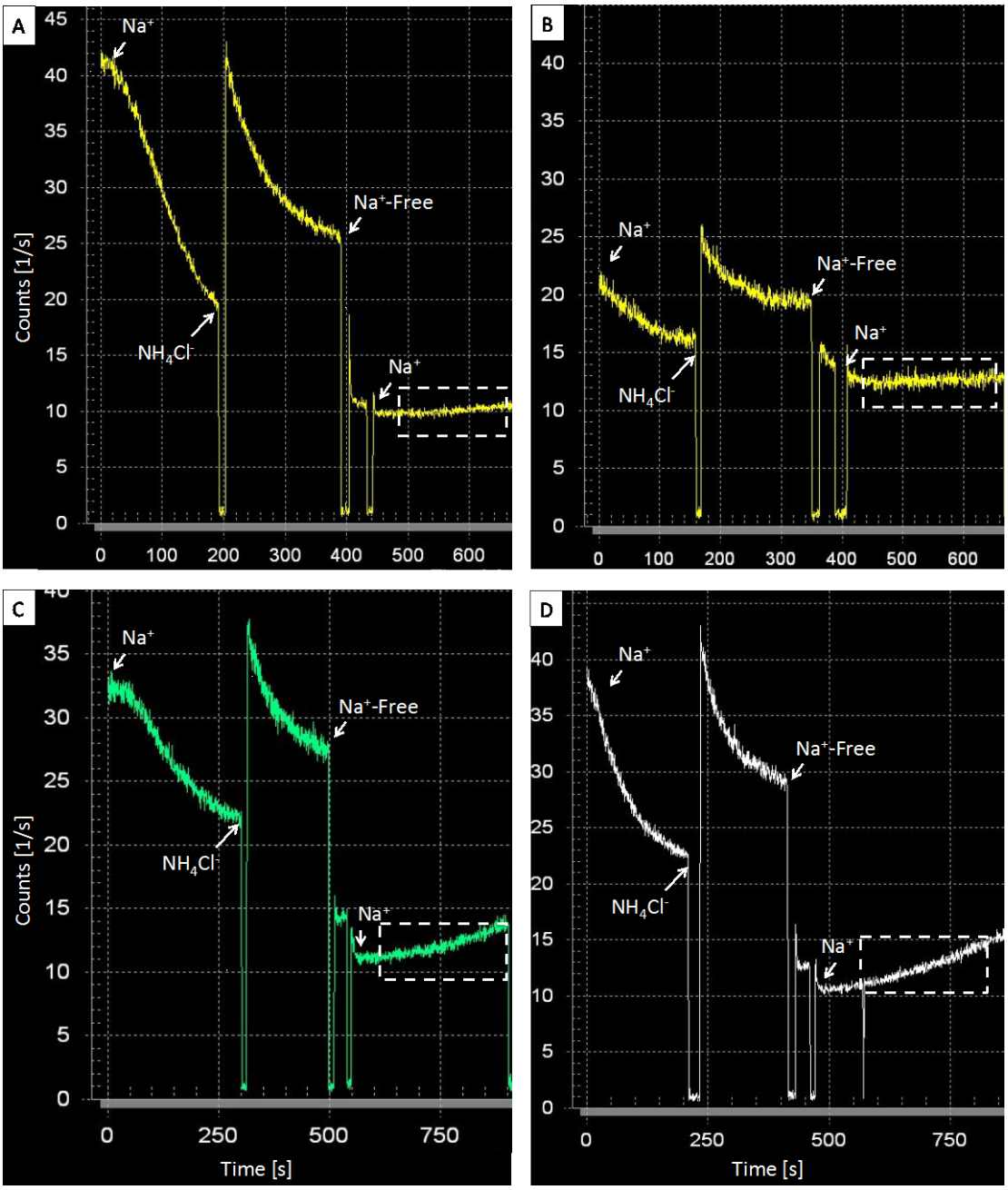

A
B
C
D
Na+
Na+-Free
NH4Cl
Counts [1/s]
Time [s]

Figure 3.6. (Previous page) Spectrofluormeter assay of Nhe activity. A) Control AP-1 untransfected cell trace B) Trout *nhe2* transfected AP-1 cell trace C) Trout *nhe3a* transfected AP-1 cell trace D) Trout *nhe3b* transfected AP-1 cell trace. Cells were attached to circular coverslips and immersed in Na^+ buffer for 3 min, 50mM NH_4Cl^- was added to induce initial cell alkalization, followed by immersion into Na+ free buffer resulting in cell acidification. Nhe activity is measured by the rate of alkalization upon final exposure to Na^+ buffer. Control cells showed no signs of increasing pH_i. Alkalization or increasing pH_i upon the addition of Na^+ was also absent or not significant in the transfected cells. Expected time of alkalization area highlighted by dashed box.

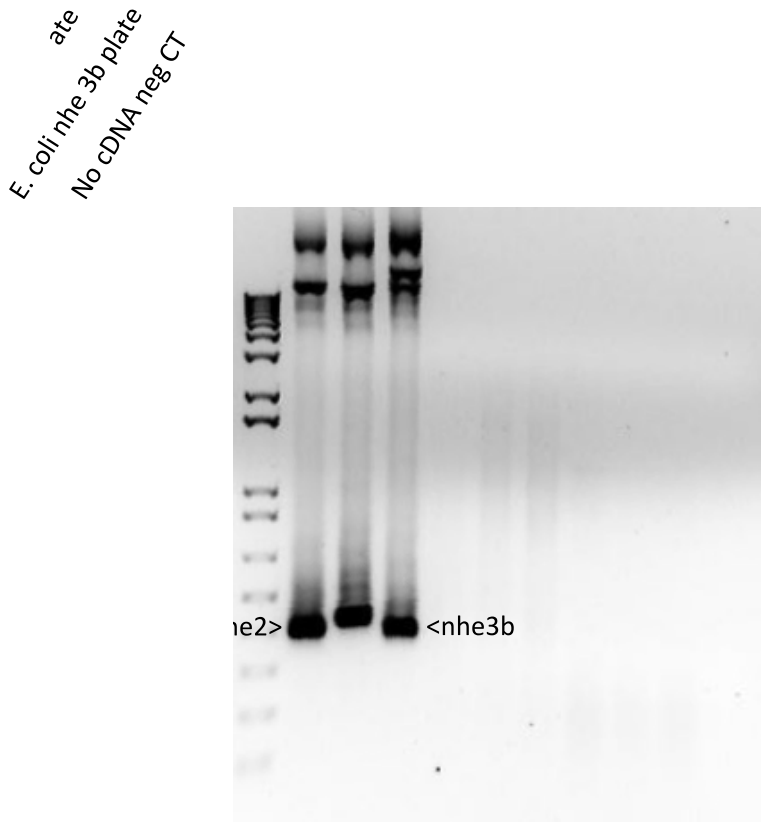


Figure 3.7. Agarose gel electrophoresis of RT-PCR for trout *nhe2*, *nhe3a*, and *nhe3b* gene expression. A) Colony PCR: Amplification of products resulting from *E.coli* colonies stabbed following transformation of bacteria with *nhe* isoforms. B) Gene expression testing comparing expression of nhe isoforms in the isolated pDisplay plasmid cDNA from transformed colonies (i.e. pDisp nhe2), in synthesized cDNA of RNA isolated from AP-1 cells transfected with each individual isoform (i.e. AP1-nhe2), or untransfected AP-1 cells(i.e. Untf AP-1 cell), and negative control sample containing PCR reaction mixture without any template cDNA.

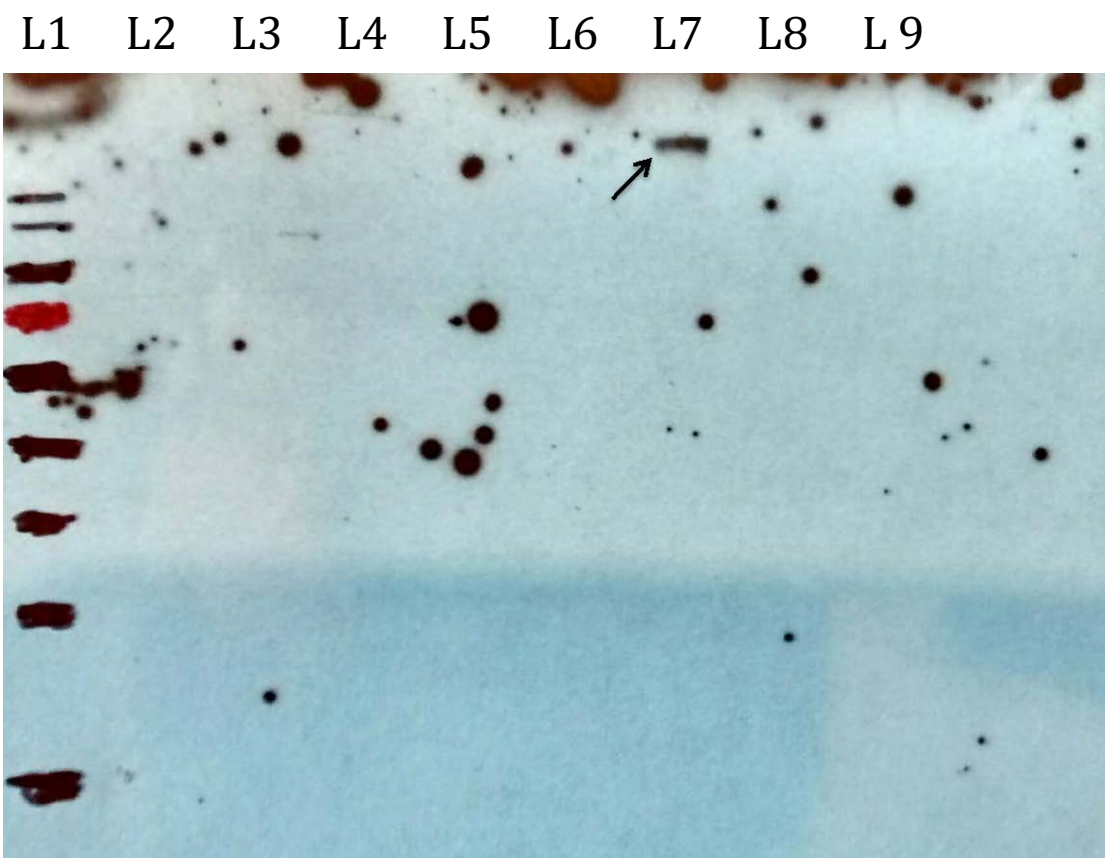


Figure 3.8. Western Blot of AP-1 cells transfected with trout *nhe*. A single band indicated by the arrow indicated expression of an HA tagged positive control protein sample in lane 8 (arrow). Lanes 2-7 contained protein samples from 2 groups each of AP-1 cells transfected with *nhe2*, *nhe3a*, and *nhe3b*, while lane 9 contained an untransfected AP-1 cell protein sample. No immunoreactivity was demonstrated in any of the experimental samples.

Table 3.1 Primer sets used for cloning trout nhe2, nhe3a, nhe3b

Gene	Primer Sequence
nhe2	
F7 Forward	5′ - CGTACTATGGGAGGATTTAGCC- 3′
R2 Reverse	5′ - CTGGTCGACCTAGGGTTTCCGA- 3′
F9 Forward	5′ - ATACCCGGGATGCGTACTATGGGAGGATTAG- 3′
R10 Reverse	5′ - TATGTCGACGGGTTTCCTAGTCTTCCAC- 3′
nhe3a	
F8 Forward	5′ - TACCCGGGATGGCATGCAGAACTTGC- 3′
R10 Reverse	5′ CGCGTCGACAAAATGACATAACATGAAATTACAC- 3′
nhe3b	
F3 Forward	5′ - CCAAGATTGATCAAAGCTCAAG- 3′
R3 Reverse	5′ - CGTGTCGACCTTGTGGGTTCC- 3′
F1 Forward	5′ - TACCCGGGATGCCAGCTCTG- 3′
R6 Reverse	5′ - CGTGTCGACCTTGTGGGTTCC- 3′
pDisplay	
T7 Promotor Forward	5′ - TAATACGACTCACTATAGGG- 3′
pDisplay Reverse	5′ - ATGATAAGGGAGATGATGG- 3′

Table 3.2 Primer sets used for verifying expression of nhe2, nhe3a, nhe3b in AP-1 cells

Gene	Primer Sequence	Product Length (bp)
nhe2		
F7 Forward	5′ - ATTGGGCTGATTGTAGGGGC - 3′	395
R2 Reverse	5′ - TCGTTGAACAGGCACTCTCC - 3′	
nhe3a		
F8 Forward	5′ - ACCAAGGCCGATGTGGATTT - 3′	421
R10 Reverse	5′ CACCCCTGACCTTTTGTGGT - 3′	
nhe3b		
F3 Forward	5′ - TGCATGAGCAGGACCTGAAG - 3′	386
R3 Reverse	5′ - TCTCATGGGGAATGAACCGC - 3′	

Discussion

Sodium transport in freshwater fish is a highly researched topic with two separate pathways of branchial Na^+ uptake against a concentration gradient. One method involves the influx of Na^+ through an apically located ENaC indirectly electrogenically coupled to an H^+-ATPase (Avella and Bornancin, 1989). This mechanism is supported especially under dilute media conditions in which the external Na^+ concentration is low and would complicate the proposed exchange of Na^+/H^+ (Na^+ influx and H^+ efflux), as the concentration gradient would favour the reversal of this process. However, there is no genetic evidence for an ENaC existing in teleost fish. Recently, our lab has proposed that the missing epithelial channel responsible for Na^+ uptake in fish gills is an ASIC, which would function under unfavourable thermodynamic conditions. The second proposed method and the subject of this study is in fact the direct exchange of Na^+/H^+ via an electroneutral Nhe also at the apical surface of the gill. Expression patterns of either mRNA and/or protein demonstrate the presence of Nhe isoforms in many model freshwater fish species including the rainbow trout (Ivanis et al., 2008; Chapter 1), Osorezan dace (Hirata et al., 2013), killifish (Edwards et al., 2005), tilapia (Inokuchi et al., 2009), and zebrafish (Yan et al., 2007). Despite the thermodynamic constraints, Nhe seems to be well expressed in many of the freshwater fish species and various studies have attempted to characterize its function with pharmacology and fluxes. The pharmacology data however have been based on inhibition profiles of mammalian transporters, as

only a few specific fish transporter studies exist (Ito et al., 2014; Guffey et al., 2015).

The goal of this study was to focus on the rainbow trout Nhe isoforms and their pharmacological interactions by cloning them into a cell line system, which could be utilized to characterize a specific pharmacological profile for each of the isoforms. Such characterization would help better interpret both future and past fish physiological studies involving Na^+ uptake. Isoforms *nhe2*, *nhe3a*, and *nhe3b* were cloned from trout gill and kidney tissues and transfections of AP-1 cells were performed, in order to measure activity of Nhe in the presence of various pharmacological inhibitors, namely amiloride, EIPA, and DAPI.

Cloning of each isoform and insertion into pDisplay plasmids was successful with the exception of trout *nhe2*. Unfortunately, following additional sequencing it was discovered that the trout gill *nhe2* sequence had 3 deletions in it, which might have resulted in the translation into an amino acid sequence representing an inactive protein transcript. *Nhe3a* and *nhe3b* sequences were confirmed and nearly identical to previously published sequence data for trout *nhe* isoforms. However, a single nucleotide difference may result in sequence mutations leading to an inactive protein or regulatory function problems.

Expression analysis of isolated RNA (RT-PCR), as well as isolated protein (Western blot analysis), showed a lack of expression of the trout Nhe isoforms in both RNA as well as protein, using the HA-tag which would be co-expressed as a proxy. The lack of expression clearly explains the lack of Nhe activity shown in both the pHi imaging and the results of Spectrofluormeter Nhe activity assay.

Without proper expression of the nhe isoforms to the plasma membrane of the AP-1 cell, no activity can be measured.

The negative results of this study are somewhat perplexing and the lack of actual experimentation with the pharmacological inhibitors is especially frustrating. Excluding trout *nhe2*, the sequence and insertion into pDisplay has been successfully confirmed for these trout *nhe* isoforms. The transfection process of the AP-1 cells with the pDisplay DNA containing nhe genes for all three isoforms resulted in positive acquired resistance to the toxin agent Geneticin, which was supplemented at a relatively high dose of (800µg/mL) in the MEMα media. This concentration was repeatedly lethal to the un-transfected control AP-1 cells, indicating that the antibiotic was indeed working. Furthermore, continuous cell growth of transfected cells following acid suicide selection suggests that cells were transfected, as this method was lethal to untransfected AP-1 cells and the transfection process itself appears to be successful. The reasons for these paradoxical findings are at the present unknown, but a possibility exists by which the cells, that survived the geneticin and acid suicide selection, were protected in some manner by neighbouring cells that clustered together and took the brunt of the solution, but this seems somewhat improbable. The fact that the pDisplay plasmid was utilized in a similar manner to transfect a different cell line (HeLa) with a catfish leukocyte immune-type receptor, suggests that the technique used in this study should be quite conceivable (Montgomery et al., 2009). In order to rule out experimental error, members of the Fliegel lab performed a second transient transfection

using the same plasmid DNA and subsequent Western blot analysis yielding the same negative results.

The next steps for the success of this project will be to utilize a different expression system other than pDisplay, which has had success in AP-1 transfection. This is theoretically simple to perform with digesting the current plasmids at common restriction sites, followed by ligation and transformation of competent *E. coli* cells to produce many copies of the new plasmid containing the present nhe gene inserts. Following isolation of plasmid DNA via miniprep, another transfection can be attempted with the new expression system. Western blot analysis or PCR analysis for gene expression will need to be immediately performed in order to confirm expression prior to attempting to measure Nhe activity. If this method is unsuccessful, re-visitation of the original cloning strategy will be necessary, potentially with the inclusion of 5 prime and 3 prime untranslated regions, or including the stop codon, or potentially leaving out the start codon since there is a conjugated HA tag on the 5 prime end (Hyvönen, 2004).

Essentially, this project has great potential and should continue to be investigated in our lab, as a full pharmacological profile for trout Nhes is critical for the continued understanding of the Na^+ uptake mechanisms utilized by freshwater fish. Once stable transfected cell lines expressing the trout nhe isoforms are established the systems and materials would be in place for the inhibition experiment to be completed in the future.

CHAPTER IV: Reduced salinity tolerance in the Arctic grayling (*Thymallus arcticus*) is associated with rapid development of a gill interlamellar cell mass: implications of high saline spills on native freshwater salmonids

A version of this chapter has been published previously in *Conservation Physiology*.

Blair, S. D., Matheson, D., He, Y., & Goss, G. G. (2016). Reduced salinity tolerance in the Arctic grayling (*Thymallus arcticus*) is associated with rapid development of a gill interlamellar cell mass: implications of high-saline spills on native freshwater salmonids. *Conservation Physiology*, *4*(1), cow010.

Introduction

The oil and gas industry is a socio-economic driver for both Canada and the United States, significantly impacting the overall gross domestic product (GDP) of both countries (Honarvar *et al*., 2011). Natural gas has been pushed as a transition fuel to replace coal and reduce greenhouse gas emissions (Pacala and Socolow, 2004; Burnham *et al*., 2012). Consequently, pressure is mounting to develop deep shale reserves in northern areas including Canada (Alberta, BC, Northwest and Yukon Territories) and Russia (Economides and Wood, 2009; BP 2015; Melikoglu 2014; Grace and Hart, 1986). Oil and gas extraction technology (e.g. hydraulic fracturing, in situ development) results in large volumes of highly saline (up to 300 ppt -10X seawater) and organic-contaminated flowback wastewater, with up to a 600-fold increase in [Na^+] compared to local lakes and rivers (Allen 2008). One of the clear risks of this industry is the potential for accidental release of these high saline waters through a spill occurring at an on-site location or during transport to disposal wells (e.g. pipeline or trucking). Although oil and gas companies are taking measures including contamination avoidance, water use and management, and reclamation (CAPP 2015); spills are still common, especially involving incidental releases of hyper-saline water resulting from hydraulic fracturing (Gross *et al*., 2013; Vengosh *et al*., 2014). Indeed, in a recent review of hydraulic practices, Goss *et al*. (2015) documented a total of 113 spills of saline flowback fluid into flowing waters in the period 2005 - 2012 alone. Unfortunately, these resource rich geographic areas directly overlap with the habitat for threatened native freshwater salmonids (Arctic

grayling, mountain whitefish, bull trout, westslope cutthroat trout, and inconnu) (Northcote 1995; Walker 2005; McPhail and Troffe, 1998; Reiman *et al*., 1997; Rodtka 2009; Costello 2006; Howland *et al*., 2001). A sudden influx of high saline water into a stream would pose an immediate osmoregulatory perturbation for aquatic organisms and it is essential to understand the potential impacts on these native species, especially since most regulatory guidelines are routinely based on the responses of the euryhaline rainbow trout (USEPA 2002; Environment Canada 1990).

The process of osmoregulation is vital for all organisms, whereby animals must maintain a relatively constant internal concentration of ions irrespective of the external environment (Evans *et al*., 2005). In freshwater, fish are required to compensate the loss of salts (Na^+, Cl^-) by active salt *absorption* across the gills against the gradient presented by the hypotonic environment they inhabit. In contrast, in hypertonic marine environments, salts are *excreted* across the gills (Dymowska *et al*., 2012). Euryhalinity is defined by the ability to osmoregulate across a wide salinity range, and characterizes some members of the Salmonidae family. Sodium potassium ATPase (Nka) plays an important role in maintaining systemic, as well as cellular, ionic homeostasis and its various isoforms have been identified to strongly influence the ability to tolerate seawater [*nkaα1a* as the freshwater isoform and *nkaα1b as the seawater isoform* (Richards *et al*., 2003; Bystriansky 2006; Nilsen *et al*., 2007; Bystriansky 2011)]. Indeed, a recent phylogenetic analysis of the Salmonidae family has identified *nkaα1a* and *nkaα1b* sequences in all three subfamilies: Salmoninae, Coregoninae,

Thymallinae (salmon and trout, whitefish, and grayling, respectively) (Dalziel *et al*., 2014).

Arctic grayling (*Thymallus arcticus*) is a native salmonid species that inhabits freshwater environments in post-glacial North America, and unlike other salmonids does not undergo smoltification (Stamford and Taylor, 2004). Grayling inhabit lakes and rivers in the northern regions of Canada that directly overlap with large oil and gas reserves. Current status reports indicate Alberta Arctic grayling populations are in serious decline, and as of 2015 a province wide zero limit regulation has been implemented, requiring all grayling fishing to be catch and release only (AEP 2015). Given the limited amount of understanding of the physiology of the Arctic grayling, the risks imposed, and the threatened status, it is imperative to examine the salinity tolerance limits for grayling to ensure proper conservation strategies for this important native species. The objective of this study was to evaluate the physiological responses of the Arctic grayling to acute higher saline exposure. We compared the response to that of the euryhaline rainbow trout (*Oncorhynchus mykiss*) because trout are currently used for setting regulatory limits and remediation strategies. We hypothesized that Arctic grayling would demonstrate a reduced salinity tolerance when exposed to short term higher saline waters, as demonstrated by an inability to compensate a similar osmotic stress compared to the rainbow trout.

Methods

Animal Collection

In collaboration with Alberta Environment and Parks (AEP), Arctic grayling (*Thymallus arcticus*) were collected via angling (fly-fishing) from Marten Creek, Alberta. Grayling were transported from Marten Creek to the University of Alberta's bio-secure aquatics facility utilizing an AEP hatchery truck carrying a 1000L tank containing oxygenated river water (~9.76 mg/L O_2, ~20.4°C), chilled with ice bags. Fish were transferred from the truck and placed into a main holding tank (825 L) with aerated flow-through dechlorinated Edmonton tap water 10°C. Grayling (20.6 ± 0.4 cm, 69.5 ± 3.6 g, means ± S.E.M.) were maintained and allowed to acclimate in captivity for 2 months prior to experimentation. Rainbow trout (21.5 ± 0.4 cm, 111.8 ± 5.8 g) were reared to size in house from embryos generously donated by the Allison Creek Fish Hatchery, (Alberta) and maintained in tanks supplied with the same facility water as the Arctic grayling. Trout and Arctic grayling were fed daily with crushed trout chow (Nu-Way®, Hi-Pro Feeds, Okotoks, AB, Canada). Grayling were supplemented with thawed *Artemia sp*. three times per week. Fish were fasted 4-5 days prior to salinity exposure experimentation.

Salinity Exposures and Sampling

Preliminary testing of exposures of grayling and trout to higher salinities was necessary to achieve a starting threshold salinity concentration and time point allowing blood parameters to be experimentally measured. A

concentration of 50% seawater (17 ppt) with a 96 hr time course was chosen since this was the highest concentration tolerated by grayling for a 96h exposure. Identical recirculating systems (180 L) containing either freshwater (0-0.1 ppt) or 17 ppt saline water were constructed and chilled to 10°C. The saline water was produced by dissolving a salt mixture (Instant Ocean) in dechlorinated Edmonton tap water to 17ppt. Salinity was measured daily by both a handheld refractometer (Atago) and digital salinity probe (YSI Model 85, Yellow Springs, Ohio) and maintained by addition of freshwater or artificial saline water at 17ppt. Fasted fish were acutely transferred to experimental tanks (0 or 17ppt saline water). Lethal sampling (n=8) of fish occurred in control (FW) at 24 h, and in 17 ppt at 24 hr and 96 hr exposure time points. Anesthetized fish (MS-222 200 mg L^{-1} buffered with $NaHCO_3$ 400 mg L^{-1}, time < 3 min) were weighed, length recorded, and blood (1 mL) was immediately sampled via caudal blood puncture using non-heparinized syringes. Blood was centrifuged (2 min - 12000 X g) and the resulting serum frozen and stored at -80°C for later blood osmotic and ion analysis. The 2nd and 3rd gill arches were excised and either rapidly frozen in liquid nitrogen, or processed for microscopy.

Serum Na^+, Cl^-, Osmolality

Serum samples from grayling and rainbow trout were diluted to appropriate volume with ultrapure water for analysis of [Na^+] via atomic absorption spectrophotometry (Model 3300, Perkin Elmer, CT, USA). Serum [Na^+] was calculated as millimoles L^{-1} against a NaCl standard curve. Analysis of

serum [Cl^-] was performed by direct analysis on a digital chloridometer (Labconco, Kansas City, MO, USA). Total serum osmolality was measured on a freezing point depression osmometer (Micro Osmette, Precision Systems).

RNA Extraction, cDNA synthesis, and Nka Isoform Expression

Excised gills were frozen in liquid nitrogen and stored at -80°C for further RNA isolation. Gill filaments were ground in liquid nitrogen with a mortar and pestle on a bed of dry ice. The resulting powdered tissue (~30 mg) was transferred to 1.5mL Eppendorf tubes containing 1mL of TRIzol®, and RNA was subsequently extracted and isolated via the TRIzol Reagent method according to the manufacturer's protocol (Ambion, Life Technologies, Carlsbad, California). Total RNA was re-suspended in 30μL of nuclease-free water, quantified and validated for purity via spectrophotometry (NanoDrop, ND-1000, Thermo Fisher Scientific, Delaware, USA). Total RNA (3 μg) was treated to remove residual DNA (DNase I, Thermo Fisher Scientific, Burlington, ON). RNA integrity was checked via gel electrophoresis and the 28S and 18S bands were visualized. Synthesis of cDNA from RNA template (1 μg) was performed using SuperScript III Reverse Transcriptase (Invitrogen) with a mix of oligo(dT) and random primers.

Quantitative real-time PCR was performed in order to measure the relative abundance of mRNA expression of Na^+/K^+ ATPase isoforms among the grayling and trout exposed to salinity at 24 and 96 hrs. The annotated partial sequences for Arctic grayling *nkaα1a* and *nkaα1b* (Dalziel *et al*., 2014) share an 89.87% nucleotide identity making common gene-specific primer design and

subsequent qPCR challenging. To confidently navigate this obstacle, we utilized the RNase H-Dependent qPCR technique and in doing so designed rhqPCR primers for grayling and trout, which are blocked and cleavable by the addition of a specific enzyme RNase H2, according to the manufacturer's protocol (Integrated DNA Technologies, Coralville, Iowa, USA) (Dobosy *et al*., 2011). This technique allows for the detection of target genes differing by only a single nucleotide due to the high specificity resulting from the enzyme cleavage of the blocked primer bound to the complimentary sequence (Dobosy *et al*., 2011). Gene specific primers *nkaα1a* and *nkaα1b* were designed in addition to *elongation factor 1α* (*ef1α*), which served as a house-keeping gene (Table 4.1). Forward and reverse primers for rainbow trout *ef1α* expression were as previously developed in our lab (Boyle *et al*., 2015); these were found to be 80% and 100% identical to the grayling *ef1α* target sequence respectively, and furthermore were tested and *ef1α* expression was shown to be stable in grayling and trout regardless of the treatment. All qPCR was performed in a light cycling PCR machine (ABI Prism 7500 sequence detection system). Samples were run in triplicate with final reaction volumes of 10µL per well containing 2X SYBR green PCR mastermix (Thermo Fischer Scientific Rockford, IL), 300nmoles of each primer, 1:20 dilution of sample cDNA, and 0.5 µL RNase H2 enzyme buffer mixture. The PCR reaction mix was denatured at 95°C for 2 min, followed by 40 thermal cycles of denaturation for 15 s at 95°C and annealing and extension for 1 min at 60°C. Dissociation curve analysis was performed after each amplification reaction to ensure that a single product was obtained. Primer efficiency curves

were run with a random grayling and trout cDNA sample separately in a 4-fold dilution pattern to evaluate replication efficiency for each species (Table 4.1). Samples were normalized relative to *ef1α*, and compared within genes to control (freshwater) levels and analyzed statistically with one-way ANOVA, and a Dunnett's multiple comparisons test (Prism 6, Graphpad Systems).

Interlamellar Cell Mass Measurements

Gills were excised from grayling and rainbow trout and fixed in a 4% paraformaldehyde (PFA) solution in phosphate buffered saline (PBS) overnight at 4°C. Gills were washed in PBS and underwent ethanol dehydration prior to paraffin wax embedding. Microtome sections of gill tissue (7 μM thick) were fixed onto Superfrost® plus microscope slides (Thermo Fisher Scientific Rockford, IL) and were hematoxylin and eosin stained and examined under light microscopy (Leica DM RXA). Digital images were collected from three randomly selected fish and two filaments from each fish with ten adjacent lamellae were imaged (QI Click, QImaging). ImageJ software (National Institutes of Health) was used to calculate interlamellar cell mass to lamellar length ratios as previously described (Ong *et al.*, 2007). Height of ILCM was measured parallel to the total lamellar length, starting from the edge of the ILCM bordering the filament to the most distal edge of the ILCM from the filament. Ratios were calculated and compared among the three treatments for both grayling and trout and analyzed via 1-way ANOVA (Prism 6, Graphpad Systems).

Results

Survival

Arctic grayling proved to be unable to cope well upon acute exposure to higher saline waters. Due to limited numbers of available animals, a preliminary set of exposures was conducted on a small number of fish to define the approximate salinity tolerance. Results indicated that short exposure to 50% (17ppt) seawater for 0 – 96 hrs was not lethal (0% lethality), however the fish were clearly distressed as shown by abnormal swimming behaviour and periodic losses of equilibrium (LOE); additionally 100% mortalities (n=2) occurred at 100 hrs post exposure. Higher salinity (25ppt- 75% seawater) resulted in 100% mortality (n=2) within 24 hrs. Therefore, a 50% seawater concentration (17ppt) and a 96h time point were chosen for final physiological analysis. Rainbow trout showed no mortality at either 50% or 75% seawater, and the strain of trout housed in our facility (supplied by Raven Brood Trout Station) have been known to survive these conditions indefinitely, with survivability similar to trout utilized in Bath and Eddy (1979).

Serum [Na^+]

Initial serum [Na^+] of freshwater Arctic grayling were slightly lower, although not significantly, from those of rainbow trout (139 ± 6.5 vs. 159 ± 0.9 mM Na^+, respectively) (ANOVA, $p < 0.05$). However, both grayling and trout demonstrated significantly elevated serum [Na^+] by 24hr exposure, increasing by ~25% and 16%, respectively from baseline levels. However, by 96 hrs, trout

serum [Na^+] had returned to near control levels, while Arctic grayling demonstrated continued elevation in [Na^+], reaching 203 ± 5.5 mM (Figure 4.1A).

Serum [Cl^-]

Average [Cl^-] for Arctic grayling and rainbow trout serum held in freshwater were nearly identical (118 ± 1.6 and 119 ± 3.4 mM Cl^-, respectively). Serum [Cl^-] rose significantly upon 50% seawater exposure, increasing by ~38% in grayling and ~23% in trout after 24 hr. At 96h, average grayling serum [Cl^-] was further elevated 65% to 196.8 mM. The serum [Cl^-] in trout returned closer to control values, falling from 146 mM Cl^- at 24 hr to 138 mM at 96h (Figure 4.1B), although the [Cl^-] was still statistically elevated at 96h (ANOVA, $p < 0.05$).

Serum Total Osmolality

Total osmolality naturally followed a similar pattern to the [Na^+] and [Cl^-] during the exposures. Arctic grayling serum osmolality rose significantly from 299 mOsm in pre-exposed fish to 374 mOsm after 24 hr exposure to 50% seawater. A similar increase was demonstrated in the trout serum osmolality, increasing from 309 to 359 mOsm. The elevated total osmolality persisted in the grayling serum, capping off at an average of 455 mOsm by 96h, however the trout serum levels significantly reduced back to control levels by 96 hrs in 50% seawater (ANOVA, $p < 0.05$) (Figure 4.1C).

Sodium Potassium ATPase Gene Expression

Gene expression data from qPCR analysis supported the expression of both isoforms of sodium potassium ATPase, *nkaα1a* and *nkaα1b* in all of the gill tissue samples analyzed. Expression patterns however for Arctic grayling indicate a significantly decreased mRNA expression of the freshwater isoform *nkaα1a* at 24 and 96h when compared to control levels (ANOVA, $p < 0.05$). A similar pattern is seen in the seawater isoform *nkaα1b*, where it also demonstrates a significant decreased relative level of expression in the gills compared to control freshwater levels at 24 and 96h (Figure 4.2).

A slightly different pattern was seen through analysis of the rainbow trout gill *nka* gene expression. Upon exposure to 50% salinity, rainbow trout expression levels of *nkaα1a* mRNA showed a decreasing trend at 24 hr, however was significantly lower than control levels at 96h, similar to that of the grayling expression (ANOVA, $p < 0.05$). In contrast to the grayling expression, gill *nkaα1b* levels in rainbow trout did not significantly decrease at either 24 or 96h exposure to 50% salinity, but rather stayed similar to control freshwater expression (ANOVA, $p > 0.05$) (Figure 4.2).

Gill Tissue Plasticity

An intriguing result arose when comparing the morphology of the gills of these fish following exposure to 50% seawater at 24 and 96h. Hematoxylin and eosin stained gill filaments indicated a significant increase in the interlamellar cell mass (ILCM) located at the base of the lamellae of the Arctic grayling

(ANOVA, $p < 0.05$) (Figure 4.3,4). ILCM height was quantified following 24 hr exposure to 50% seawater. We found a 156% increase in the ILCM of Arctic grayling (Figure 4.5). This ILCM was not seen in the rainbow trout, at any of the time points observed (Figure 4.4). The grayling ILCM by 96h was still elevated 98% above controls, with a slight reduction from the 24 hr peak (Figure 4.5).

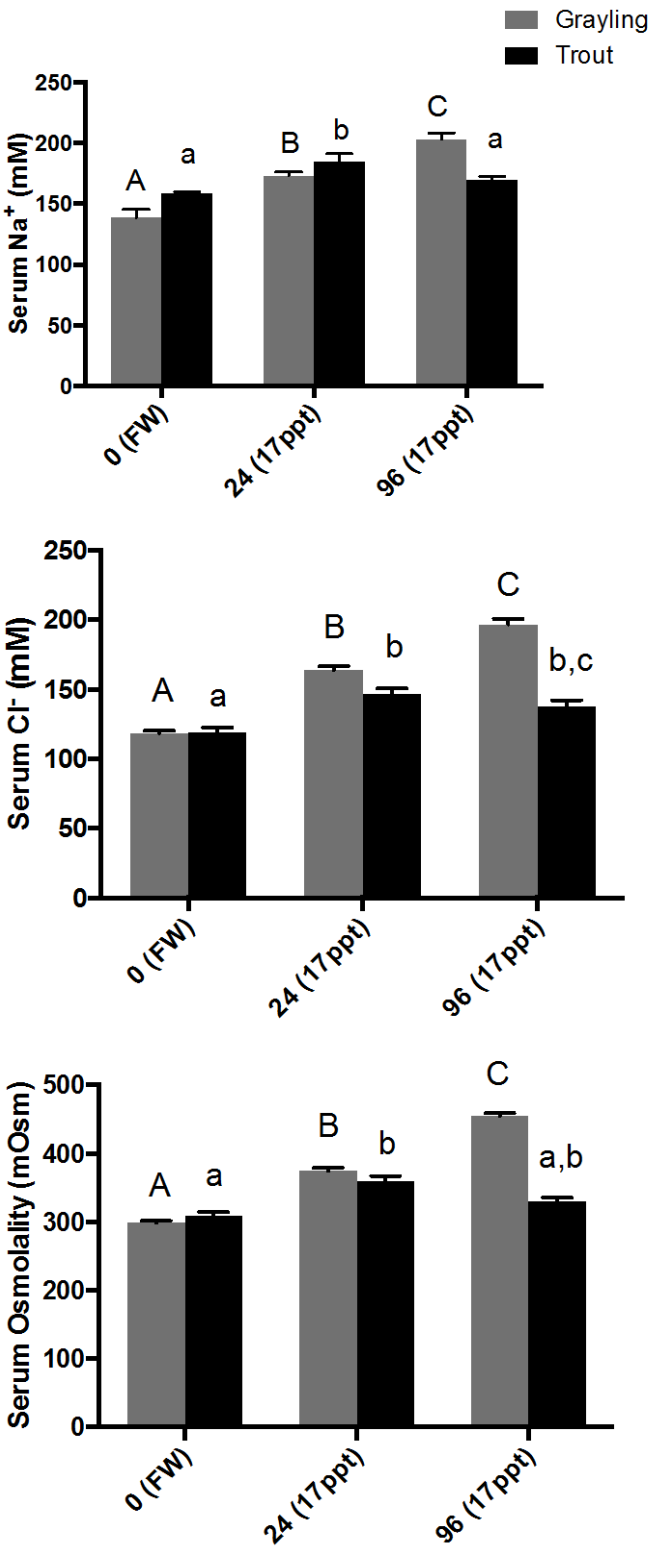

Grayling
Trout
Serum Na+ (mM)
250
200
150
100
50
0
A
a
B
b
C
a
0 (FW)
24 (17ppt)
96 (17ppt)
Serum Cl- (mM)
250
200
150
100
50
0
A
a
B
b
C
b,c
0 (FW)
24 (17ppt)
96 (17ppt)
Serum Osmolality (mOsm)
500
400
300
200
100
0
A
a
B
b
C
a,b
0 (FW)
24 (17ppt)
96 (17ppt)

Figure 4.1. (Previous page) A) Serum sodium [Na^+] B) Serum chloride [Cl^-] C) Serum osmolality [mOsm] of Arctic grayling and rainbow trout exposed to freshwater (control) and 17ppt saline water at 24 and 96h. Grayling and trout demonstrating significant increases in serum ion and osmolality at 24 hr, and by 96h grayling serum concentrations were further elevated in grayling, but rainbow trout levels returned to or near that of control values. Grayling (grey bars) and rainbow trout (black bars) values indicate mean ± S.E.M., while dissimilar letters demonstrates significance using one-way ANOVA, Tukeys multiple comparisons. Capital letters denotes grayling significance in comparison to grayling freshwater, while lowercase letters denote rainbow trout significance ($n=8$, $P<0.05$, 1-way ANOVA).

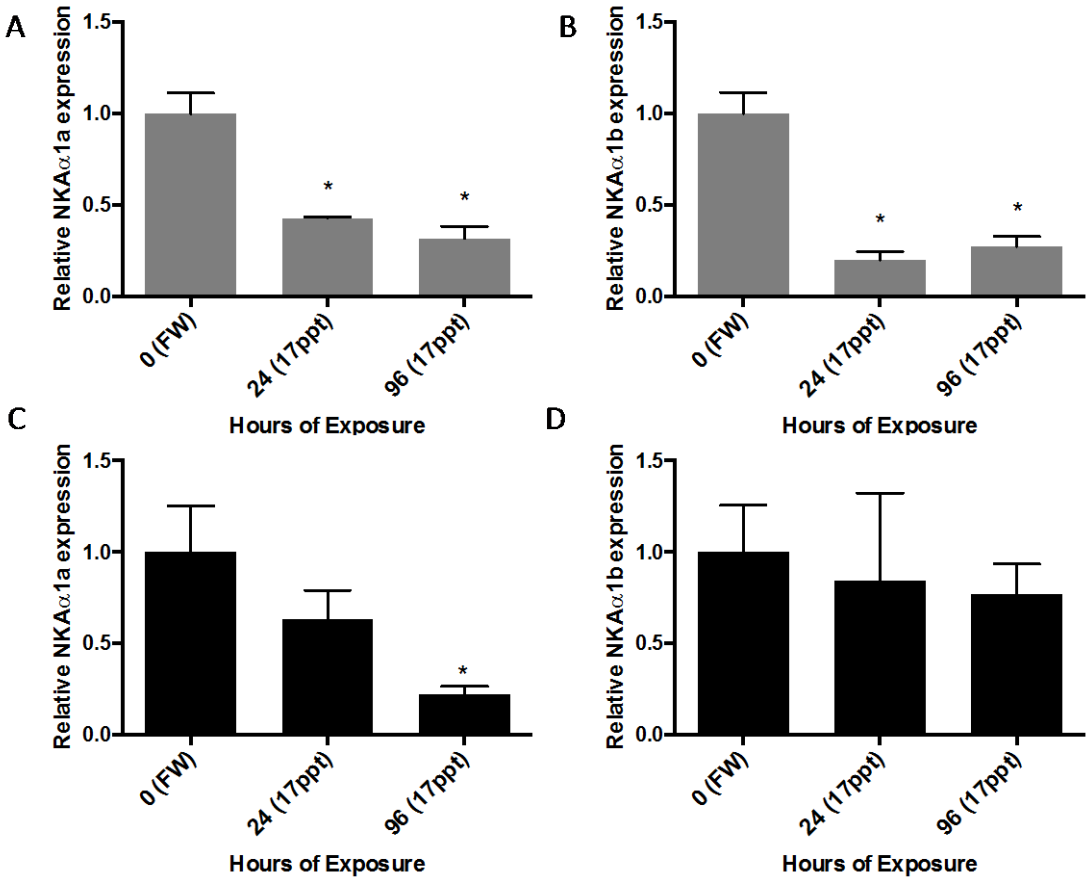


Figure 4.2. Gill expression of sodium potassium ATPase isoforms *nkaα1a* and *nakα1b* in Arctic graying (A,B) and rainbow trout (C,D) at freshwater and following 24 and 96 h exposure to 50% (17 ppt) seawater. mRNA expression is normalized to the endogenous control gene *ef1α*, and significant differences from control freshwater levels are designated with an asterisk (n=3, $P<0.05$, 1-way ANOVA, Dunnett's multiple comparisons test).

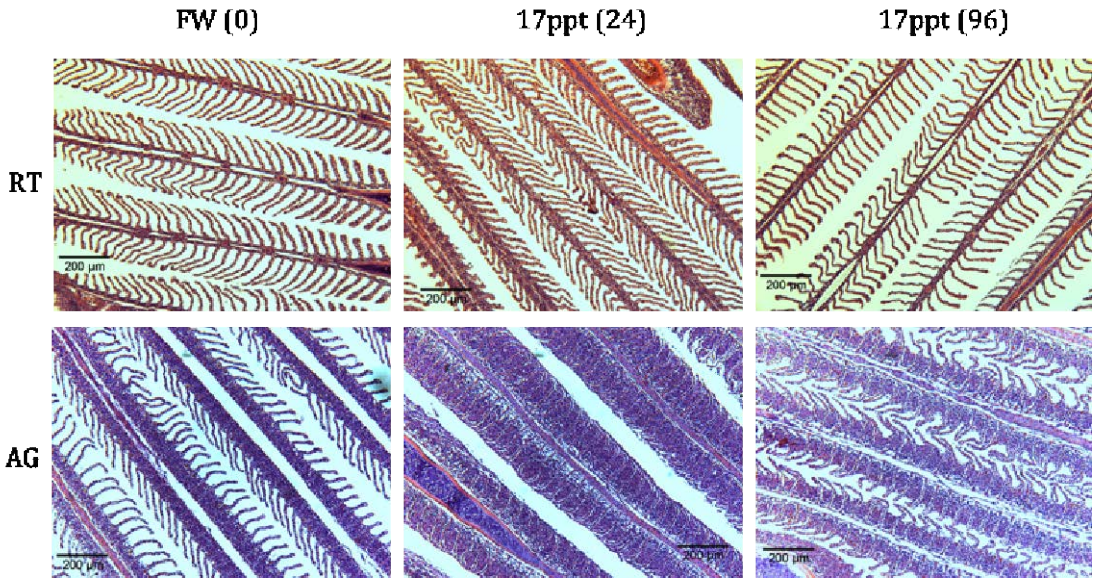


Figure 4.3. Light microscopy images showing comparison between (top row) rainbow trout and (bottom row) Arctic grayling gills under control freshwater conditions, 24 h exposure, and 96 h exposure to 50% seawater (17 ppt).

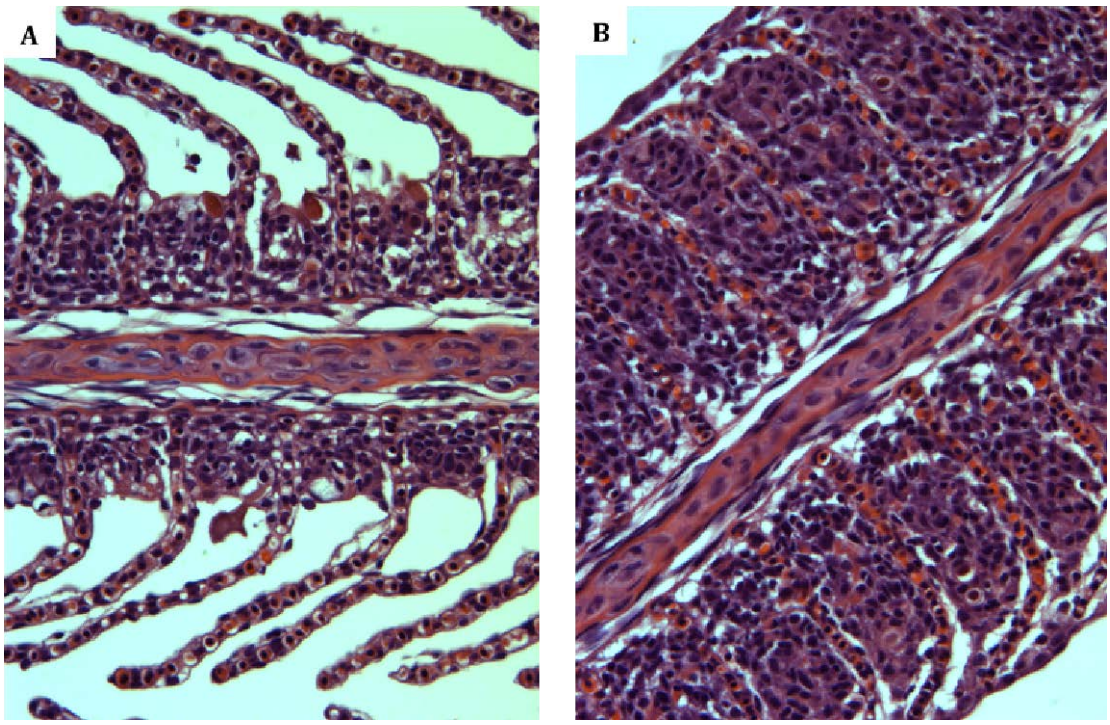


Figure 4.4. Light microscopy image showing increases in ILCM. Hematoxylin and eosin stained Arctic grayling gill filaments in (A) freshwater and (B) following 24 h exposure to 50% seawater (17ppt).

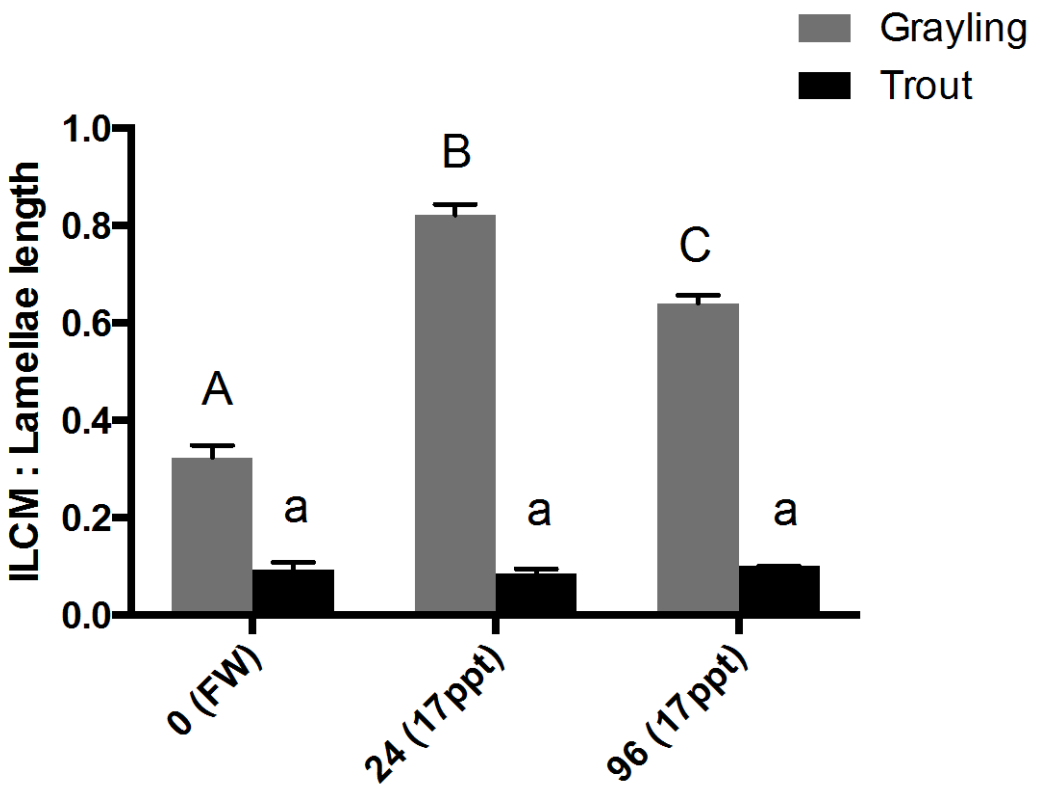


Figure 4.5. ILCM lamellar coverage of Arctic grayling and rainbow trout exposed to freshwater (control) and 17ppt saline water at 24 and 96h. Grayling (grey bars) and rainbow trout (black bars) values indicate mean ± S.E.M., with significance demonstrated by dissimilar letters using one-way ANOVA. Capital letters denotes grayling significance in comparison to grayling freshwater, while lowercase letters denote rainbow trout significance (n=3, $P<0.05$, 1-way ANOVA).

Table 4.1. Nucleotide sequences of quantitative real time PCR primers designed for the RNase H-dependent PCR. Bolded **r(X)** represents the inserted RNA base. Gene accession information from NCBI and primer efficiencies as well as subsequent amplicon lengths are given.

Gene	Accession information	qPCR primer 5'-3'	Amplicon Length	Primer Efficency
AG NKAα1a	Accession: KJ175158 GI: 645929871	F - GACGCCTCTTGGAATTGArAATTGC/3SpC3/ R - CCAGAATGACGGAGAGGArTTAAGG/3SpC3/	98	1.89
AG NKAα1b	Accession: KJ175159 GI: 645929873	F - GTGGCTGGAGAGTCCAArGCACCC/3SpC3/ R - CGTTCTGGAAGGCTTCTTTrCAACTT/3SpC3/	133	1.86
RT NKAα1a	Accession: AY319391.1 GI: 34812026	F - GCCTCTCGGAATTGAAATTGArTCACTG/3SpC3/ R - GGATGGCAGCCATCCATArGCCCAA/3SpC3/	117	1.92
RT NKAα1b	Accession: AY319390.1 GI: 34812024	F - AAAGAGATTGAGCACTTTATCCArCATCAG/3SpC3/ R - GACAGCTTCCAGCCArGCCATG/3SpC3/	107	1.95
ef1α	Accession: AF498320.1 GI: 20269865	F - CTGTTGCCTTTGTGCCCATC R - CATCCCTTGAACCAGCCCAT	82	1.96

Discussion

Research on salmonid salinity tolerance is quite extensive, as this family is well known for the anadromous fish species which naturally make the transition from fresh to saline water at some point in their life cycle (Morgan and Iwama, 1991; Staurnes *et al*., 1992; Singer *et al*., 2002; Richards *et al*., 2003; Bystriansky *et al*., 2006; Larsen *et al*., 2008; Bystriansky and Schulte, 2011; McCormick *et al*., 2013; Dalziel *et al*., 2014). In this study, we aimed to investigate the effects salinity exposure may have on Arctic grayling, a threatened salmonid species that has a strict freshwater existence in North American for the past 3-5 million years (Stamford and Taylor, 2004).

Spills of saline process-affected water into aquatic and riparian areas have been documented across North America and the possibility of saline spills into flowing water bodies containing Arctic grayling is increasing (Vengosh *et al*., 2014; Goss *et al*., 2015).

Current environmental testing for spill response, environmental impact assessment and remediation strategies use data from rainbow trout. For high concentrations of salts that commonly occur in the produced water (up to 10X seawater or ~300ppt) even a 20-fold dilution of this water would still be above the salinity threshold of Arctic grayling if they were exposed to it for greater than 96 hrs. We have not yet investigated the 24 hr salinity threshold for grayling, although our limited survivability data showed that animals (n=2) exposed to 75% SW in preliminary tests died before 24 hr. Given a high volume spill of 300 ppt produced water into a stream containing grayling, one would

expect mortality, as the osmotic stress would be the initial threat to these fish. In northern areas, fish in streams tend to congregate in deep overwintering holes as many sections of rivers and streams freeze completely in the winter. The severity of a spill of saline produced water at this time would be compounded by the high density of the fluid and the low stream flow, resulting in the settling of this water into overwintering pools and swamping the fish, which are essentially "trapped" for the winter months. Aspects of volume, concentration, and time are important factors influencing the survivability of these fish given the occurrence of a spill and need further investigation. Our study clearly demonstrates that Arctic grayling have a much greater sensitivity to saline water exposure when compared to their euryhaline salmonid relative, the rainbow trout.

Arctic grayling were unable to successfully osmoregulate even when exposed to 50% seawater as demonstrated by histological, physiological, and molecular indicators. Grayling showed significant elevations of blood serum [Na^+] and [Cl^-] at 24 hr, and these were even further elevated through the 96h-sampling period. This elevation of blood salinity would be detrimental for normal physiological function (Conte and Wagner, 1965; Jackson 1981) and is likely the proximal cause of death due to hematological failure (Maxime et al., 1991).

Comparing rainbow trout and Arctic grayling, the response in serum [ion] and osmolarity were similar in 50% seawater at 24 hr. However, rainbow trout had the physiological capacity to overcome this stress and successfully reduced the ion load by 96h while grayling were unable to compensate. A salmonid

comparison study by Bystriansky *et al*. (2006) demonstrated the differences in elevation patterns of plasma ions between three salmonid species acutely exposed to 32ppt (~100% seawater). Although some mortality did occur, all three species, Arctic char, Atlantic salmon, and rainbow trout were able to compensate the osmotic disturbance and gradually lowered initial elevated plasma ion levels, with char being the least successful out of the group (Bystriansky *et al*., 2006). Similarly, Richards *et al*. (2003) showed that rainbow trout exposed to 40% and 80% seawater gradually decrease their initial ion elevations, returning to control freshwater levels by day 5. In contrast to these studies and the rainbow trout in the present study, Arctic grayling were unable to correct the salt load and at 96h serum Na^+, Cl^-, and total osmolality were elevated significantly higher than both control and the already elevated 24 hr levels. This pattern was also observed in landlocked Arctic char, which have limited sea water tolerance, whereby upon transfer to 100% seawater (32ppt) plasma osmolality levels rose from freshwater control levels and increased significantly through day seven (Bystriansky *et al*., 2007).

Various studies have shown the link between salinity transfer and gill Nka expression and activity in salmonids (McCormick 1996; Richards *et al*., 2003, Bystriansky *et al*., 2006; McCormick *et al*., 2013). In freshwater, Nka provides the driving energy for Na^+ absorption through mitochondrion rich cells (MRCs) while in seawater adapted fish, Nka mediates the branchial excretion of Na^+ through maintaining the transepithelial electrical potential across the gill epithelium allowing for the movement of Na^+ from the blood back to the external

environment through leaky junctions between MRCs and accessory cells (Evans *et al*., 2005). In many instances, transfer of salmonids from freshwater to seawater results in the increased expression of *nkaα1b* mRNA and the decreased expression of *nkaα1a*, as seen in rainbow trout (Richards *et al*., 2003; Bystriansky *et al*., 2006), in Atlantic salmon smolts (McCormick *et al*., 2012), in brown trout (*nkaα1b* only) (Larsen *et al*., 2008); in Arctic char and Atlantic salmon (Bystriansky *et al*., 2006). This differential expression pattern has been associated with the ability of salmonids to tolerate seawater, and in the present study both grayling and trout showed a significant decrease in expression of *nkaα1a* upon transfer to seawater, similar to that of previous findings. Following exposure to seawater, *nkaα1b* mRNA expression also showed a significant decrease in the grayling gill. Their apparent inability to up-regulate the *nkaα1b* isoform may indeed lead to the grayling's lack of salinity tolerance seen in this experiment. In support, Bystriansky and colleagues (2007) showed a similar pattern in a population of land-locked Arctic char, which upon exposure to seawater (32ppt), displayed identical results to the grayling in our experiment with both isoforms demonstrating decreasing levels of expression, and concluded that this was indicative of a loss of salinity tolerance. It is possible that the relative ratio of MR cells to total cell number is altered during ILCM production, which may affect the result presented in (Figure 4.2) where relative expression to *ef1α* was demonstrated. However, Mitrovic et al (2009) in a study using hypoxia and ILCM development in goldfish demonstrated only minor changes in MR cell number (<10%) per filament over three days. Furthermore,

they demonstrated the ionocytes migrated to the surface of the ILCM rather than increase in number (Mitrovic *et al*., 2009). With that being said we are currently investigating the number of ionocytes in Arctic grayling associated with the ILCM.

Many studies utilize full strength seawater (100% or 32 - 34ppt) and longer acclimation periods (>7days) to demonstrate changes in *nka* expression patterns. For example, Richards *et al*. (2003) demonstrated the down regulation of *nkaα1a* and up-regulation of *nkaα1b* mRNA in the gills of rainbow trout exposed to 80% (~26ppt) seawater occurring at day one persisting through day five following exposure. While upon exposure to 40% (~13ppt), rainbow trout showed decreased expression *nkaα1a*, however they did not demonstrate a significant increase in *nkaα1b* expression and it was suggested that 40% seawater was insufficient to induce *nkaα1b* (Richards *et al*., 2003). This pattern supports our findings where *nkaα1b* did not increase in 50% seawater, and suggests that there must be a "threshold cue" for up-regulation of this seawater isoform to occur. In our study, grayling clearly had a substantial osmotic perturbation in their blood serum but there was no upregulation of *nkaα1b*. Whether or not they have the capacity to do so or simply have not reached a required threshold in our study remains to be determined.

Histological images of the gill filaments revealed an impressive plasticity associated with the gill tissue of the Arctic grayling resulting from exposure to saline water. Following 24 hr exposure to 50% seawater, a significant increase in ILCM of the grayling appeared, at times completely overgrowing the protruding

lamellae essentially eliminating the interlamellar region for water flow and decreasing the surface area of the gills dramatically. To our knowledge the present study represents the first evidence of a saline induced ILCM increase in any salmonid, and is intriguing with respect to the rapid time course (< 24 hrs) exposure by which it appeared in the Arctic grayling gill.

Gill plasticity or increases in ILCM are not new observations; indeed it has been well documented in the literature and can happen as a result of many factors. The classic example of gill remodeling occurs in crucian carp (*Carassius Carassius*) and goldfish (*Carassius auratus*) in response to hypoxia. In control animals, a thick ILCM exists and this is rapidly reduced upon exposure to hypoxic conditions. This plasticity is proposed to create greater surface area for oxygen uptake (Sollid *et al*., 2003; Sollid and Nilsson, 2006; Nilsson *et al*., 2012). Decreases in ILCM have also been documented in response to temperature variation in the goldfish when acclimated to 25°C compared to those acclimated to 7°C (Mitrovic and Perry, 2008). However, in response to high environmental ammonia (Sinha *et al*., 2014), crucian carp and goldfish increased their ILCM. Similarly, Wright and colleagues documented killifish (*Kryptolebias marmoratus*) increasing their ILCM in response to air exposure to prevent water loss (Ong *et al*., 2007; Turko 2011). Wright's group also found that the ILCM of the killifish was actually decreased in seawater acclimated animals compared to freshwater acclimated fish (LeBlanc *et al*., 2007). The only documented ILCM increase by a salmonid was observed in brook trout (*Salvelinus fontinalis*) in response to aluminum exposure in slightly acidic water (Mueller *et al*., 1991).

We suggest this particular gill remodeling would be a beneficial defensive mechanism upon exposure to high salinity, limiting the uptake of salts (Na^+/Cl^-) and preventing water loss across the gill epithelium by reducing the exposed lamellar surface area. As the results indicate, the ILCM although still highly elevated at 96h does become slightly more reduced than its maximum height at 24 hr. We hypothesize that the decrease in ILCM from the 24 hr point to the 96h level may be in response to the grayling blood becoming hypercapnic or hypoxic as a result of reduced gas exchange attributed to the ILCM. Theoretically, the ability to increase ILCM would be advantageous to the grayling during a short-term spill of saline produced water. However, for a longer-term spill or if faced with environmental hypoxia, the ILCM would have deleterious consequences.

We propose that the appearance of an ILCM may be able to serve as a biological marker of grayling exposed to high saline water. Given the simplicity of field fixation of moribund animals, this has the potential to be developed as an indicator of saline exposure following saline spills into grayling bearing waters. Development of the ILCM as a viable marker for exposure requires further research into the rate of ILCM formation, the persistence/reversibility after the saline exposure and the physiological consequences of the ILCM (i.e. blood O_2/CO_2 concentrations).

In conclusion, the present study has demonstrated a significantly reduced salinity tolerance by the Arctic grayling in comparison to the rainbow trout. From a conservation physiology perspective, these data reveals the high sensitivity of grayling to an environmental spill of produced water. Our study

highlights the lethal effects on Arctic grayling exposed to saline water at just 50% seawater (17ppt). Due to the non-anadromous ancestral life history and having a strictly freshwater existence for over 3 million years in North America, we suggest that under these acute salinity conditions Arctic grayling have lost the ability to execute the necessary osmoregulatory mechanisms to cope with higher salinity environments. As a main by-product of hydraulic fracturing, high saline produced water poses a great risk to freshwater habitats and the aquatic organisms. With the expansion of oil and gas industry, there is an increased risk of spills of produced water. We must be aware of the conservation impact that spills may have on native salmonid species that cannot physiologically compensate for such osmoregulatory challenges. Investigations on the physiological responses to acute salinity of other native freshwater salmonids (bull trout, mountain whitefish, cutthroat trout) have not been conducted. Compared to rainbow trout, which are utilized as an environmental regulatory species, Arctic grayling are unable to compensate for the osmotic stressors, which would result from a high saline produced water spill. Given these new data, collaboration between fisheries and the oil and gas industry will be vital in the long term conservation strategies in regards to the Arctic grayling in their native habitat. Increasing research aimed at the fisheries located in these industrially impacted regions will be critical in coming to informed conclusions on the sustainable coexistence of these combined resource interests.

CHAPTER V: Physiological and Morphological Investigation on Arctic Grayling (*Thymallus arcticus*) Gill Filaments and Interlamellar Cell Mass with High Salinity Exposure

Introduction

Arctic grayling (*Thymallus arcticus*) are the only salmonid representative of the sub-family Thymallinae native to North America. Historically their range spread west of Hudson Bay across the northern halves of British Columbia, Alberta, Saskatchewan, and Manitoba, as well as populations found in Michigan and Montana (NatureServe 2004). The southern reaches of the population have been declining in the past century; most notably, the Alberta and Montana populations, and the complete extirpation of the Michigan populations. In Alberta, the exact reason for this decline is unknown and is likely the result of a multifaceted network of factors, including but not limited, to habitat fragmentation, overharvest, climate change, and watershed effects brought on by the oil and gas industry (Walker, 2005).

Research has informed us of the negative effects of habitat fragmentation via road construction and culverts on river populations of fish, and there are current practices being implemented to reconnect fish to their spawning areas and reduce negative effects of culverts on fish passage (Park et al., 2008; MacPherson et al., 2012). Overharvest effects have been directly addressed in Alberta by the implementation of a zero harvest regulation for grayling province wide (AEP 2015). Current investigations on temperature effects on grayling are being performed and will help to elucidate thermal sensitive areas and the populations at risk (Jessica Reilly: AEP personal communication). Although some regulations exist, oil and gas industrial practices can result in large volumes of hypersaline water, which upon incidental release into waterbodies

can result in osmoregulatory challenges for the aquatic organisms. However information on the potential risks concerning Arctic grayling and other native species under these conditions is extremely limited and regulations do not yet exist to accommodate their specific osmoregulatory needs.

The ability to osmoregulate is in direct relation to the function of the gill or branchial epithelium of the fish (Evans et al., 2005). In general, teleost fish gills are composed of two sets of four gill arches. Each arch is composed of many laterally branching filaments, which contain an afferent and efferent blood vessel that branch individually to the many folds of the epithelial surface or lamellae, and allow for a countercurrent exchange of gasses and ions with the water (Evans et al., 2005). The branchial epithelium is made up of pavement cells, mucous cells, mitochondrial rich cells (MR cells), the latter of which are responsible for the bulk of ion transport and are found on the trailing edge of the filament as well as near the base of the lamellae facing the interlamellar space where water flow occurs (Evans et al., 2005; Perry, 1997). The structure of the branchial epithelium is well characterized in a number of species, although it has not been well characterized in Arctic grayling.

Osmoregulation is critical for freshwater homeostatic balance and is hindered by osmotic stress such as a sudden change in environmental salinity. Hydraulic fracturing practices result in large volumes of hypersaline water, up to 10X that of seawater (300ppt) (Allen, 2008). It has been documented that spills of this produced water do occur in some regularity, 51 incidents of varying volumes of saline water releases have been reported from Jan 2016 to June 2016

(AER 2016) in Alberta alone. A small percentage of hypersaline releases may affect standing or flowing water, and under these circumstances osmotic stress on the aquatic organisms can be an immediate result. The degree of damage a release may have is proportional to the volume introduced and length of time of the release.

Recently I have demonstrated that even a short-term exposure to a salinity level of 50% seawater or 17ppt (a concentration which is the result of a 20-fold dilution of common hypersaline produced water), is still beyond the tolerance level of Arctic grayling (Blair et al., 2016). It was shown that due to the grayling's non-anadromous behaviour and the inability to up-regulate the necessary seawater isoform sodium-potassium ATPase alpha1b (*nkaα1b*) there was a reduced tolerance to hypersaline waters as demonstrated by their moribund state and considerably increased osmolality and serum ion levels. Additionally, exposure to 17ppt saline water resulted in gill remodeling and the appearance of an ILCM that was not present in their salmonid relative, the rainbow trout, under the same treatments (Blair et al., 2016). The presence of an ICLM has been noted in other instances in which fish were exposed to some type of ionoregulatory or osmoregulatory perturbations including: hypoxia-induced changes in ILCM in goldfish (Sollid et al., 2003; Sollid and Nilsson, 2006; Nilsson et al., 2012); temperature variations in goldfish (Mitrovic and Perry, 2009); high environmental ammonia on crucian carp (Singha et al 2014); air exposure and salinity on killifish (Ong et al., 2007; Turko et al., 2011; LeBlanc et al., 2010) and increases to ILCM of brook trout exposed to aluminum (Mueller et

al., 1991). We hypothesized that the ILCM appearance in the Arctic grayling in response to salinity could be a defense mechanism limiting the uptake of salts (Na^+/Cl^-) under hypertonic environments.

The question remains that if an Arctic grayling exposed to salinity for a short-term 48 hrs or less, was able to reach freshwater, could normal serum ion levels be recovered and the resultant ILCM be reduced? It was thus of interest to evaluate this ILCM, in relation to time of development and ability to recover if the fish was allowed to enter back into a more favourable hypotonic freshwater environment following exposure. This present study set out to further investigate the general gill morphology and identify cell types of the epithelium and ILCM of Arctic grayling. Furthermore, I aimed to explore that in the event a release of hypersaline water was quickly ameliorated and environmental salinity returned to normal, whether the potentially lethal effects seen in Chapter 4 could be reversed.

Methods

Animal Collection

Arctic grayling (18.1 ± 0.4 cm, 49.7 ± 3.9 g, means ± S.E.M.) were collected in July, 2015 from a tributary found in the Athabasca River watershed in collaboration with Alberta Environment and Parks (AEP) using conventional angling methods (fly-fishing). Fish were transferred in an Alberta fish transportation truck to the University of Alberta, where they were held in a bio-secure aquatics facility. Fish were maintained in a 600 L circular tank supplied

with flow-through dechlorinated Edmonton city tap water (10°C) and constantly aerated. Fish were allowed to acclimate for 3 months prior to experimentation. Arctic grayling were fed a mixture of *Artemia* and *Mysis* shrimp, every other day.

Experimental tanks

Two identical systems (freshwater and a saline) were utilized for the experimental tanks for the exposures. Each system consisted of three (60 L) in-line tanks, a top header tank, an exposure tank (housed fish), and a sump tank, which re-circulated 180 L of water maintained at 10°C with an attached chiller unit. Both systems were initially supplied with dechlorinated Edmonton city tapwater, which was aerated with air stones placed in each exposure tank. Dissolved oxygen levels were maintained above 10 mg/L throughout the experiment. The saline system water was made up to 17ppt (50% sea water) with the addition of a soluble salt mixture (Instant Ocean). This salinity concentration was chosen based on our previous research revealing sub-lethal effects at this salinity, which are the focus of the present study. When necessary, salinity levels were maintained by adding salts and/or freshwater following measurement using a handheld digital salinity probe (YSI Model 85; Yellow Springs, OH, USA).

Salinity Exposure and Sampling

Arctic grayling were fasted four days prior to experimentation and underwent six different treatments. Fish (n=5 for each treatment) were acutely

transferred to either 17ppt saline water and held for 12, 24, and 48 hrs, or to the freshwater system for 24 hrs (control) before lethal sampling. In order to observe the Arctic grayling's ability to recover following the 24 and 48 hr acute saline exposure, fish were transferred back to a freshwater environment where they were allowed to recover for an additional 24 hrs before undergoing lethal sampling.

Fish were anesthetized with a lethal dose of MS-222 (200 mg L^{-1}) buffered with $NaHCO_3$ (400 mg l^{-1}), weights and fork lengths were taken, followed by immediate blood and tissue sampling. Blood was drawn from caudal puncture using non-heparinized syringes and deposited into 1.5ml Eppendorf tubes and centrifuged (2 min, 12,000 x g). Serum was separated and frozen at -80°C for future analysis. The second and third gill arches were excised from each side of the fish and further processed for light microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) analysis.

Physiological Serum Levels

Arctic grayling serum from each of the six treatments was thawed and analyzed for total osmolarity (mOsm), (Na^+), and (Cl^-) concentrations. Following essential dilution in ultrapure water, analysis serum [Na^+] was performed using an atomic absorption spectrophotometer (Varian 220 FS). Serum [Cl^-] was measured via digital chloridometer (Labconco, Kansas City, MO, USA). Total serum osmolarity analysis was performed using a freezing point depression

osmometer (Micro Osmette; Precision Systems). Statistical analysis was performed with one-way ANOVA, followed by Tukeys multiple comparisons test (Graphpad Systems: Prism 6).

Light Microscopy

For consistency purposes the right third gill arch from each grayling was excised and fixed in a 4% PFA in PBS (pH 7.4) for 24 hrs at 4°C. Gill tissue processing followed a standard histological protocol consisting of washes in PBS, followed by a dehydration series of increasing ethanol concentrations, and paraffin wax embedding. Gills were mounted in wax blocks and serial sectioned with a microtome yielding 7µm thick sections to be mounted on Superfrost plus microscope slides (Thermo Fischer Scientific, Rockford, IL, USA). Five slides per gill were obtained containing 3-4 serial sections on each slide. Slides were stained with either Periodic acid-Schiff (PAS) stain for mucous cells localization, or hematoxylin and eosin for general histological analysis and viewed on compound light microscope (Leica DM RXA). A single slide per fish was randomly selected and digital images were captured (QI Click, QImaging). Three filaments from each fish were selected, and subsequently 10 lamellae lengths and interlamellar cell mass measurements per filament were taken using ImageJ software (National Institutes of Health). As previously described (Ong et al., 2007; Blair et al., 2016), ratios of interlamellar cell mass to lamellae height were calculated for each fish (n=5) per treatment and averages were compared as

above via one-way ANOVA, with Tukeys Multiple Comparisons test (Graphpad Systems: Prism 6).

Electron Microscopy

For SEM and TEM analysis, gill filaments were dissected from the left third gill arch. For SEM, pairs of filaments were dissected away from the middle of the gill arch, while individual filaments were teased apart under a dissecting microscope for TEM analysis. Both samples were fixed overnight at room temperature in a buffered 0.15 M sodium cacodylate solution (pH 7.4) containing 2.5% glutaraldehyde and 3% paraformaldehyde. Filaments underwent three ten-minute washes in 0.15M sodium cacodylate buffer, followed by dehydration in a series of ethanol washes. Following complete dehydration the SEM filaments were placed in a serial series of increasing concentrations of hexamethyldisilazane (HMDS) and allowed to air dry. SEM filaments were mounted on to SEM stubs and sputter coated with a Au and Pd mixture for microscopy analysis.

TEM filaments were exposed to 1 hr wash in 0.15 M sodium cacodylate buffer containing 1% osmium tetroxide, and an additional buffer wash step prior to dehydration through the graded ethanol series. TEM filaments were individually embedded into SPURR Resin blocks and sectioned using an ultramicrotome (Reichert-Jung Ultracut_E Ultramicrotome). Thin sections (~80 nm) were placed on copper grids and stained with a uranyl acetate and lead citrate stain. Examination and imaging of sections was conducted with a

transmission electron microscope (Philips-FEI, Morgagni 268), equipped with a Gatan Orius CCD camera.

RESULTS

Physiological Serum Analysis

Similar to our previous study, serum [Na^+], serum [Cl^-], and serum [mOsm] significantly ($P<0.05$) and progressively increased when Arctic grayling were exposed to 17ppt (50% seawater) at 12 hr, 24 hr, and 48 hr time points. At 48 hrs of exposure Arctic grayling serum levels reached elevations of 204.54 ± 5.27 mM Na^+, 184.40 ± 4.29 mM Cl^-, and 411.0 ± 5.0 mOsm (mean ± S.E.M.), an increase of 20%, 55%, and 44% respectively, from freshwater levels (Figure 5.1A, 1B, 1C).

Fish that were allowed a 24 hr recovery period in freshwater following the exposure to 17 ppt for 24 hr, showed significant decreases in serum [Cl^-], and serum [mOsm] ($P<0.05$). Although serum [Na^+] levels also demonstrated a decreased trend, statistical significance was hindered by sample variability in the 24 hr + recovery sample. Fish that were exposed for to 17 ppt for 48 hr and then allowed to recover in freshwater for 24 hr also demonstrated a similar pattern of shifting back towards osmotic balance. Significant differences were seen between samples from 48 hr exposure and the 48 hr + recovery samples, with serum [Na^+] decreasing by 10%, serum [Cl^-] decreasing by 21%, and total serum osmolality decreasing by 18%. Although 24 hr recovery in freshwater resulted in significant decreases from both the 24 hr and 48 hr exposure periods,

levels were still significantly elevated above those of fish held in control freshwater, for each of the measured serum levels (Figure 5.1).

Interlamellar Cell Mass

Measurements of lamellar length and height of interlamellar cell mass were taken at each of the sampling time points. Compared to freshwater values, significant elevations in ILCM were present just 12 hrs after exposure to 17 ppt water ($P<0.05$). The ILCM remained elevated at the 24 and 48 hr time points, however the 24 hr recovery period in freshwater resulted in significant reductions in ILCM height for both the 24 hr and 48 hr exposures ($P<0.05$, one-way ANOVA) (Figure 5.1D). This salinity-induced interlamellar cell mass began to decrease and slough off following 24 hr recovery in freshwater (Figure 5.2)

In addition to the increase in interlamellar cell mass, it was also apparent that during exposure to 17 ppt, the gills demonstrated an apparent increased in mucous cells on the apical side of the epithelium revealed by PAS staining. These cells were present in higher numbers in the 48 hr salinity exposed animals than in the freshwater control animals or in the freshwater recovery animals following the initial 48 hr exposure to 17 ppt (Figure 5.3).

Electron Microscopy

For SEM and TEM analysis, we utilized the salmonid relative the rainbow trout exposed to 17 ppt for 24 hrs to perform morphological comparisons of the Arctic grayling branchial epithelia. In brief, as the morphology of trout gills has

been described in great detail previously (Goss et al 1994; Evans et al 2008; Perry 1997), the lamellae of the rainbow trout expand to the edge of the filament, with a relatively small area of epithelial cells on either side making up both the leading and trailing edge (Figure 5.4A). Rainbow trout gill epithelial morphology consists of a covering layer of pavement cells, with a maze-like formation of interconnected uniform microplicae on their surface. Mitochondrial rich cells with two distinct surface morphologies are found interspersed amongst the pavement cells, one surfaced characterized by many fingerlike microvilli projections, while the other has a tightly packed multicursal labyrinth configuration of microvilli (Figure 5.4B).

Comparable to the rainbow trout gill structure, the Arctic grayling filament is composed of lamellae in similar orientation, however they extend laterally to a lesser extent leaving more surface area on the leading and trailing edges (Figure 5.5A). Large epithelial pavement cells cover the filament with their apical surfaces either flat or ridged with shortened microplicae in a less continuous fashion that those of the trout (Figure 5.5B). Differentiated MRC types commonly appear in pairs and their surface characteristics are almost identical to those of trout with one type having a more dotted appearance of short fingerlike microvilli, while the other surface portrays a compact maze-like quality (Figure 5.5B). Exposure to salinity results in reduced lamellar surface area, with the ILCM dominating (Figure 5.6A). Swelling mucous cells protrude from the Arctic grayling epithelium, and are noticeably more prevalent upon

exposure to 17ppt. There were no morphological changes to the MR cells in terms of apical crypts characteristic of salinity acclimation (Figure 5.6B).

Transmission electron micrographs of Arctic grayling gill sections revealed the presence of different cell types near the apical surface depending on the environmental salinity, as well as various cells present in the interlamellar cell mass. Representative freshwater samples show both healthy mitochondria rich cells, mucous cells, and apparent eosinophil (containing large granulocytes) at the apical surface of the filament in the interlamellar space (Figure 5.7A). Upon exposure to 17ppt at 12 hrs and persisting through 48 hrs, the interlamellar cell mass is identifiable and is characterized by an increased number of mucous cells throughout and near the surface of the elevated ILCM, as well as what appeared to be mucous cells or eosinophils bursting or releasing their granulocytes (Figure 5.7B). These bursting cells were not present in any freshwater samples. Furthermore, MR cells were not found in the interlamellar cell mass in any of the salinity exposed samples. Representative micrographs of gills from fish recovering in freshwater for 24 hrs post-salinity exposures, show the ILCM dominated by mucous cells, as well as non-descript dead cells, near the surface of the ILCM (as indicated by the denatured formation of the nucleus). However, on the edges of the filament (trailing edge), the surface contains healthy MR cells and mucous cells (Figure 5.7C, D, E). The 48 hr exposure plus recovery samples show apparent apoptotic cells with irregular nuclei and increased number of granulocytes or eosinophils (Figure 5.7F). Figure 8 shows

higher magnifications of the various cell types and internal organelles present in the ILCM of Arctic grayling.

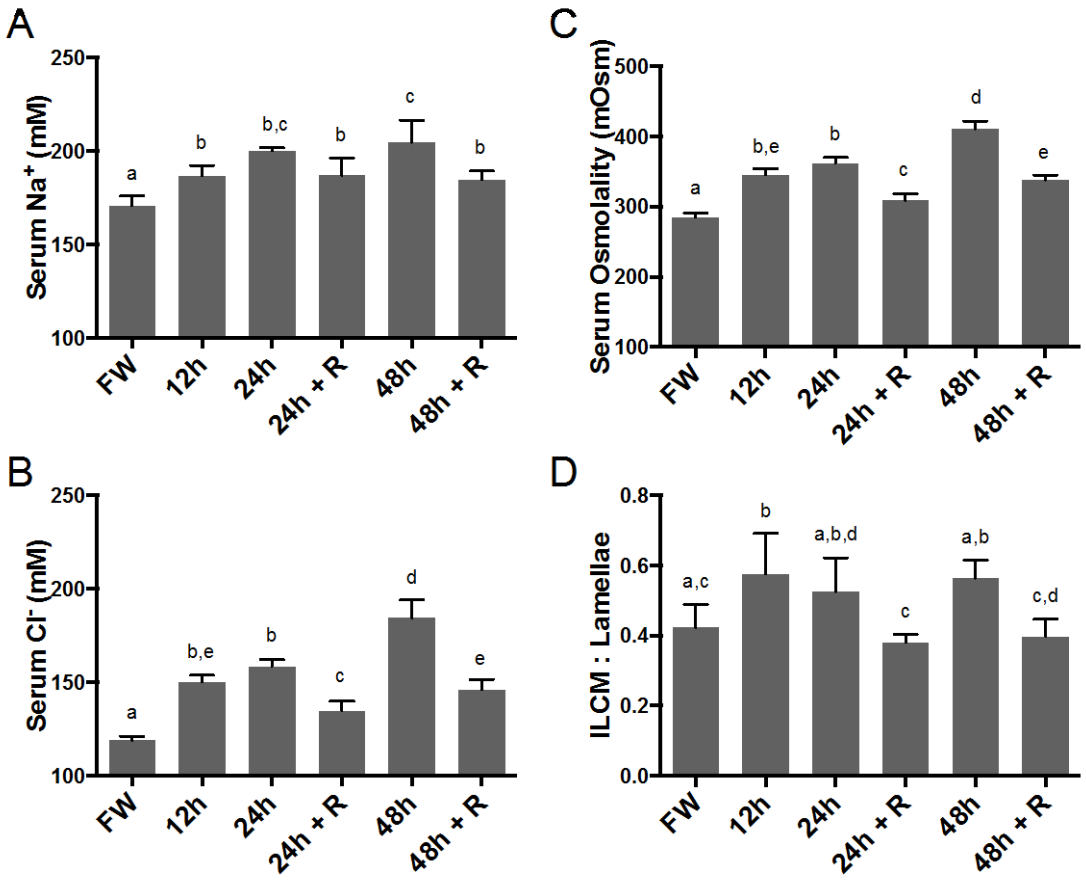


Figure 5.1. Physiological serum and ILCM data of Arctic grayling exposed to 17 ppt and following a 24 hr recovery period. (+R) indicates fish allowed to recover in freshwater for 24 hrs, following exposure to 17 ppt for 24 and 48 hr time points. A) Serum sodium [Na^+], B) Serum chloride [Cl^-], C) Total serum osmolality [mOsm], D) Height to length ratio of ILCM to lamellae of the various treatment groups. Data are presented as mean ± S.E.M., while dissimilar letters designate statistically significant differences between groups as demonstrated by one-way ANOVA, with Tukey's multiple comparisons test (n=5, $P < 0.05$, ANOVA).

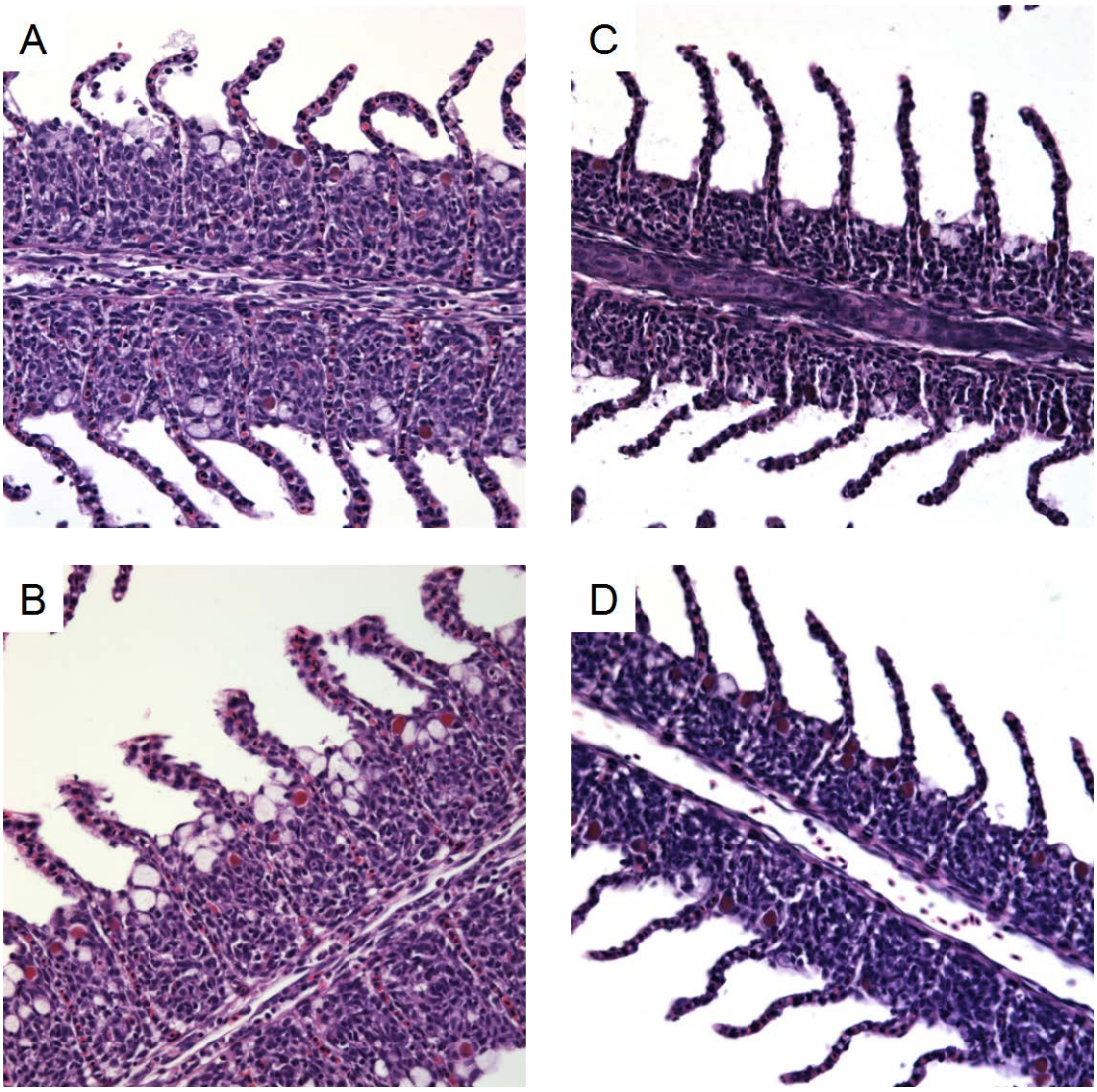


Figure 5.2. Representative images of Hematoxylin and Eosin stained filaments of Arctic grayling exposed to 17 ppt for 24 and 48 hrs and those allowed to recover in freshwater for 24 hrs, demonstrating reduction of ILCM. A) 24 hr exposed, elevated ILCM; B) 48 hr exposed, elevated ILCM; C) 24 hr + recovery in freshwater with reduced ILCM (24 hr +R); D) 48 hr + recovery in freshwater with reduced ILCM (48 hr +R). Images taken through 40X objective lens on a compound light microscope (Leica DM RXA).

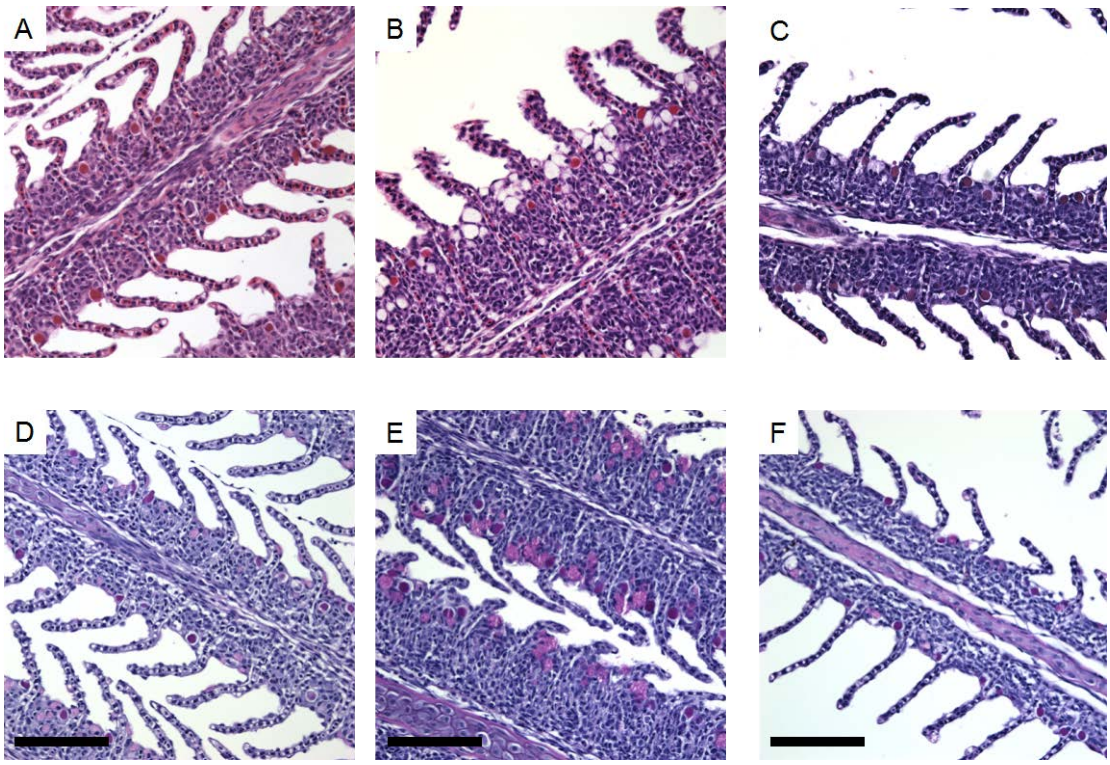
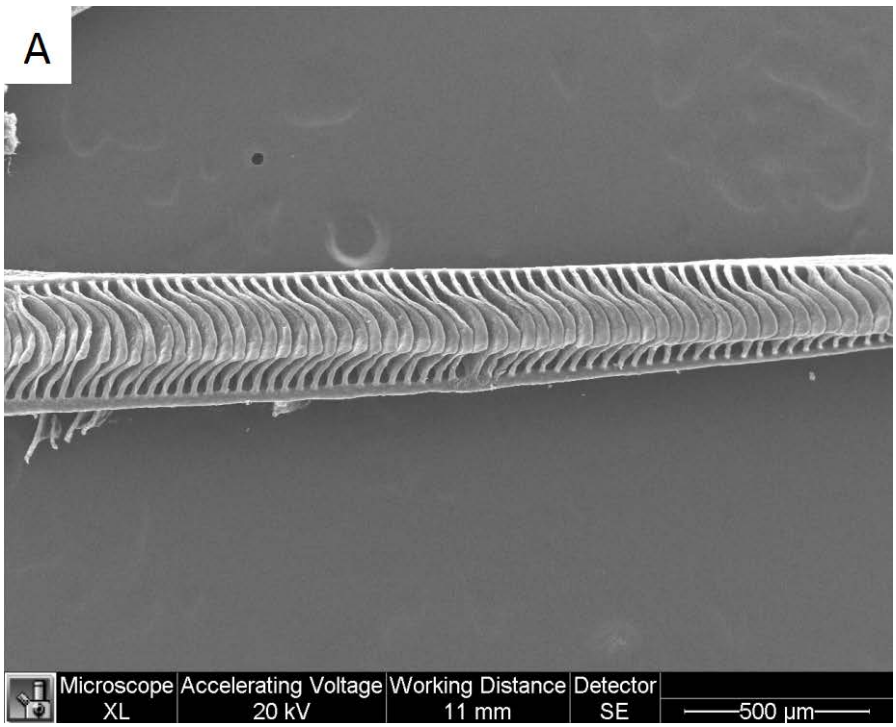


Figure 5.3. Hematoxylin and Eosin (Top Panel) and PAS stained (Bottom Panel) Arctic grayling gills showing increased number of mucous cells in fish exposed to 17ppt for 48 hr compared to fish held in freshwater and following 24 hr recovery post exposure. A,D) Freshwater; B,E) 48 hr exposed; C,F) 48 hr exposed + recovery.


A
Microscope
XL
Accelerating Voltage
20 kV
Working Distance
11 mm
Detector
SE
500 µm

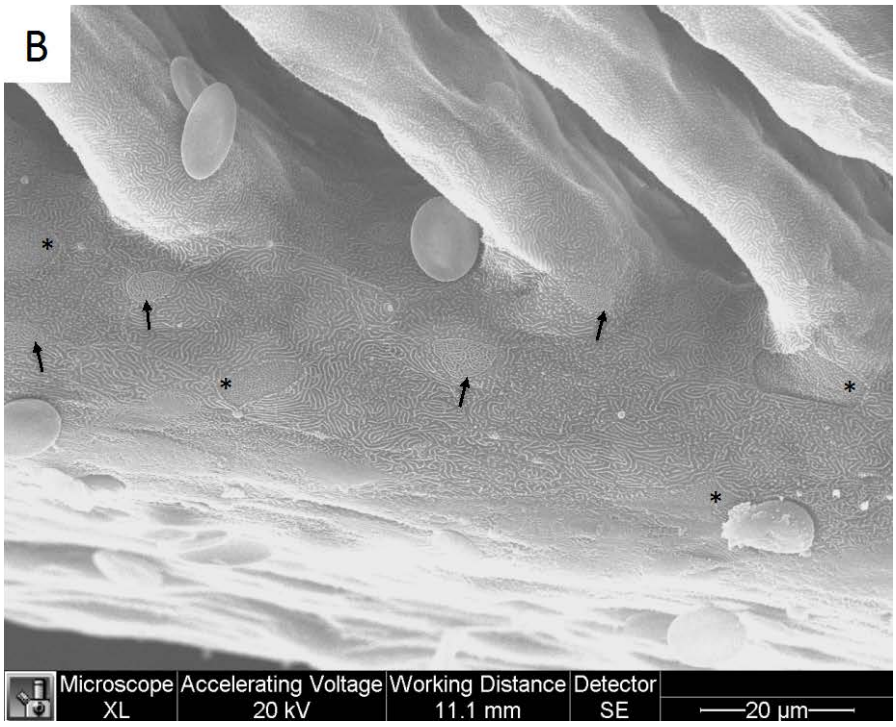

B
Microscope
XL
Accelerating Voltage
20 kV
Working Distance
11.1 mm
Detector
SE
20 µm

Figure 5.4. (Previous page) Scanning electron micrograph of rainbow trout gills. A) Single gill filament with exposed protruding lamellae following 24 hr exposure to 17ppt salinity. B) Higher magnification of trailing edge of filament near base of protruding lamellae. Apical surface reveals pavement cells covered with of interconnected uniform microplicae, and 2 types of MR cells interspersed among pavement cells, one with finger like projections (*) and one with tightly packed microvilli forming with a maze like appearance (arrows).

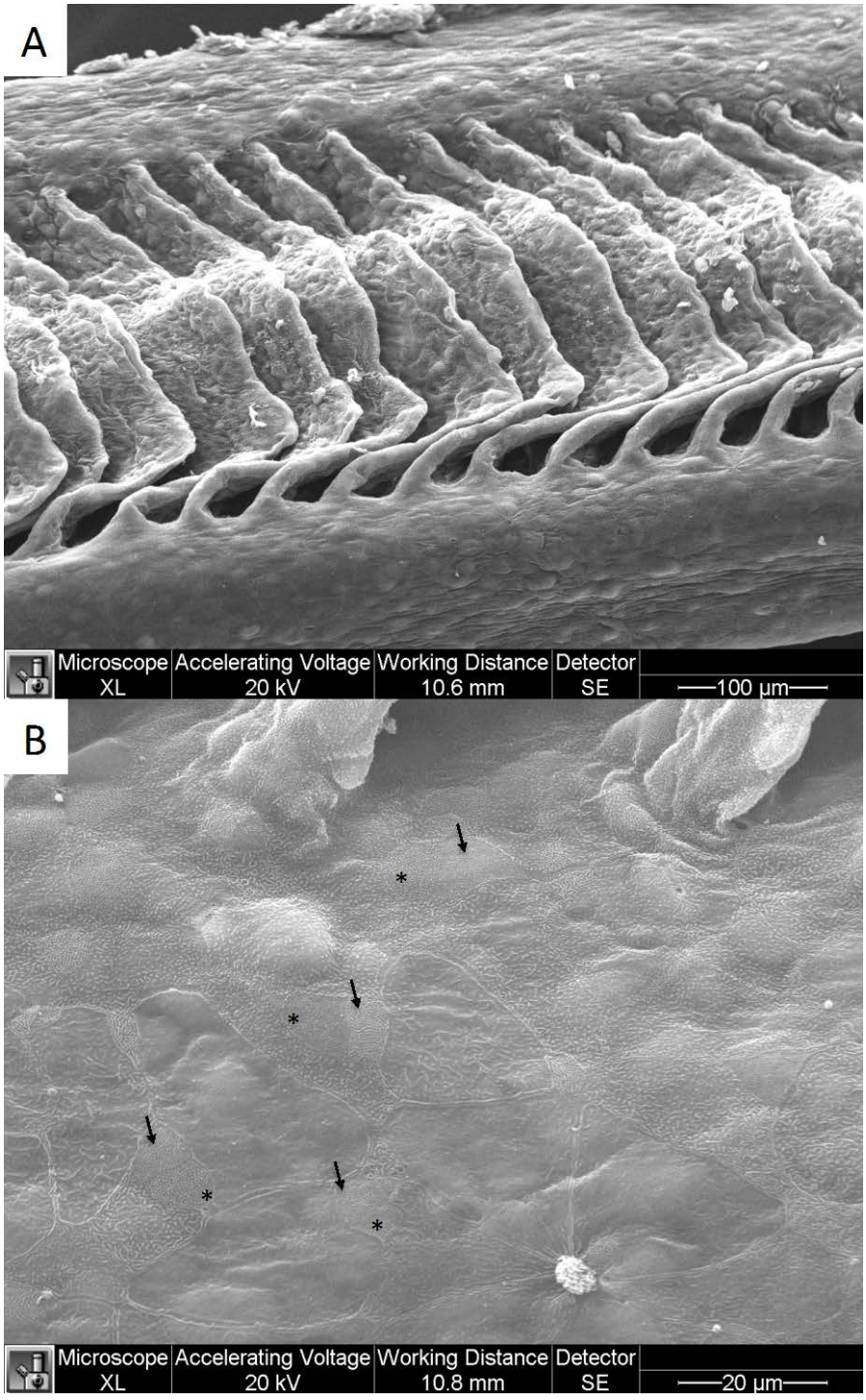

A
Microscope
XL
Accelerating Voltage
20 kV
Working Distance
10.6 mm
Detector
SE
100 µm
B
Microscope
XL
Accelerating Voltage
20 kV
Working Distance
10.8 mm
Detector
SE
20 µm

Figure 5.5. (Previous page) Scanning electron micrograph of Arctic grayling gills. A) Single gill filament with exposed protruding lamellae under control freshwater conditions. B) Higher magnification of trailing edge of filament near base of protruding lamellae. Apical surface reveals large flat pavement cells or covered with shortened unconnected microplicae, along with 2 types of MR cells similar to that of trout interspersed among pavement cells in pairs, one with finger like projections (*) and one with tightly packed microvilli forming with a maze like appearance (arrows).

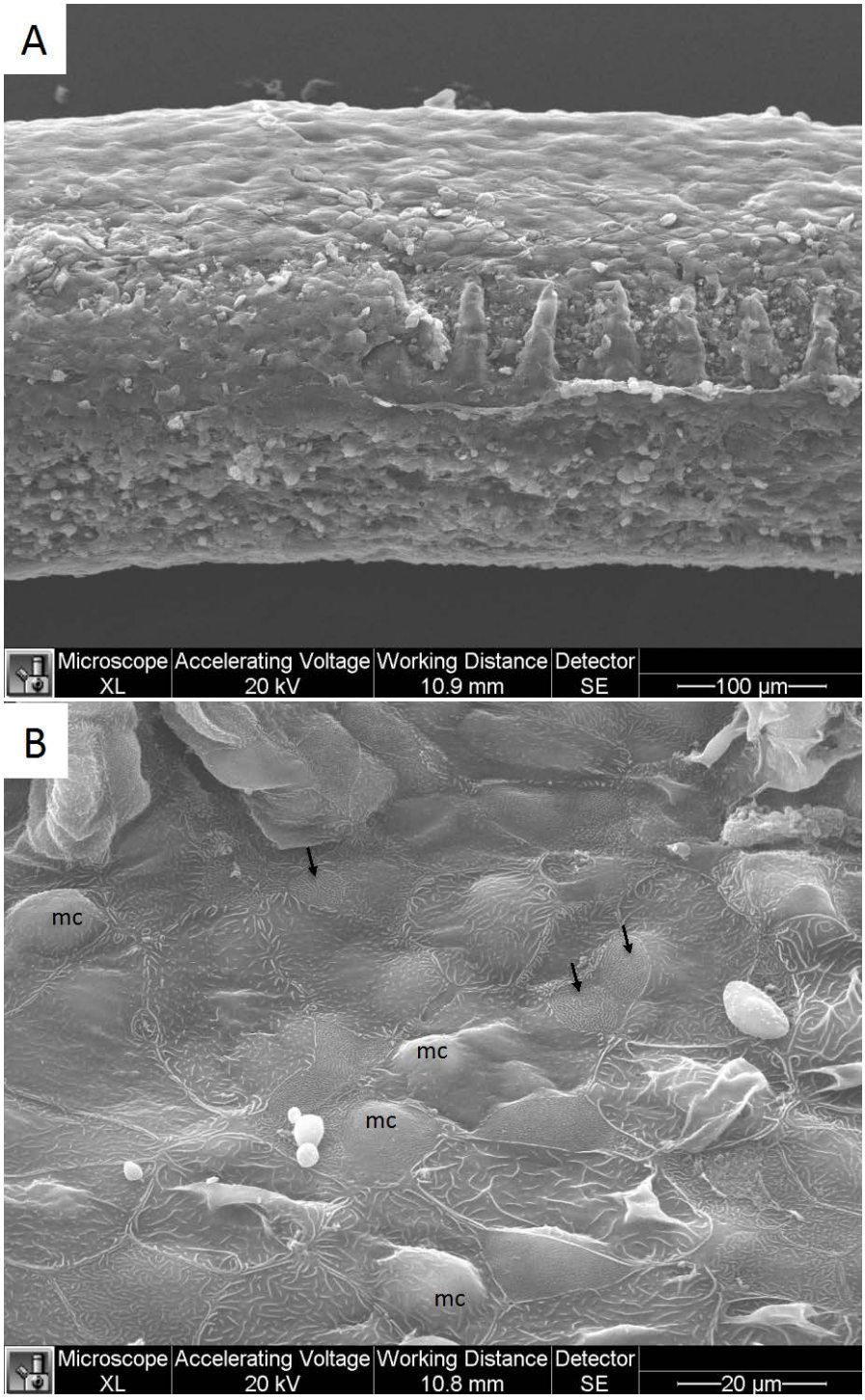

A
Microscope
XL
Accelerating Voltage
20 kV
Working Distance
10.9 mm
Detector
SE
100 µm
B
mc
mc
mc
mc
Microscope
XL
Accelerating Voltage
20 kV
Working Distance
10.8 mm
Detector
SE
20 µm

Figure 5.6. (Previous page) Scanning electron micrograph of Arctic grayling gills. A) Single gill filament with limited number of exposed lamellae and increased ILCM. B) Higher magnification of trailing edge of filament near base of protruding lamellae. Apical surface of the trailing edge reveals large protruding mucous cells (mc) as well as a dehydrated like appearance of apical surface of pavement cells. MR cells (arrow) still apparent on trailing edge of filament, however no apical crypt development was seen.

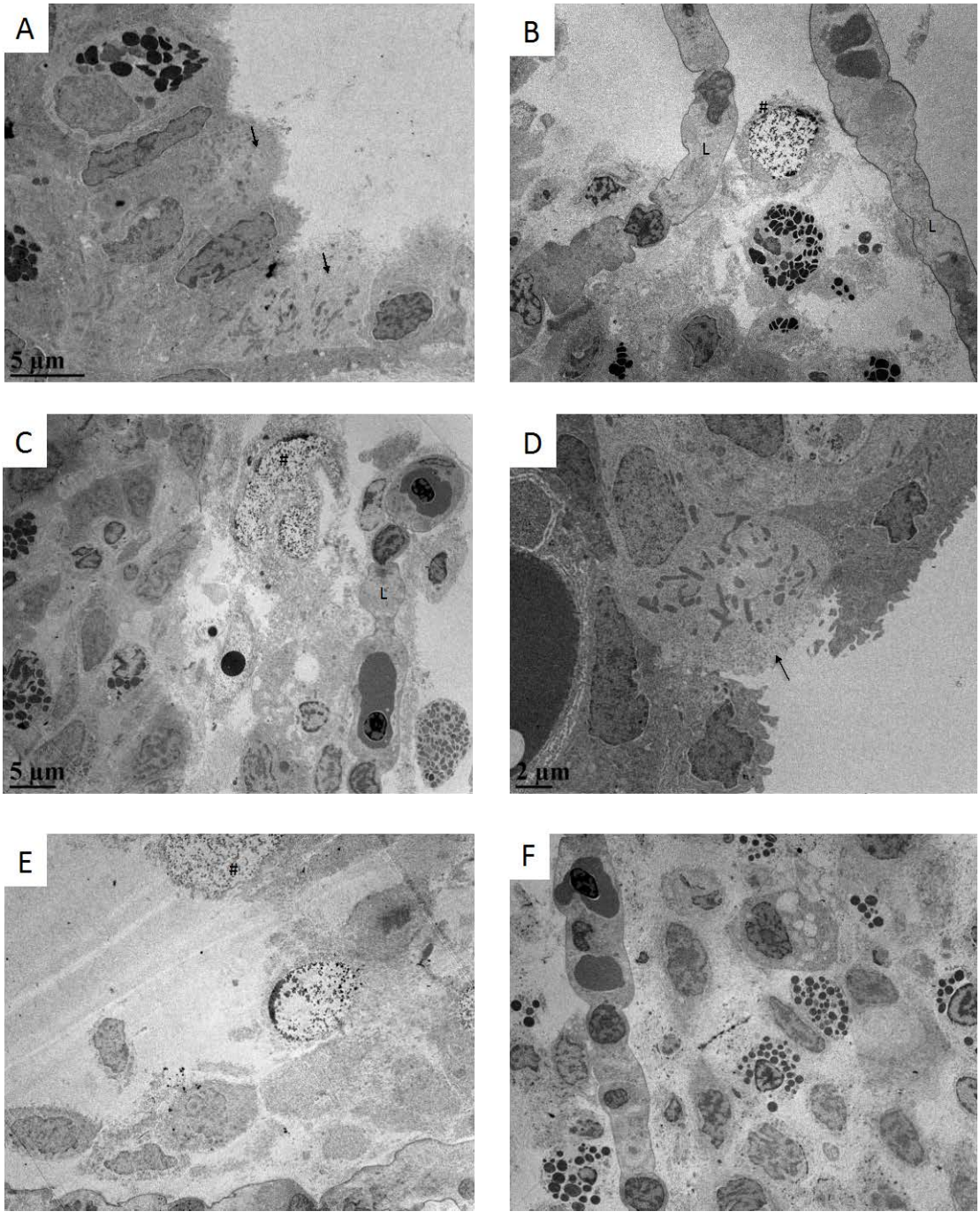

A
5 µm
B
#
L
L
C
#
L
5 µm
D
2 µm
E
#
F

Figure 5.7. (Previous page) Transmission electron micrographs of Arctic grayling ILCM under various salinity exposures. A) FW gill interlamellar space, with MR cells present (arrow), granulocytes (black granules), B) 12h salinity exposure with lamellae on either side (L) and with elevated ILCM containing granulocytes and apparent bursting/releasing cells on apical surface (#), no MR cells on apical surface, C) 24 hr salinity exposure with granulocytes and again bursting or releasing cells next to end of lamellae, D) Trailing edge following 24 hr recovery, with exposed MR cell, E) 48 hr exposed ILCM, F) 48R recovery, ILCM still slightly elevated but cells seem to be undergoing apoptosis (irregular nucleus) and many granulocytes present.

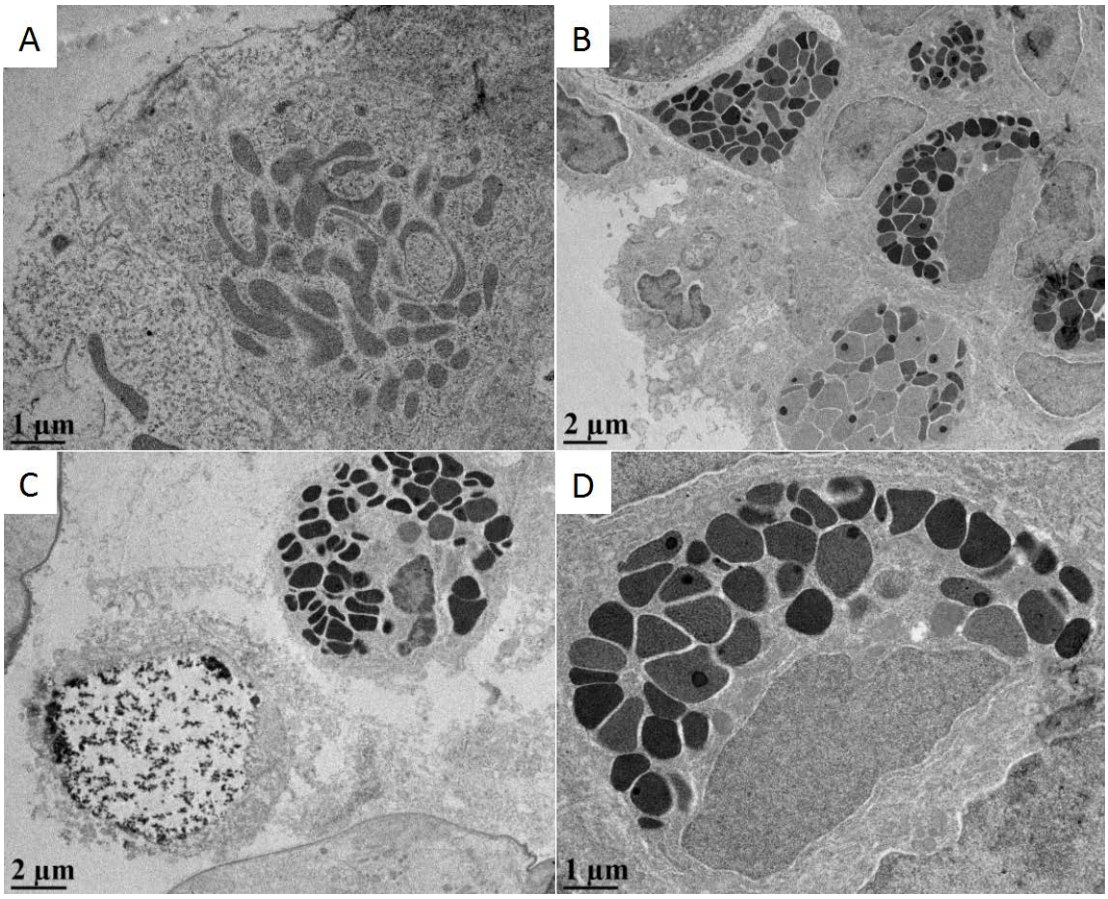


Figure 5.8. Higher magnification of various cell types found within the Arctic grayling ILCM. A) Mitochondria rich cell, B) Granulocytes/Eosinophils and apoptotic cells, C) Granulocyte and bursting/releasing cell, D) Enhanced granulocyte.

Discussion

The present study was performed to fill a gap in the teleost literature regarding the unique and important salmonid species, the Arctic grayling. Physiological data demonstrate the stenohaline behaviour of this salmonid, while novel morphological evidence effectively provides insight into the cell and tissue alterations occurring during an osmotic perturbation encountered by this organism. In doing so, we also answered the significant ecological and conservational question of possible freshwater recovery following an environmental exposure to high salinity. To our knowledge this is the first study to provide general branchial morphological data on the Arctic grayling, in addition to characterizing the gill remodeling that occurs following exposure and recovery from acute elevated environmental salinity.

Changes in serum levels of measured ions and total osmolality occur within twelve hrs of acute exposure to 17ppt salinity. Significant and persistent elevations in these levels through 48 hrs post exposure indicate osmotic imbalance on the fish and measures must be taken to alleviate this stress. In most salmonids, osmoregulation to return to a homeostatic level occurs within 2-4 days following osmotic exposure, (Finstad et al., 1988; Richards et al., 2003; Bystriansky et al., 2006; McCormick et al., 1989). We know from previous experiments, that Arctic grayling cannot successfully osmoregulate upon 17ppt exposure displaying serum levels highly elevated above normal exposure values, becoming moribund around 96-100 hrs (Blair et al., 2016). However, in our current study, these fish were allowed to return to freshwater and consequently,

show decreased serum ion and total osmolality levels by the end of the 24 h recovery period. Due to the reversed gradient, moving from a hypertonic to a hypotonic environment, aids in the excretion and diffusive loss of these blood salts back to the water, allowing for the grayling's serum levels to begin returning to normal freshwater parameters.

The presence of the ILCM was previously shown to appear upon 24 hrs exposure to 17ppt salinity, however in this study increases were seen already at 12 hrs post exposure. This rapid gill remodeling of increasing ILCM is one of the fastest reported to date, although decrease of ICLM was reported within 1 day in goldfish exposed to hypoxia (Mitrovic et al., 2009). Again when Arctic grayling are allowed to recover in freshwater for 24 hrs, ICLM decreased in comparison to the 24 hr and 48 hr exposures to 17 ppt salinity. This demonstrates the ability for rapid reduction of the ILCM when favourable conditions return, which would allow for increased surface gill area for oxygen uptake and salt excretion. The ILCM development and reduction continues to support our temporary defense mechanism hypothesis describing this gill plasticity as a way to protect the fish against environmental salinity resulting in immediate lethal effects. The ILCM has been shown to develop or decrease when environmental conditions induce the need for it to do so. Sollid and colleagues (2003) demonstrate this ability in crucian carp gills which normally lack protruding lamellae under normoxia conditions, however when exposed to hypoxia, the interlamellar cell mass begins to decrease exposing the secondary lamellae which greatly increases the surface area for oxygen uptake from the environment. This gill remodeling was

associated with a lack of cell mitosis and increases of apoptotic cells within the ILCM under hypoxia conditions (Sollid et al., 2003). Apoptosis would be necessary for the shedding of the ILCM allowing the lamellae to be exposed, including during recovery in freshwater by Arctic grayling following salinity exposure.

Much of gill ionoregulation takes place on the apical surface of the gill epithelium in the interlamellar space containing the mitochondria rich cells under freshwater control conditions. Electron microscopy reveals that during salinity exposure, this area is characterized by a lack of MR cells on the apical surface and that the increased ILCM is composed of undifferentiated cells, pavement cells, mucous cells and granulocyte containing cells resembling eosinophils. The presence of MR cells was still apparent on the trailing edge of the filament, which likely allowed for the maintenance of basal ion transport during the increased ILCM event. The appearance of the granulocyte cells and mucous cells is indicative of gill irritation or stress, and we surmise, based on the increased presence of eosinophils, is the result of a localized immunologic response. As demonstrated by Collins et al. (1995), just as eosinophils are mobilized, trafficked, and regulated from the bone marrow into the lung in the presence of an allergen, the gills of fish may also undergo similar influx of leucocytes following immune stimulation from an environmental irritant, in this case salinity. IL-5 is the specific cytokine responsible for this trafficking in the previous example (Rankin et al., 2000), and although not yet cloned from any bony fish, predicted mRNA sequences for IL-5 receptors do exist for some fishes

including, but not limited to, the teleost channel catfish (XM_017458042.1), Atlantic salmon (XM_014155984.1), and the northern pike (XM_010905115.1). The predicted presence of these receptors alludes to the possibility that a similar immune mechanism could be present in the gills of fish and the ILCM plasticity and presence of granulocytes could be the downstream result of this process. In light of the presence of eosinophils in the ILCM of Arctic grayling exposed to salinity a precise assay or immune response indicator must be utilized in order to determine if the appearance or disappearance of the ILCM is associated with the immune system of the Arctic grayling.

In the event of a spill of hypersaline-processed water, the osmotic perturbations to the aquatic organisms present are a real threat and fish kills do occur. In 2007, hydraulic fracturing fluids overflowed a retention pond and spilled into Acorn Fork Creek (Knox County, Kentucky) raising stream conductivity from 200 µS/cm (<1 ppt) to over 35,000 µS/cm (>25 ppt) (Papoulias and Velasco, 2013). High salinity along with low pH and resulted in the mortality stress or displacement of many aquatic invertebrates and fish including the threatened Blackside Dace, *Chrosomus cumberlandensis,* which much like the Arctic grayling need cool clear water to thrive. Other affected fish included creek chub, which demonstrated extreme gill lamellar hyperplasia (we refer to as increased ILCM) which in this study was associated with a combination of effects resulting from decreased pH, metal toxicity, and increased conductivity (salinity) (Papoulias and Velasco, 2013). While these results are quite similar to the changes seen in the present study, we have demonstrated the

gill remodeling can be elicited by salinity alone in the case of these Arctic grayling. This may have implications for other studies associated with highly saline waters from hydraulic fracturing spills including the interpretation of results and consistent need for salt controls during physiological experiments.

It is again important to note volume, concentration, and time of a spill when interpreting the imminent negative consequences to the environment and organisms. As our study demonstrates if spills result in environmental salinity increasing to 17 ppt, Arctic grayling can potentially survive this perturbation if freshwater is regained within 48 hrs. It can be theorized that under environmental spill conditions an "acute transfer" of freshwater organisms into salinity can indeed occur given a large immediate spill, although a slower transition is more likely under the circumstances of a pipeline break or leak. In turn, the sudden recovery to freshwater could also be feasible given the leak was quickly fixed or flow was shut off, however a protracted recovery to full freshwater is more likely and plasma ion levels as well as ILCM is believed to reflect that transition.

Our freshwaters retain a constant risk of becoming more saline due to anthropogenic forces including, but not limited to, hydraulic fracturing, which has adverse consequences on the aquatic organisms. For this reason increased regulations and higher standards need to be met and enforced regarding salinity effects on freshwater environments (Canedo-Arguelles et al., 2016). Currently, the rainbow trout is used as a regulatory species for effluent testing and acute toxicity (USEPA 2002). However, it may be necessary in some situations for

regulations to be based on the local sensitive species near potential spill areas rather than the rainbow trout, which demonstrates higher salinity tolerance. Without discounting this clear obligation, many of our freshwater species are indeed able to cope with minor osmotic changes or perturbations which plague their habitat, within a certain species-specific tolerance range. The Arctic grayling maintains the ability to develop a potentially protective interlamellar cell mass in order to alleviate these threats to a certain limit. This ILCM seems to be associated with some type of immune response indicated by the presence of granulocyte containing cells within the resulting mass. Furthermore, upon the return of favourable conditions, the grayling blood serum ion levels decreased along with marked reductions in the ILCM, which is hypothesized to be linked to cell apoptosis. Further research into the exact mechanisms behind the development and reduction of the ILCM is necessary. Based on this evidence and other current examples in the literature, more evidence of gill plasticity or remodeling will likely be found in many more teleost fish.

CHAPTER VI: GENERAL DISCUSSION

General Summary

Members of the family Salmonidae employ various cellular mechanisms allowing them to inhabit environments that vary drastically in their salt content from very dilute freshwater streams to the hypertonic waters of the oceans. Furthermore, some salmonid species are capable of transitioning from these opposing environments quite readily, while others are not fully capable of tolerating both extremes. As a result of both freshwater and marine fish maintaining their internal osmotic balance relatively the same regardless of the exterior media, two very different processes are undertaken. Freshwater fish are exposed to strong hypotonic gradients and must balance the uptake of water and diffuse loss of ions by producing large amounts of dilute urine and by active absorption of ions (such as Na^+) across the gills. The Nhe is suggested to be one possible route of Na^+ acquisition, despite thermodynamic concerns over its ability to function under low pH and low external Na^+ conditions. In contrast, in a hypertonic marine environment, fish must balance the osmotic H_2O loss by constantly drinking and offsetting the subsequent salt load by producing small quantities of concentrated urine and actively excreting ions across the gill. Nka is one of the major ion transporters in the gill responsible for the excretion of Na^+. Past cellular models of both freshwater and marine fish have incorporated both of these proteins based on expression studies and pharmacological evidence, however the exact mechanisms involved in the handling of Na^+ were still not clear. I chose to focus the bulk of my Ph.D. research on clarifying some of the conflicting data surrounding rainbow trout Nhe isoforms; and from a

conservation physiology perspective, assess whether a strictly freshwater salmonid still maintained the capability of tolerating salinity by way of expression of Nka in order to assess the risk associated with hypersaline spills in Alberta.

Thesis Contributions

In this thesis I have contributed to the field of fish physiology by demonstrating for the first time expression patterns of all three nhe isoforms in rainbow trout at both the embryonic stage and juvenile stage, and cloned all three isoforms from adult trout tissues. I have demonstrated Na^+ uptake patterns and associated nhe expression at time points throughout embryonic development reared in hard water and in soft water conditions. I have also provided evidence supporting a prominent role of Nhe3b in Na^+ uptake under these low Na^+ soft water conditions. I have set the framework for a full pharmacological profile characterization of all three nhe isoforms, and although my personal attempts at this characterization were not successful, I am confident that finalizing this project is highly achievable in the near future with minor adjustments. My further work in the area of conservation physiology has led to the first documentation of a salinity-induced ILCM in salmonids. It was established that Arctic grayling have a reduced salinity tolerance putting them at higher lethality risk to hypersaline spills than other more tolerant fish. Additionally, I have demonstrated that Arctic grayling can recover following salinity exposure given the opportunity to return to freshwater within 48 hrs.

This recovery is aided by the ICLM serving as a protective mechanism during acute exposure and I further investigated the ILCM with various types of microscopy in order to identify the cell types responsible for its assemblage. While, somewhat separate from the theme of Na^+ transport, but in line with the conservation of the native Arctic grayling, I administered thermotolerance testing specific to an Alberta population of these fish with expectation that this CTM data will be useful for further management practices by Alberta Environment and Parks (see Appendix A). My first Chapter has laid out the background and acted as a literature review for the research topics of the subsequent data chapters. For the remainder of this final discussion chapter, I will review the previous data chapters, incorporating the important new information and discuss these in terms of the larger impact to the fields of both fish physiology and conservation physiology. Finally, I will highlight some future directions for each of these research areas.

Rainbow Trout Nhe Expression and Role in Na^+ Uptake

In Chapter II, I utilized $^{22}Na^+$ fluxes, pharmacological inhibitors, and gene expression data to characterize Na^+ uptake between trout embryos and larvae reared in hard water or low Na^+ soft water. This experiment was designed to reveal whether environmental freshwater Na^+ levels influenced differential expression patterns of nhe isoforms or other modes of Na^+ uptake in developing rainbow trout embryos. Although previous studies indicated the presence of Nhe isoforms, these lacked a functional relevance for the three isoforms being

simultaneously expressed and which ones were involved in Na^+ uptake. My data suggested that embryos from both treatments maintained similar internal Na^+ levels throughout development and the uptake rates during flux experiments were higher in the low Na^+ soft water-reared embryos. Furthermore, *nhe3b* expression was correlated with Na^+ uptake rates in soft water-reared embryos, while *nhe2* expression was low and not consistently quantifiable, and *nhe3a* was absent in embryo and larvae. A cohort of these embryos were allowed to grow into juveniles for six months and a week long acclimation to the same water conditions revealed specific tissue localization of each isoform with *nhe2* and *nhe3b* confined to the gills, while *nhe3a* was localized to the kidney (which clarified its absence in embryo and larvae as the kidney would be underdeveloped at this time). This pattern of increased *nhe3b* expression seen in the embryos was also found in the juveniles where it was 75-fold higher than *nhe2* in the gills regardless of treatment conditions. This was the first study to date to localize and quantify the expression patterns of all three known nhe isoforms in rainbow trout. It was concluded that *nhe3b* is the dominant nhe isoform expressed in young rainbow trout and plays a significant role in Na^+ uptake. It is clear that *nhe2* and *nhe3a* are also expressed in rainbow trout and their role and functional significance is still to be determined (see future directions section).

The aim of Chapter III was to perform a pharmacological profile on the trout Nhe isoforms. This would allow for the characterization of activity inhibition of each isoform by some of the commonly used Na^+ uptake

pharmacologic agents including: amiloride, EIPA, and DAPI. I accomplished the initial steps for these characterizations and cloned each of the genes into an expression vector system to be used for cell line transfection. Following cloning, multiple attempts at transfections were performed and cell viability results (G418 selection and acid suicide selection) indicated that the AP-1 cells were successfully transfected with each trout *nhe* gene. Further expression data and activity assays indicated that there was in fact no expression of any of the Nhe isoforms, thus false positive results were previously achieved. This chapter currently acts as a methods chapter with negative results, however I would not assess this chapter to be unsuccessful or non-informative. Contrary to that, in addition to the personal benefit of learning each of these techniques, this chapter clearly outlines that alternate steps are needed for successful outcome and has highlighted the problematic experimental protocols. All three trout *nhe* isoforms were cloned into pDisplay plasmids and upon transfection the AP-1 cells showed no protein expression. This lack of expression may indicate that the pDisplay expression system was not an ideal choice for this technique. Future work will be performed in the lab to finish this experiment. This data, when finalized, would have a very high impact on the field of fish physiology and will help interpret past, present and future experiments focused on Na^+ uptake mechanisms.

Arctic Grayling Tolerances and Conservation Physiology

The ability to tolerate seawater is a trait shared by many species of salmonids, as their common ancestors experienced marine environments. This ability however has been reduced in a number of salmonids including landlocked freshwater subspecies. Consequently, the aim of Chapter IV was to investigate the salinity tolerance of a threatened salmonid native to Alberta, the Arctic grayling. I demonstrated that indeed the Arctic grayling of Alberta have a greatly reduced salinity tolerance, surviving acute exposure to 17 ppt (50% seawater) for 96 hr (or less) prior to mortality occurring. Fish exposed to 75% seawater died within 24 hrs indicating a severely reduced salinity tolerance, which was linked to an inability to up-regulate the seawater *nka* isoform *nkaα1b*, which is consistent with a similar expression pattern in other species (Bystryanski et al., 2007). Moreover, I provided evidence of exposure associated gill remodeling by the Arctic grayling, as the first documentation of a salinity-induced ICLM among salmonids. It is theorized that this ILCM serves as a protective mechanism, able to be employed as a result of either an immunological response or directly from an environmental cue. Although this ILCM can provide temporary protection from water loss and decreases surface area for undesired salt or ion uptake, the decreased respiratory activity is likely a negative consequence and along with the osmotic pressures would lead to fish mortality, especially in rivers or streams with low oxygen tensions. An important salmonid comparison was made between the Arctic grayling and rainbow trout exposed to the exact same saline conditions; revealing successful

osmoregulatory behaviour and the lack of an ILCM employed by the trout. Implications of these data include the extra vigilance necessary when discussing the risks of hypersaline water spills and the species affected. Rainbow trout, an industrial and governmental regulatory model species, would not be affected by a spill of hypersaline water depending on concentration, at least when compared with other native salmonids (e.g. Arctic grayling). Thus, utilizing a more sensitive freshwater species would be necessary during effluent testing when salinity is a concern. Additionally, the ILCM, which was evident in salinity exposed Arctic grayling, has the potential to be used as a biological marker for saline spill affected waters. However, further research is needed to investigate the viability and feasibility of this option. From a conservation physiology standpoint, this data will be useful in determining management risks for the future of Arctic grayling populations in Alberta.

In Chapter V of my thesis, I aimed to answer some resounding questions stemming from the results of Chapter 4. These included: 1) Does the ILCM appear earlier than 24 hr post exposure to 17 ppt? 2) Can Arctic grayling recover both internal osmotic balance, as well as the resulting ILCM, given the opportunity to re-enter freshwater following a short exposure? 3) What is the morphology and cellular make up of the ILCM? For this experiment I utilized Arctic grayling from a different river system in Alberta in order to confirm that the previous results were not specific to a certain region. The exposure concentrations were matched to the previous experiment, with the addition of a 12 hr exposure, as well as two (24 hr in freshwater) recovery time points

following 24 hr and 48 hr of salinity exposure. Osmotic imbalance and ILCM was observed 12 hrs post-exposure and was similar to the initial study with internal ion concentrations significantly increasing compared to controls, however given the sub-lethal time points chosen (<48 hr) no mortalities occurred. Recovery in freshwater (24 hr) did in fact allow the Arctic grayling to begin to alleviate the osmotic stress as levels rapidly fell back to or near control concentrations, which is positive news regarding spills lasting less than 48 hrs or if fish are able to escape the plume of hypersaline water quickly.

In order to investigate the associated ILCM more closely, I utilized various microscopy techniques and demonstrated that the ILCM is composed of undifferentiated cells, pavement cells, increased number of mucous cells (shown by PAS stain), and apparent eosinophils or granulocytes. The increase of mucous cells and granulocytes is indicative of an immune-related response resulting in hyperplasia of cells on the filament in the interlamellar cell space. Light microscopy histological analysis indicated an increased number of mucous cells in the saline exposed fish, as well as the response of the ILCM to decrease or shed following post-exposure freshwater recovery. SEM and TEM micrographs revealed a large number of granulocytes in the actual ILCM growing between lamellae, as well as the presence of cells bursting or releasing their contents. Further characterization and staining is necessary for exact identification of various specific cell types. To my knowledge this was the first electron microscopy performed specifically on the ILCM of any salmonid and can serve as a future reference for ILCM studies, which I theorize will increase in number in

the next few years as more and more fish will be examined for this type of gill tissue plasticity under various environmental conditions.

Future Directions

The results and data presented in this Ph.D. thesis have added to the knowledge base in the field of fish physiology especially regarding the Na^+ regulation mechanisms in salmonids. Thankfully however, for each question answered, several more arise that lead to future research directions. In this section I highlight some future directions building on the data I presented for upcoming students in the lab who wish to expand on these findings.

Functional Role of Trout Gill nhe2 and nhe3b

In Chapters II and III, I demonstrated the expression patterns of trout *nhe* isoforms *nhe2* and *nhe3b* in the embryonic and larval stage, and specific tissue distribution in the juvenile, with subsequent cloning from adult trout tissues. The fact that *nhe2* and *nhe3b* mRNA are expressed at the gill and protein expression has also been confirmed (Ivanis et al 2008; Hiroi and McCormick 2012) allows us to question the relative functional significance of these two isoforms at the gill. In mammals, it has been suggested that NHE3 is the primary isoform responsible for Na^+ absorption effecting electrolyte and acid base balance, as well as blood pressure homeostasis, while the role of NHE2 appears to be mainly secretory, regulating cell volume without reabsorption roles

(Orlowski and Grinstein 2004). It is possible a similar allocation of function resides within the trout gill epithelial cells expressing these transporters.

Furthermore, it has not been clearly demonstrated that Nhe2 and Nhe3b are localized to the same gill cells, therefore they may indeed be performing two different functional roles and isolated to separate cell types much like differential expression patterns of the various isoforms of Nka found in the zebrafish ionocytes (Hwang et al 2011). In order to clarify these roles, the development of isoform specific antibodies would necessary. Although functional protein expression is superior, *in situ* hybridization with isoform specific probes could also be utilized to show differential expression of mRNA in the gills of trout and would be quicker and more economically feasible given the complexity surrounding antibody production.

Along with specific antibodies and probes, functional characterization of these isoforms would be benefited with the use of isoform specific pharmacological inhibitors. These could be used in conjunction with varying environmental conditions (low Na^+, high Na^+, low pH, high pH, etc.) and the analysis of flux data to show which isoform is responsible for mitigating certain osmotic and ionic perturbations. This would ideally be paired with mRNA or protein expression as in Chapter II of this thesis.

Is Salinity Tolerance an Inherited Aspect of Phenotypic Plasticity?

I have demonstrated in Chapters IV and V a reduced salinity tolerance of the freshwater salmonid, the Arctic grayling. This reduced tolerance is associated with the inability to up-regulate the necessary seawater isoform *nkaα1b*, even though this gene is expressed throughout the salmonid species, including Arctic grayling (Dalziel et al 2014). Based on the freshwater life history of the Arctic grayling and the lack of osmoregulation in saline waters, is it possible that these fish no longer have an environmental stress response that results in higher expression and in-turn have lost their tolerance? This brings about the idea of genetic plasticity, a currently popular topic in environmental physiology, whereby the environment may have the ability to induce one genotype to produce different phenotypes (Agrawal 2001). The aspect of epigenetics or the ability to inherit nuclear information without changes to the DNA sequence (Holliday 2006) may be the subject of investigation for changes in species salinity tolerances. This definition has been extended to epigenetic inheritance whereby heritable changes in gene expression unrelated to genetic variation occur (Richards 2006). It would be of great interest to acclimate grayling to higher salinities from freshwater at a slow rate in order to observe if prolonged acclimation would allow for tolerance to be reestablished. Alternatively, exposure to sub-lethal salinities at different life history stages (e.g. embryo/larval stage) may alter the salinity tolerance. To assess salinity tolerance as an aspect influenced by epigenetics, a different model organism that possessed a faster generation time than the Arctic grayling could be used. A

euryhaline organism would be of benefit, such as one with both freshwater and marine residing populations. The mummichog or killifish (*Fundulus heteroclitus*) would be an appropriate species to select for this experiment given its population stratification in different osmotic environments and previous results of genetic plasticity related to salinity (Whitehead et al., 2011). A potential experimental protocol might include repeated exposure of the freshwater parental generation to salinity and then compare the salinity tolerance of the offspring to the offspring of parental individuals that did not undergo salinity exposures. Interestingly, a similar experiment was conducted on fertilized eggs of the dessert pupfish (*Cyprinidon macularius*), whereby they were reared in either their spawning salinities or waters of differing salinities. Following hatch, the young were monitored for growth rates and food conversion; those reared in the spawning salinities fared far better than those transferred to a different salinity level following fertilization (Kinne 1962). It is hypothesized that environmental stressors such as salinity exposure can result in inherited epigenetics in the offspring, preparing them for similar scenarios. Since grayling do retain the genetic markers for salinity tolerance (Nka isoforms), it is possible that salinity tolerance can be regained and a relevant investigation would be a valuable. Additionally, there are other threatened native Alberta salmonids (e.g. mountain whitefish) that may also show similar patterns of reduced salinity tolerance and these ought to be further investigated.

Extent and Regulation of Interlamellar Cell Mass

Arctic grayling exposed to salinity (only 50% seawater) developed an ILCM within 12 hrs. This relatively quick form of gill remodeling is likely a protective mechanism employed by these fish to avoid environmental perturbations. However, this ILCM has been documented to develop or reduce as a result of temperature, air exposure, metal toxicity, and oxygen levels in a variety of other fish species. Taken together, is the development of an ILCM a common mechanism throughout all fish species? Is it exclusive to freshwater fish? Is it found only in teleosts? Have we as fish physiologists missed this mutual process as a result of excluding gill histology in a large number of experiments? These are some of the questions that should be addressed in order to include the development of an ILCM in the arsenal of coping mechanisms shared by fish in response to environmental stressors.

Questions remain as to the function and the exact trigger of the ILCM response in fish species. A reasonable theory for the ability of Arctic grayling to quickly produce an ILCM may be a protective function. For Arctic grayling, which reside in streams that are filled with glacial sediments and are subject to sudden flooding that turns some rivers from clear to turbid quite quickly; the ILCM may be a response to changes in sediment load. To assess this hypothesis, specific sedimentation experiments need to be carried out in conjunction with histological analysis in order to determine if stream sediment can cause the development of an ILCM.

In this thesis, I have demonstrated the presence of granule-containing cells, resembling eosinophils in the ILCM suggesting that at least some of the ILCM growth may be associated with an immune response. Further work is needed to establish this theory with more refined histamine or other cytokine release assays and increased cell identification. Is the ILCM stimulated by an early cortisol response? Wilson et al., (2002) demonstrated a plasma cortisol spike at 12 hrs upon seawater adaptation of freshwater Coho salmon. It is possible that a spike in cortisol could elicit a gill remodeling response driven by hyperplasia. However, another hormone may be responsible for the ILCM development. Insulin-like growth factor (IGF) is also present in teleosts and recent evidence points to its association with cell hyperplasia in zebrafish gills (Wood et al., 2005; Shive et al., 2015). It is clear that salinity does not trigger an ILCM in fish with high salinity tolerance like the rainbow trout; therefore the ILCM is likely a direct result of an irritant specific to each individual species or event. A diagram incorporating various aspects of the physiology surrounding the ILCM was developed based on these ideas (Figure 6.2). Further research into the induction of the ILCM across various species of fish will increase our understanding of this gill remodeling mechanism.

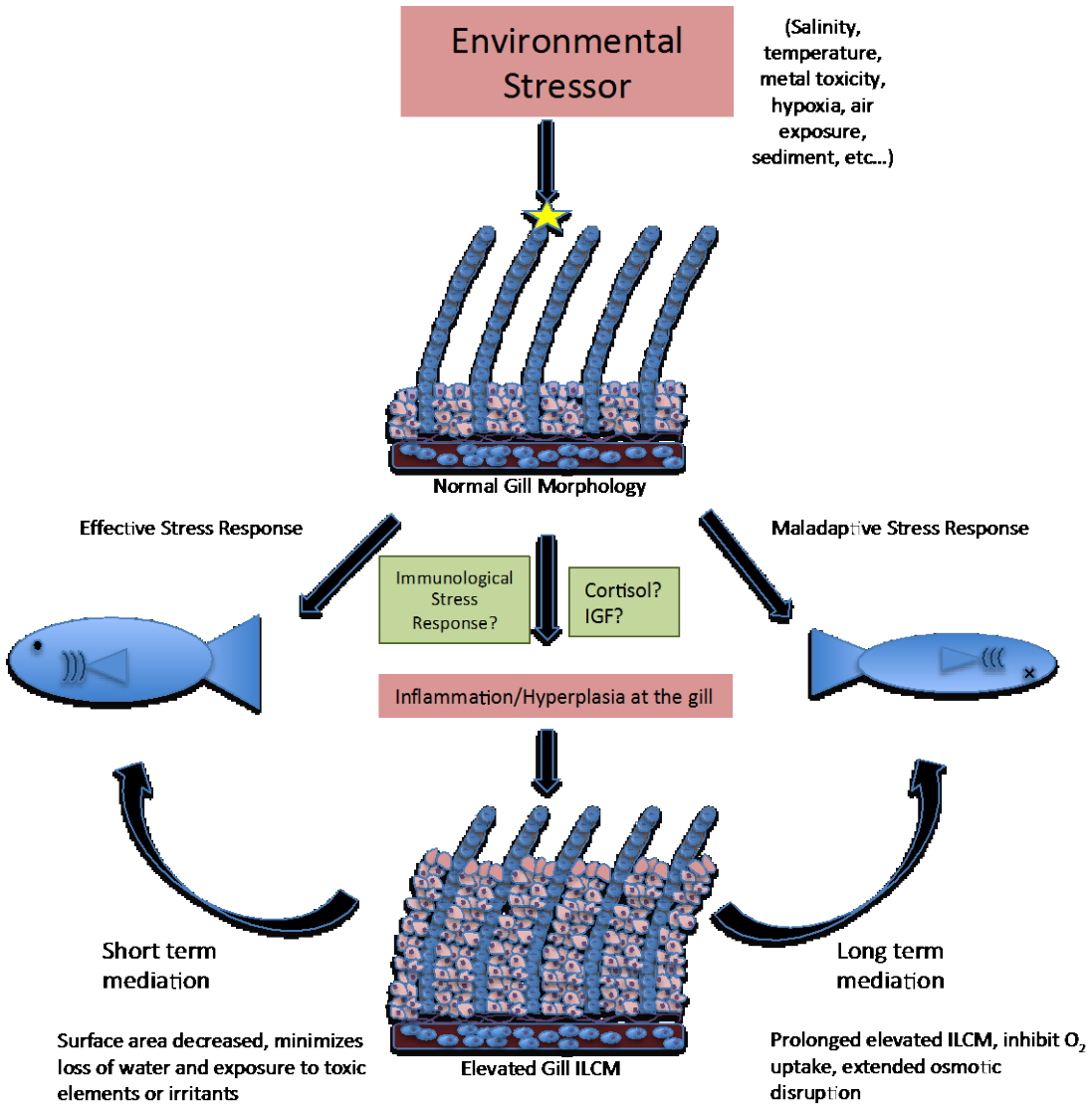


Figure 6.1. Theorized process of development of the ILCM in response to environmental stressors.

Conservation physiology perspectives regarding produced water spills

It was my aim that the physiological data I presented in my Ph.D. thesis would be utilized to benefit the conservation of the threatened Alberta native Arctic grayling. These data should make us aware of the conservation impact that hypersaline spills may have on native salmonid species that cannot physiologically compensate for such osmoregulatory challenges. There were two valid criticisms of my experimentation using 17ppt saline water as a proxy for spills of hydraulic fracturing flowback fluid or hypersaline produced water: 1) What is the environmentally relevant salinity concentration that fish would be exposed to in a real spill in an Arctic grayling stream in Alberta? 2) Hydraulic fracturing fluid is a composed of a mix of many different salts, organics, solvents, acids, and other chemicals; why are you solely using salinity as the stressor?

Firstly, the salinity concentration of the water body following a spill would depend on the volume of the spill, the size of the water body, the flow rate of the river or stream, the original concentration of the fluid itself, and the length of time that the spill persists. This would be difficult to calculate and the only way to get an indication of the salinity following a spill would be to sample the water with a salinity or conductivity probe immediately following a spill. Under normal circumstances this proves difficult because a) it takes time to report a spill, b) the company's private contractors or researchers usually perform the sampling, and c) the resulting data would be proprietary or used as evidence in environmental damage prosecutions, thus unavailable to the public. However, there are some instances where measurements were made following spills

including the Kentucky spill when researchers from the US Geological survey were able to sample and measure conductivity some time after a spill occurred in Acorn Creek (Papoulias and Velasco, 2013). At the time of the measurements approximately 1 week after the wells were fracked, conductivity at one of the sites measured 35,000 μS/cm (between 20-25 ppt); much higher than the 17 ppt standard I used in the salinity tolerance tests on grayling. It is expected that the initial concentration in the river in the first few days following the spill was even more elevated given the week of dilution that occurred prior to sampling. Thus, our study remains valid and likely underestimates the actual concentrations fish are exposed to following a spill of hypersaline water.

The second point made concerning the 17 ppt saline water used to demonstrate the harmful effects of hydraulic fracturing fluid or hypersaline produced water spilling into an environment was that salinity is only a minor aspect of this flowback fluid, which is actually a complex mixture of chemicals with varying compositions of salts. The fact that the mixture of chemicals is likely more harmful than salinity alone is an arguable point. I nevertheless maintain that the osmotic stress will be the most immediate and most significant stress that the fish will encounter in the event of a spill. However, the presence of organics and other chemicals within the mixture has the potential to compound the lethal and sublethal effects of the hypersaline fluid. A direct exposure of Arctic grayling to hydraulic flowback water diluted to the same 17ppt salinity is a route of further investigation that ought to be taken. I theorize that mortality would set in quicker than when exposed to elevated

salinity alone. The direct toxicity of chemicals and the potential for some chemicals to inhibit physiological process, possibly even halting the development of the ICLM, is likely to speed the mortality outcome. Even a 20-fold dilution of some flowback fluid is still above the salinity tolerance threshold of Arctic grayling. A direct exposure is feasible as our lab has unique access to fracturing flowback fluids and is an experiment that would further my conservation physiology aim in supporting this native salmonid.

General Conclusion

The salmonids are a fascinating group of fish extending to all parts of the globe, armed with a suite of ionoregulatory mechanisms allowing them to survive in a variety of aquatic habitats including freshwater and marine environments. Their importance in this world extends beyond that of a food source, science, culture, economics, sport fishing, and ecology. Members of the Salmonidae family are subject to a variety of environmental stressors, many of them resulting from anthropogenic processes. The continued understanding of their physiology, tolerances, and regulatory mechanisms is critical for their future conservation.

REFERENCES

AEP (Alberta Environment and Parks) (2015) Alberta guide to sportfishing regulations. http://www.albertaregulations.ca/fishin-gregs/management.html.

AER (Alberta Energy Regulator) (2016) Compliance Board. https://www.aer.ca/compliance-and-enforcement/incident-reporting

Agrawal AA (2001) Phenotypic plasticity in the interactions and evolution of species. *Science* 294: 321–326.

Allen EW (2008) Process water treatment in Canada's oil sands industry: I. Target pollutants and treatment objectives. *J Environ Eng Sci* 7: 123–138.

Allin CJ, Wilson RW (2000) Effects of pre-acclimation to aluminium on the physiology and swimming behaviour of juvenile rainbow trout (*Oncorhynchus mykiss*) during a pulsed exposure. *Aquat Toxicol* 51: 213–224.

Amiri, BM, Baker DW, Morgan JD, Brauner CJ (2009) Size dependent early salinity tolerance in two sizes of juvenile white sturgeon *Acipenser transmontanus*. *Aquaculture* 286: 121-126

Avella M, Bornancin M (1989) A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J Exp Biol* 142: 155–175.

Ayson FG, Kaneko T, Hasegawa S, Hirano T (1994) Development of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia, Oreochromis mossambicus, in fresh water and seawater. *J Exp Zool* 270: 129–135.

Barrett K (2001) Changes in ion content and transport during development of embryonic rainbow trout. *J Fish Biol* 59: 1323–1335.

Bath RN, Eddy FB (1979) Salt and water balance in rainbow trout (*Salmo gairdneri*) rapidly transferred from fresh water to sea water. *J Exp Biol* 83: 193–202.

Becker CD, Genoway RG (1979) Evaluation of the critical thermal maximum for determining thermal tolerance of freshwater fish. *Environ Biol Fishes* 4: 245–256.

Beitinger TL, Bennett WA, McCauley RW (2000) Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environ Biol Fishes* 58: 237–275.

Benos DJ (1982) Amiloride: a molecular probe of sodium transport in tissues and cells. *Am J Physiol-Cell Physiol* 242: C131–C145.

Berners J (1880) A Treatyse of Fysshynge Wyth an Angle. E. Stock.

Berthelot C, Brunet F, Chalopin D, Juanchich A, Bernard M, Noël B, Bento P, Da Silva C, Labadie K, Alberti A (2014) The rainbow trout genome provides novel insights into evolution after whole-genome duplication in vertebrates. *Nat Commun* 5.

Blair SD, Matheson D, He Y, Goss GG (2016) Reduced salinity tolerance in the Arctic grayling (*Thymallus arcticus*) is associated with rapid development of a gill interlamellar cell mass: implications of high-saline spills on native freshwater salmonids. *Conserv Physiol* 4: cow010.

Blanco G, Mercer RW (1998) Isozymes of the Na-K-ATPase: heterogeneity in structure, diversity in function. *Am J Physiol-Ren Physiol* 275: F633–F650.

Boisen AM, Amstrup J, Novak I, Grosell M (2003) Sodium and chloride transport in soft water and hard water acclimated zebrafish (*Danio rerio*). *Biochim Biophys Acta BBA - Biomembr* 1618: 207–218.

Bolin B (1986) Scope 29-The greenhouse effect, climatic change, and ecosystems.

Boyle D, Clifford AM, Orr E, Chamot D, Goss GG (2015) Mechanisms of Cl^- uptake in rainbow trout: Cloning and expression of slc26a6, a prospective Cl−/HCO3− exchanger. *Comp Biochem Physiol A Mol Integr Physiol* 180: 43–50.

BP (British Petroleum) (2015) BP statistical review of world energy 2015. http://www.bp.com/en/global/corporate/about-bp/energy-economics/statistical-review-of-worldenergy.html.

Brett JR (1952) Temperature tolerance in young Pacific salmon, genus Oncorhynchus. *J Fish Board Can* 9: 265–323.

Brix KV, Grosell M (2012) Comparative characterization of Na^+ transport in *Cyprinodon variegatus variegatus* and C*yprinodon variegatus hubbsi*: a model species complex for studying teleost invasion of freshwater. *J Exp Biol* 215: 1199–1209.

Burnham A, Han J, Clark CE, Wang M, Dunn JB, Palou-Rivera I (2012) Life-cycle greenhouse gas emissions of shale gas, natural gas, coal, and petroleum. *Environ Sci Technol* 46: 619–627.

Bystriansky JS, Richards JG, Schulte PM, Ballantyne JS (2006) Reciprocal expression of gill Na^+/K^+-ATPase -subunit isoforms 1a and 1b during seawater acclimation of three salmonid fishes that vary in their salinity tolerance. *J Exp Biol* 209: 1848–1858.

Bystriansky JS, Frick NT, Richards JG, Schulte PM, Ballantyne JS (2007) Failure to up-regulate gill Na^+,K^+-ATPase α-subunit isoform α1b may limit seawater tolerance of land-locked Arctic char (*Salvelinus alpinus*). *Comp Biochem Physiol A Mol Integr Physiol* 148: 332–338.

Bystriansky JS, Schulte PM (2011) Changes in gill H+-ATPase and Na+/K+-ATPase expression and activity during freshwater acclimation of Atlantic salmon (*Salmo salar*). *J Exp Biol* 214: 2435–2442.

Cameron JN (1976) Branchial ion uptake in arctic grayling: resting values and effects of acid-base disturbance. *J Exp Biol* 64: 711–725.

Campbell SK, Butler VL (2010) Archaeological evidence for resilience of Pacific Northwest salmon populations and the socioecological system over the last~ 7,500 years.

Canedo-Arguelles M, Hawkins CP, Kefford BJ, Schafer RB, Dyack BJ, Brucet S, Buchwalter D et al. (2016) Saving freshwater from salts. *Science* 351: 914-916.

CAPP (Canadian Association of Petroleum Producers) (2015) Water http://www.capp.ca/responsible-development/water.

Claiborne JB, Edwards SL, Morrison-Shetlar AI (2002) Acid-base regulation in fishes: cellular and molecular mechanisms. *J Exp Zool* 293: 302–319.

Claiborne JB, Perry E, Bellows S, Campbell J (1997) Mechanisms of acid-base excretion across the gills of a marine fish. *J Exp Zool* 279: 509–520.

Collins PD, Marleau S, Griffiths-Johnson DA, Jose PJ, Williams TJ (1995) Cooperation between interleukin-5 and the chemokine eotaxin to induce eosinophil accumulation in vivo. *J Exp Med* 182.4: 1169-1174.

Columbia River Inter-Tribal Fish Commission (2016) http://www.critfc.org

Conte F, Wagner H (1965) Development of osmotic and ionic regulation in juvenile steelhead trout *Salmo gairdneri*. *Comp Biochem Physiol* 14: 603–620.

Cooke SJ, Sack L, Franklin CE, Farrell AP, Beardall J, Wikelski M, Chown SL (2013) What is conservation physiology? Perspectives on an increasingly integrated and essential science. *Conserv Physiol* 1: cot001–cot001.

Costello AB (2006) Status of the westslope cutthroat trout (*Oncorhynchus clarkii lewisii*) in Alberta. Alberta Sustainable Resource Development (No. 61). Wildlife Status Report.

Counillon L, Scholz W, Lang HJ, Pouyssegur J (1993) Pharmacological characterization of stably transfected Na^+/H^+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Mol Pharmacol* 44: 1041–1045.

Craig PM, Wood CM, McClelland GB (2007) Gill membrane remodeling with soft-water acclimation in zebrafish (*Danio rerio*). *Physiol Genomics* 30: 53–60.

Crête-Lafrenière A, Weir LK, Bernatchez L (2012) Framing the Salmonidae family phylogenetic portrait: a more complete picture from increased taxon sampling. *PLoS One* 7: e46662.

Dalziel AC, Bittman J, Mandic M, Ou M, Schulte PM (2014) Origins and functional diversification of salinity-responsive Na^+, K^+ ATPase α1 paralogs in salmonids. *Mol Ecol* 23: 3483–3503.

Daufresne M, Roger MC, Capra H, Lamouroux N (2004) Long-term changes within the invertebrate and fish communities of the Upper Rhone River: effects of climatic factors. *Glob Change Biol* 10: 124–140.

Dittman A, Quinn T (1996) Homing in Pacific salmon: mechanisms and ecological basis. *J Exp Biol* 199: 83–91.

Dobosy JR, Rose SD, Beltz KR, Rupp SM, Powers KM, Behlke MA, Walder JA (2011) RNase H-dependent PCR (rhPCR): improved specificity and single nucleotide polymorphism detection using blocked cleavable primers. *BMC Biotechnol* 11: 80.

Donowitz M, Ming Tse C, Fuster D (2013) SLC9/NHE gene family, a plasma membrane and organellar family of Na^+/H^+ exchangers. *Mol Aspects Med* 34: 236–251.

Doyle WL, Gorecki D (1961) The so-called chloride cell of the fish gill. *Physiol Zool* 34: 81–85.

Dymowska AK, Hwang PP, Goss GG (2012) Structure and function of ionocytes in the freshwater fish gill. *Respir Physiol Neurobiol* 184: 282–292.

Dymowska AK, Schultz AG, Blair SD, Chamot D, Goss GG (2014) Acid-sensing ion channels are involved in epithelial Na^+ uptake in the rainbow trout Oncorhynchus mykiss. *AJP Cell Physiol* 307: C255–C265.

Economides MJ, Wood DA (2009) The state of natural gas. *J Nat Gas Sci Eng* 1: 1–13.

Eddy FB, Ward MR, Talbot C, Primmett D (1990) Ionic movements across the chorion in newly shed salmon eggs (Salmo salar L.). *J Comp Physiol B* 159: 771–776.

Eddy F, Talbot C (1985) Sodium balance in eggs and dechorionated embryos of the Atlantic salmon *Salmo salar L*. exposed to zinc, aluminium and acid waters. *Comp Biochem Physiol Part C Comp Pharmacol* 81: 259–266.

Edwards SL, Marshall WS (2013) Principles and patterns of osmoregulation and euryhalinity in fishes. *Fish Physiol* 32: 1–44.

Edwards SL, Tse CM, Toop T (1999) Immunolocalisation of NHE3-like immunoreactivity in the gills of the rainbow trout (*Oncorhynchus mykiss*) and the blue-throated wrasse (*Pseudolabrus tetrious*). *J Anat* 195: 465–469.

Edwards SL, Wall BP, Morrison-Shetlar A, Sligh S, Weakley JC, Claiborne JB (2005) The effect of environmental hypercapnia and salinity on the expression of NHE-like isoforms in the gills of a euryhaline fish (*Fundulus heteroclitus*). *J Exp Zoolog A Comp Exp Biol* 303A: 464–475.

Eigler J, Kelter J, Renner E (1967) Wirkungscharakteristika eines neuen Aeylguanidins—Amiloride-HCL (MK 870)—an der isolierten Haut von Amphibien. *Klin Wochenschr* 45: 737–738.

Environment Canada (1990) Biological test method: Acute lethality test using rainbow trout, environmental protection series. Report EPS 1/RM/9, July 1990, amended May 2007. Ottawa, Ontario, Canada.

Epstein FH, Katz AI, Pickford GE (1967) Sodium-and potassium-activated adenosine triphosphatase of gills: role in adaptation of teleosts to salt water. *Science* 156: 1245–1247.

Esaki M, Hoshijima K, Nakamura N, Munakata K, Tanaka M, Ookata K, Asakawa K, Kawakami K, Wang W, Weinberg ES, *et al.* (2009) Mechanism of development of ionocytes rich in vacuolar-type H^+-ATPase in the skin of zebrafish larvae. *Dev Biol* 329: 116–129.

Evans DH (1987) The fish gill: site of action and model for toxic effects of environmental pollutants. *Environ Health Perspect* 71: 47.

Evans DH, Piermarini PM, Choe KP (2005) The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol Rev* 85: 97–177.

FAO (Food and Agriculture Organization of the United Nations) (2016) Fisheries and Aquaculture Department. World review of fisheries and aquaculture http://www.fao.org/fishery/en

Fenwick JC, Bonga SW, Flik G (1999) In vivo bafilomycin-sensitive Na^+ uptake in young freshwater fish. *J Exp Biol* 202: 3659–3666.

Ficke AD, Myrick CA, Hansen LJ (2007) Potential impacts of global chimate change on freshwater fisheries. *Rev Fish Biol Fisheries* 17: 581-613.

Finstad B, Staurnes M, Reite OB (1988) Effect of low temperature on sea-water tolerance in rainbow trout, *Salmo gairdneri*. *Aquaculture* 72: 319-328.

Folland CK, Rayner NA, Brown S, Smith T, Shen S, Parker D, Macadam I, Jones P, Jones R, Nicholls N (2001) Global temperature change and its uncertainties since 1861. *Geophys Res Lett* 28: 2621–2624.

From J, Rasmussen G (1991) Growth of rainbow trout, *Oncorhynchus mykiss* (*Walbaum*, 1792) related to egg size and temperature. *Dana* 9: 31–38.

Fry FEJ (1947) Effects of the environment on animal activity. University of Toronto press.

Fry FEJ (1967) Responses of vertebrate poikilotherms to temperature. *Thermobiol Acad Press N Y* 375–409.

Fu C, Wilson JM, Rombough PJ, Brauner CJ (2010) Ions first: Na^+ uptake shifts from the skin to the gills before O_2 uptake in developing rainbow trout, *Oncorhynchus mykiss*. *Proc R Soc B Biol Sci* 277: 1553–1560.

Goss GG, Alessi D, Allen D, Gehman J, Brisbois J, Kletke S, Sharak AZ, Notte C, Thompson DY, Hong K (2015). Unconventional wastewater management: a comparative review and analysis of hydraulic fracturing wastewater management practices across four North American basins. *Canadian Water Network* Report, Waterloo, Ontario, Canada.

Goss GG, Jiang L, Vandorpe DH, Kieller D, Chernova MN, Robertson M, Alper SL (2001) Role of JNK in hypertonic activation of Cl^--dependent Na^+/H^+ exchange in Xenopus oocytes. *Am J Physiol-Cell Physiol* 281: C1978–C1990.

Goss GG, Perry SF, Wood CM, Laurent P (1992) Mechanisms of ion and acid-base regulation at the gills of freshwater fish. *J Exp Zool* 263: 143–159.

Goss GG, Wood CM (1991) Two-substrate kinetic analysis: a novel approach linking ion and acid-base transport at the gills of freshwater trout, *Oncorhynchus mykiss*. *J Comp Physiol B* 161: 635–646.

Grace JD, Hart GF (1986) Giant gas fields of northern West Siberia. *AAPG Bull* 70: 830–852.

Gresh T, Lichatowich J, Schoonmaker P (2000) An estimation of historic and current levels of salmon production in the Northeast Pacific ecosystem: evidence of a nutrient deficit in the freshwater systems of the Pacific Northwest. *Fisheries* 25: 15–21.

Grinstein S, Woodside M, Waddell T, Downey G, Orlowski J, Pouyssegur J, Wong D, Foskett J (1993) Focal localization of the NHE-1 isoform of the Na^+/H^+ antiport: assessment of effects on intracellular pH. *EMBO J* 12: 5209.

Gross SA, Avens HJ, Banducci AM, Sahmel J, Panko JM, Tvermoes BE (2013) Analysis of BTEX groundwater concentrations from surface spills associated with hydraulic fracturing operations. *J Air Waste Manag Assoc* 63: 424–432.

Guffey SC, Fliegel L, Goss GG (2015) Cloning and characterization of Na^+/H^+ exchanger isoforms NHE2 and NHE3 from the gill of Pacific dogfish *Squalus suckleyi*. *Comp Biochem Physiol B Biochem Mol Biol* 188: 46–53.

Gurley RR (1902) The Habits of Fishes. *Am J Psychol* 13: 408.

Hair NL, Edwards SL, Morrison-Shetlar A, Claiborne JB. (2002) Relative expression of mRNA for NHE-2 in the gills of the long-horned sculpin, *Myoxocephalus octodecimspinosus*. Bull Mt Desert Is Biol Lab 41:21–22.

Hari RE, Livingstone DM, Siber R, BURKHARDT-HOLM P, Guettinger H (2006) Consequences of climatic change for water temperature and brown trout populations in Alpine rivers and streams. *Glob Change Biol* 12: 10–26.

Hartmann DL, Klein Tank AM, Rusticucci M, Alexander LV, Brönnimann S, Charabi YAR, Dentener FJ, Dlugokencky EJ, Easterling DR, Kaplan A (2013) Observations. Presented at the Cambridge University Press.

Hirata T, Kaneko T, Ono T, Nakazato T, Furukawa N, Hasegawa S, Wakabayashi S, Shigekawa M, Chang M-H, Romero MF, *et al.* (2003) Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am J Physiol - Regul Integr Comp Physiol* 284: R1199–R1212.

Hiroi J, McCormick SD (2007) Variation in salinity tolerance, gill Na^+/K^+-ATPase, Na+/K+/2Cl- cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. *J Exp Biol* 210: 1015–1024.

Hiroi J, McCormick SD (2012) New insights into gill ionocyte and ion transporter function in euryhaline and diadromous fish. *Respir Physiol Neurobiol* 184: 257–268.

Holliday R (2006) Epigenetics: A Historical Overview. *Epigenetics* 1: 76–80.

Honarvar A, Canadian Energy Research Institute (2011) Economic impacts of new oil sands projects in Alberta (2010-2035). CERI, Calgary, Alta. www.americanpetroleuminstitute.net/~/media/files/news/2011/economi_impacts_of_new_oil_sands_projects_alberta.pdf

Hoogerwerf WA, Tsao SC, Devuyst O, Levine SA, Yun CH, Yip JW, Cohen ME, Wilson PD, Lazenby AJ, Tse C-M, *et al.* (1996) NHE2 and NHE3 are human and rabbit intestinal brush-border proteins. *Am J Physiol-Gastrointest Liver Physiol* 270: G29–G41.

Howland K (2001) Contrasts in the hypo-osmoregulatory abilities of a freshwater and an anadromous population of inconnu. *J Fish Biol* 59: 916–927.

Hwang PP, Lee TH (2007) New insights into fish ion regulation and mitochondrion-rich cells. *Comp Biochem Physiol A Mol Integr Physiol* 148: 479–497.

Hwang PP, Lee TH, Lin LY (2011) Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *AJP Regul Integr Comp Physiol* 301: R28–R47.

Hwang PP, Lee TH, Weng CF, Fang MJ, Cho GY (1999) Presence of Na-K-ATPase in mitochondria-rich cells in the yolk-sac epithelium of larvae of the teleost *Oreochromis mossambicus. Physiol Biochem Zool* 72: 138–144.

Hyvonen M (2004) Guide to expression construct cloning. camelot.bioc.cam.ac.uk/~marko/methods/cloning.pdf

Inokuchi M, Hiroi J, Watanabe S, Hwang P-P, Kaneko T (2009) Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. *J Exp Biol* 212: 1003–1010.

Ip YK, Wilson JM, Loong AM, Chen XL, Wong WP, Delgado ILS, Lam SH, Chew SF (2012) Cystic fibrosis transmembrane conductance regulator in the gills of the climbing perch, *Anabas testudineus*, is involved in both

hypoosmotic regulation during seawater acclimation and active ammonia excretion during ammonia exposure. *J Comp Physiol B* 182: 793–812.

Ito Y, Kato A, Hirata T, Hirose S, Romero MF (2014) Na^+/H^+ and Na^+/NH_4^+ exchange activities of zebrafish NHE3b expressed in *Xenopus* oocytes. *AJP Regul Integr Comp Physiol* 306: R315–R327.

Ivanis G, Esbaugh AJ, Perry SF (2008) Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol* 211: 2467–2477.

Jackson A (1981) Osmotic regulation in rainbow trout (*Salmo gairdneri*) following transfer to sea water. *Aquaculture* 24: 143–151.

Jorgensen PL, Håkansson KO, Karlish SJD (2003) Structure and mechanism of Na,K-ATPase: Functional sites and their interactions. *Annu Rev Physiol* 65: 817–849.

Kandasamy RA, Frank HY, Harris R, Boucher A, Hanrahan JW, Orlowski J (1995) Plasma membrane Na^+/H^+ exchanger isoforms (NHE-1,- 2, and- 3) are differentially responsive to second messenger agonists of the protein kinase A and C pathways. *J Biol Chem* 270: 29209–29216.

Kaneko T, Shiraishi K, Katoh F, Hasegawa S, Hiroi J (2002) Chloride cells during early life stages of fish and their functional differentiation. *Fish Sci* 68: 1–9.

Keleher CJ and Rahel FJ (1996) Thermal limits to salmonid distributions in the Rocky mountain region and potential habitat loss due to global warming: A geographic information systems (GIS) approach. *Trans Amer Fish Soc* 125: 1-13.

Keys AB (1931) Chloride and water secretion and absorption by the gills of the eel. *Z Für Vgl Physiol* 15: 364–388.

Kinne O (1962) Irreversible nongenetic adaptation. *Comp Biochem Physiol* 5: 265–282.

Kienzle SW (2016) Alberta Climate Records Kienzle Hydrology Lab at the Department of Geography, University of Lethbridge, Alberta, Canada. www.albertaclimaterecords.com

Kirschner LB, Greenwald L, Kerstetter TH (1973) Effect of amiloride on sodium transport across body surfaces of freshwater animals. *Am J Physiol Content* 224: 832–837.

Kleyman TR, Cragoe EJ (1988) Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol* 105: 1–21.

Krogh A (1937) Osmotic regulation in fresh water fishes by active absorption of chloride ions. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 24: 656–666.

Krogh A (1938) The active absorption of ions in some freshwater animals. *Z Für Vgl Physiol* 25: 335–350.

Krogh A, Leitch I (1919) The respiratory function of the blood in fishes. *J Physiol* 52: 288.

Kumai Y, Perry SF (2011) Ammonia excretion via Rhcg1 facilitates Na^+ uptake in larval zebrafish, *Danio rerio*, in acidic water. *AJP Regul Integr Comp Physiol* 301: R1517–R1528.

LaPerriere JD, Carlson RF (1973) Thermal tolerances of interior Alaskan Arctic grayling (*Thymallus arcticus*). University of Alaska, Institute of Water Resources.

Larsen PF, Nielsen EE, Koed A, Thomsen DS, Olsvik PA, Loeschcke V (2008) Interpopulation differences in expression of candidate genes for salinity tolerance in winter migrating anadromous brown trout (*Salmo trutta L.*). *BMC Genet* 9: 12.

Laurent P, Dunel S (1980) Morphology of gill epithelia in fish. *Am J Physiol-Regul Integr Comp Physiol* 238: R147–R159.

Law G, Kreh L (2003) A concise history of fly fishing. Globe Pequot Press.

LeBlanc DM, Wood CM, Fudge DS, Wright PA (2010) A fish out of water: Gill and skin remodeling promotes osmo- and ionoregulation in the mangrove killifish *Kryptolebias marmoratus*. *Physiol Biochem Zool* 83: 932–949.

Liew HJ, Sinha AK, Nawata CM, Blust R, Wood CM, De Boeck G (2013) Differential responses in ammonia excretion, sodium fluxes and gill permeability explain different sensitivities to acute high environmental ammonia in three freshwater teleosts. *Aquat Toxicol* 126: 63–76.

Lin H, Randall D (1991) Evidence for the presence of an electrogenic proton pump on the trout gill epithelium. *J Exp Biol* 161: 119–134.

Lin H, Randall D (1995) 9 Proton pumps in fish gills. *Fish Physiol* 14: 229–255.

Lohr SC, Byorth PA, Kaya CM, Dwyer WP (1996) High-temperature tolerances of fluvial Arctic grayling and comparisons with summer river temperatures of the Big Hole River, Montana. *Trans Amer Fish Soc* 125: 933-939.

Ma B, Jiang H, Sun P, Chen J, Li L, Zhang X, Yuan L (2016) Phylogeny and dating of divergences within the genus *Thymallus* (Salmonidae: Thymallinae) using complete mitochondrial genomes. *Mitochondrial DNA Part A* 27: 3602–3611.

MacCrimmon HR (1971) World distribution of rainbow trout (*Salmo gairdneri*). *J Fish Board Can* 28: 663–704.

MacPherson LM, Sullivan MG, Foote AL, Stevens CE (2012) Effects of culverts on stream fish assemblages in the Alberta foothills. *North Am J Fish Manag* 32: 480–490.

Marshall WS (2002) Na^+, Cl^-, Ca_2^+ and Zn_2^+ transport by fish gills: retrospective review and prospective synthesis. *J Exp Zool* 293: 264–283.

Maxime V, Pennec JP, Peyraud C (1991) Effects of direct transfer to seawater on respiratory and circulatory variables and acid-base status in rainbow trout. *J Comp Phys B* 161: 557-568.

McCormick SD (1996) Effects of growth hormone and insulin-like growth factor I on salinity tolerance and gill Na^+, K^+-ATPase in Atlantic salmon (*Salmo salar*): interaction with cortisol. *Gen Comp Endocrinol* 101: 3–11.

McCormick SD, Regish AM, Christensen AK (2009) Distinct freshwater and seawater isoforms of Na^+/K^+-ATPase in gill chloride cells of Atlantic salmon. *J Exp Biol* 212: 3994–4001.

McCormick SD, Regish AM, Christensen AK, Bjornsson BT (2013) Differential regulation of sodium-potassium pump isoforms during smolt development and seawater exposure of Atlantic salmon. *J Exp Biol* 216: 1142–1151.

McCullough DA, Bartholow JM, Jager HJ, Beschta RL, Cheslak EF, Deas ML, Ebersole JL ,Foott JS et al. (2009) Research in thermal biology: burning questions for coldwater stream fishes. *Rev Fish Sci* 12: 90-115.

McPhail JD, Troffe PM (1998) The mountain whitefish (*Prosopium williamsoni*): a potential indicator species for the Fraser System.

Melikoglu M (2014) Shale gas: Analysis of its role in the global energy market. *Renew Sustain Energy Rev* 37: 460–468.

Milligan CL, Wood CM (1986) Intracellular and extracellular acid-base status and H^+ exchange with the environment after exhaustive exercise in the rainbow trout. *J Exp Biol* 123: 93–121.

Mitrovic D, Dymowska A, Nilsson GE, Perry SF (2009) Physiological consequences of gill remodeling in goldfish (*Carassius auratus*) during exposure to long-term hypoxia. *AJP Regul Integr Comp Physiol* 297: R224–R234.

Mitrovic D, Perry SF (2009) The effects of thermally induced gill remodeling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *J Exp Biol* 212: 843–852.

Mohseni O, Stefan HG (1999) Stream temperature/air temperature relationship: a physical interpretation. *J Hydrol* 218: 128–141.

Montgomery, BCS, Mewes J, Davidson C, Burshtyn DN, Stafford JL (2009) Cell surface expression of channel catfish leukocyte immune-type receptors (IpLITRs) and recruitment of both Src homology 2 domain-containing protein tyrosine phosphatase (SHP)-1 and SHP-2. *Dev Comp Immunol* 22: 570-582.

Morgan JD, Iwama GK (1991) Effects of salinity on growth, metabolism, and ion regulation in juvenile rainbow and steelhead trout (*Oncorhynchus mykiss*) and fall chinook salmon (*Oncorhynchus tshawytscha*). *Can J Fish Aquat Sci* 48: 2083–2094.

Mueller ME, Sanchez DA, Bergman HL, McDonald DG, Rhem RG, Wood CM (1991) Nature and time course of acclimation to aluminum in juvenile brook trout (*Salvelinus fontinalis*). II. Gill histology. *Can J Fish Aquat Sci* 48: 2016–2027.

Murtazina R, Booth BJ, Bullis BL, Singh DN, Fliegel L (2001) Functional analysis of polar amino-acid residues in membrane associated regions of the NHE1 isoform of the mammalian Na^+/H^+ exchanger. *Eur J Biochem* 268: 4674–4685.

Nakada T, Hoshijima K, Esaki M, Nagayoshi S, Kawakami K, Hirose S (2007) Localization of ammonia transporter Rhcg1 in mitochondrion-rich cells of yolk sac, gill, and kidney of zebrafish and its ionic strength-dependent expression. *AJP Regul Integr Comp Physiol* 293: R1743–R1753.

NatureServe (2004) http://explorer.natureserve.org/servlet/NatureServe?searchName=Thymallus+arcticus

Nawata CM, Hung CC, Tsui TK, Wilson JM, Wright PA, Wood CM (2007) Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. *Physiol Genomics* 31: 463–474.

NCBI (2016) National Center for Biotechnology Information, U.S. National Library of Medicine. 8600 Rockville Pike, Bethesda MD 20894 USA. www.ncbi.nlm.nih.gov

Niemi EG, Whitelaw E, Lindahl D, Fifield A, Gall M (1999) Salmon and the economy: a handbook for understanding the issues in Washington and Oregon.

Nilsen TO, Ebbesson LOE, Madsen SS, McCormick SD, Andersson E, Bjornsson BT, Prunet P, Stefansson SO (2007) Differential expression of gill Na^+,K^+-ATPase - and -subunits, Na^+,K^+,$2Cl^-$ cotransporter and CFTR anion channel in juvenile anadromous and landlocked Atlantic salmon *Salmo salar*. *J Exp Biol* 210: 2885–2896.

Nilsson GE, Dymowska A, Stecyk JAW (2012) New insights into the plasticity of gill structure. *Respir Physiol Neurobiol* 184: 214–222.

Northcote TG (1995) Comparative biology and management of Arctic and European grayling (Salmonidae, Thymallus). *Rev Fish Biol Fish* 5: 141–194.

Ong KJ, Stevens ED, Wright PA (2007) Gill morphology of the mangrove killifish (*Kryptolebias marmoratus*) is plastic and changes in response to terrestrial air exposure. *J Exp Biol* 210: 1109–1115.

Orlowski J (1993) Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger. *J Biol Chem* 268: 16369–16377.

Orlowski J, Grinstein S (2004) Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflgers Arch Eur J Physiol* 447: 549–565.

Pacala S, Socolow R (2004) Stabilization wedges: solving the climate problem for the next 50 years with current technologies. *science* 305: 968–972.

Papoulias DM, Velasco AL (2013) Histopathological analysis of fish from Acorn Fork Creek, Kentucky, exposed to hydraulic fracturing fluid releases. *Southeastern Naturalist* 12.4: 92-111.

Park D, Sullivan M, Bayne E, Scrimgeour G (2008) Landscape-level stream fragmentation caused by hanging culverts along roads in Alberta's boreal forest. *Can J For Res* 38: 566–575.

Parks SK, Tresguerres M, Galvez F, Goss GG (2010) Intracellular pH regulation in isolated trout gill mitochondrion-rich (MR) cell subtypes: Evidence for Na+/H+ activity. *Comp Biochem Physiol A Mol Integr Physiol* 155: 139–145.

Parks SK, Tresguerres M, Goss GG (2008) Theoretical considerations underlying Na^+ uptake mechanisms in freshwater fishes. *Comp Biochem Physiol Part C Toxicol Pharmacol* 148: 411–418.

Perry SF (1997) The chloride cell: structure and function in the gills of freshwater fishes. *Annu Rev Physiol* 59: 325–347.

Perry SF, Gilmour KM (2006) Acid–base balance and CO_2 excretion in fish: Unanswered questions and emerging models. *Respir Physiol Neurobiol* 154: 199–215.

Perry S, Randall D (1981) Effects of amiloride and SITS on branchial ion fluxes in rainbow trout, *Salmo gairdneri*. *J Exp Zool* 215: 225–228.

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. *Nucleic Acids Res* 29: e45–e45.

Philpott CW (1980) Tubular system membranes of teleost chloride cells: osmotic response and transport sites. *Am J Physiol-Regul Integr Comp Physiol* 238: R171–R184.

Pouysségur J, Sardet C, Franchi A, L'Allemain G, Paris S (1984) A specific mutation abolishing Na^+/H^+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc Natl Acad Sci* 81: 4833–4837.

Preest MR, Gonzalez RJ, Wilson RW (2005) A Pharmacological examination of Na^+ and Cl^- transport in two species of freshwater fish. *Physiol Biochem Zool* 78: 259–272.

Quinn TP, Myers KW (2005) Anadromy and the marine migrations of Pacific salmon and trout: Rounsefell revisited. *Rev Fish Biol Fish* 14: 421–442.

Rankin SM, Conroy DM, Williams TJ (2000) Eotaxin and eosinophil recruitment: implications for human disease. *Molec Med Today* 6: 20-27.

Reid SD (2003) Localization and characterization of phenamil-sensitive Na^+ influx in isolated rainbow trout gill epithelial cells. *J Exp Biol* 206: 551–559.

Richards EJ (2006) Inherited epigenetic variation—revisiting soft inheritance. *Nat Rev Genet* 7: 395–401.

Richards JG, Semple JW, Bystriansky JS, Schulte PM (2003) Na^+/K^+-ATPase - isoform switching in gills of rainbow trout (*Oncorhynchus mykiss*) during salinity transfer. *J Exp Biol* 206: 4475–4486.

Rieman BE, Lee DC, Thurow RF (1997) Distribution, status, and likely future trends of bull trout within the Columbia River and Klamath River basins. *North Am J Fish Manag* 17: 1111–1125.

Rodtka M. (2009). Status of the bull trout (*Salvelinus confluentus*) in Alberta: Update 2009. Alberta Sustainable Resource Development, Edmonton, Alberta.

Rombough P (2007) The functional ontogeny of the teleost gill: Which comes first, gas or ion exchange? *Comp Biochem Physiol A Mol Integr Physiol* 148: 732–742.

Rombough PJ (1999) The gill of fish larvae. Is it primarily a respiratory or an ionoregulatory structure? *J Fish Biol* 55: 186–204.

Rotin D, Grinstein S (1989) Impaired cell volume regulation in Na^+-H^+ exchange-deficient mutants. *Am J Physiol* 257: C1158–C1165.

Rounsefell GA, U.S. Fish and Wildlife Service (1958) Anadromy in North American Salmonidae. U.S. Government Printing Office.

Schwartz A, Lindenmayer GE, Allen JC (1975) The sodium-potassium adenosine triphosphatase: pharmacological, physiological and biochemical aspects. *Pharmacol Rev* 27: 3–134.

Seidelin M, Madsen SS, Blenstrup H, Tipsmark CK (2000) Time-course changes in the expression of Na^+, K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. *Physiol Biochem Zool* 73: 446–453.

Seo MY, Mekuchi M, Teranishi K, Kaneko T (2013) Expression of ion transporters in gill mitochondrion-rich cells in Japanese eel acclimated to a wide range of environmental salinity. *Comp Biochem Physiol A Mol Integr Physiol* 166: 323–332.

Shen AC, Leatherland JF (1978) Structure of the yolksac epithelium and gills in the early developmental stages of rainbow trout (*Salmo gairdneri*) maintained in different ambient salinities. *Environ Biol Fishes* 3: 345–354.

Shih TH, Horng JL, Liu ST, Hwang PP, Lin LY (2012) Rhcg1 and NHE3b are involved in ammonium-dependent sodium uptake by zebrafish larvae

acclimated to low-sodium water. *AJP Regul Integr Comp Physiol* 302: R84–R93.

Shive HR, West RR, Embree LJ, Sexton JM, Hickstein DD (2015) Expression of *KRAS* ^{G12V} in zebrafish gills induces hyperplasia and *CXCL8* -associated inflammation. *Zebrafish* 12: 221–229.

Silva P, Solomon R, Spokes K, Epstein FH (1977) Ouabain inhibition of gill Na-K-ATPase: relationship to active chloride transport. *J Exp Zool* 199: 419–426.

Singer TD, Clements KM, Semple JW, Schulte PM, Bystriansky JS, Finstad B, Fleming IA, McKinley RS (2002) Seawater tolerance and gene expression in two strains of Atlantic salmon smolts. *Can J Fish Aquat Sci* 59: 125–135.

Sinha AK, Matey V, Giblen T, Blust R, De Boeck G (2014) Gill remodeling in three freshwater teleosts in response to high environmental ammonia. *Aquat Toxicol* 155: 166–180.

Skou JC (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. *Biochim Biophys Acta* 23: 394–401.

Slepkov ER, Rainey JK, Sykes BD, Fliegel L (2007) Structural and functional analysis of the Na^+/H^+ exchanger. *Biochem J* 401: 623.

Smith GR, Stearley RF (1989) The classification and scientific names of rainbow and cutthroat trouts. *Fisheries* 14: 4–10.

Smith HW (1929) The excretion of ammonia and urea by the gills of fish. *J Biol Chem* 81: 727–742.

Smith HW (1930) The absorption and excretion of water and salts by marine teleosts. *Am J Physiol Content* 93: 480–505.

Smith HW (1931) The absorption and excretion of water and salts by the elasmobranch fishes. *Am J Physiol Content* 98: 279–295.

Sollid J (2003) Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J Exp Biol* 206: 3667–3673.

Sollid J, Nilsson GE (2006) Plasticity of respiratory structures — Adaptive remodeling of fish gills induced by ambient oxygen and temperature. *Respir Physiol Neurobiol* 154: 241–251.

Stamford MD, Taylor EB (2004) Phylogeographical lineages of Arctic grayling (*Thymallus arcticus*) in North America: divergence, origins and affinities

with Eurasian Thymallus: multilple phylogeographic lineages of of Arctic grayling. *Mol Ecol* 13: 1533–1549.

Staurnes M, Sigholt T, Lysfjord G, Gulseth OA (1992) Difference in the seawater tolerance of anadromous and landlocked populations of Arctic char (*Salvelinus alpinus*). *Can J Fish Aquat Sci* 49: 443–447.

Stevens ED, Fry F (1974) Heat transfer and body temperatures in non-thermoregulatory teleosts. *Can J Zool* 52: 1137–1143.

Takei Y, Hiroi J, Takahashi H, Sakamoto T (2014) Diverse mechanisms for body fluid regulation in teleost fishes. *AJP Regul Integr Comp Physiol* 307: R778–R792.

Tipsmark CK, Breves JP, Seale AP, Lerner DT, Hirano T, Grau EG (2011) Switching of Na^+, K^+-ATPase isoforms by salinity and prolactin in the gill of a cichlid fish. *J Endocrinol* 209: 237–244.

Towle DW, Baksinski A, Richard NE, Kordylewski M (1991) Characterization of an endogenous Na^+/H^+ antiporter in *Xenopus laevis* oocytes. *J Exp Biol* 159: 359–369.

Turko AJ, Earley RL, Wright PA (2011) Behaviour drives morphology: voluntary emersion patterns shape gill structure in genetically identical mangrove rivulus. *Anim Behav* 82: 39–47.

Ura K, Soyano K, Omoto N, Adachi S, Yamauchi K (1996) Localization of Na^+, K^+-ATPase in tissues of rabbit and teleosts using an antiserum directed against a partial sequence of the α-subunit. *Zoolog Sci* 13: 219–227.

USEPA (US Environmental Protection Agency) (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th ed. USEPA, Office of Water, Washington, DC. EPA-821-R-02-012.

USEPA (US Environmental Protection Agency) (2015) Review of state and industry spill data: characterization of hydraulic fracturing-related spills. Office of Research and Development, Washington, DC. EPA/601/R-14/001.

Varsamos S, Nebel C, Charmantier G (2005) Ontogeny of osmoregulation in postembryonic fish: A review. *Comp Biochem Physiol A Mol Integr Physiol* 141: 401–429.

Velsen FPJ, Pacific Biological Station (1972-) (1987) Temperature and Incubation in Pacific salmon and rainbow trout: Compilation of data on median hatching time, mortality and embryonic staging. Department of

Fisheries and Oceans, Fisheries Research Branch, Pacific Biological Station.

Vengosh A, Jackson RB, Warner N, Darrah TH, Kondash A (2014) A critical review of the risks to water resources from unconventional shale gas development and hydraulic fracturing in the United States. *Environ Sci Technol* 48: 8334–8348.

Vincent RE (1962) Biogeographical and ecological factors contributing to the decline of Arctic grayling, *Thymallus arcticus* Pallas, in Michigan and Montana. agris.fao.org

Walker JR (2005) Status of the Arctic grayling (*Thymallus arcticus*) in Alberta. Alberta Sustainable Resource Development, Edmonton, Alberta. Report No. 57. ISBN: 0-7785-3675-0.

Wall B, Morrison-Shetlar AI, Claiborne JB. (2001a) Effect of environmental salinity and hypercapnia on NHE2-like and NHE3-like protein expression in the gill of the mummichog (*Fundulus heteroclitus*). Bull Mt Desert Is Biol Lab 40: 58–59.

Wang H, Singh D, Fliegel L (1998) Functional role of cysteine residues in the Na^+/H^+ exchanger effects of mutation of cysteine residues on targeting and activity of the Na^+/H^+ exchanger. *Arch Biochem Biophys* 358: 116–124.

Wells P, Pinder A (1996) The respiratory development of Atlantic salmon. II. Partitioning of oxygen uptake among gills, yolk sac and body surfaces. *J Exp Biol* 199: 2737–2744.

West RL, Smith MW, Barber WE, Reynolds JB, Hop H (1992) Autumn migration and overwintering of Arctic grayling in coastal Streams of the Arctic National Wildlife Refuge, Alaska. *Trans Am Fish Soc* 121: 709–715.

Whitehead A, Roach JL, Zhang S, Galvez F (2011) Genomic mechanisms of evolved physiological plasticity in killifish distributed along an environmental salinity gradient. *Proc Natl Acad Sci* 108: 6193–6198.

Wikelski M, Cooke SJ (2006) Conservation physiology. *Trends Ecol Evol* 21: 38–46.

Wilson JM, Laurent P (2002) Fish gill morphology: inside out. *J Exp Zool* 293: 192–213.

Wilson JM, Whiteley NM, Randall DJ (2002) Ionoregulatory changes in the gill epithelia of coho salmon during seawater acclimation. *Physiol Biochem Zool* 75: 237–249.

Wilson MV (1977) Middle eocene freshwater fishes from British Columbia. Royal Ontario Museum.

Witters H, Berckmans P, Vangenechten C (1996) Immunolocalization of Na^+, K^+-ATPase in the gill epithelium of rainbow trout, *Oncorhynchus mykiss*. *Cell Tissue Res* 283: 461–468.

Wood AW, Duan C, Bern HA (2005) Insulin-like growth factor signaling in fish. *Int Rev Cytol* 243: 215–285.

Wright PA, Wood CM (2009) A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. *J Exp Biol* 212: 2303–2312.

Wright P, Wood C (1985) An analysis of branchial ammonia excretion in the freshwater rainbow trout: effects of environmental pH change and sodium uptake blockade. *J Exp Biol* 114: 329–353.

Wu SC, Horng JL, Liu ST, Hwang PP, Wen ZH, Lin CS, Lin LY (2010) Ammonium-dependent sodium uptake in mitochondrion-rich cells of medaka (*Oryzias latipes*) larvae. *AJP Cell Physiol* 298: C237–C250.

Yan JJ, Chou MY, Kaneko T, Hwang PP (2007) Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *AJP Cell Physiol* 293: C1814–C1823.

Yu FH, Shull GE, Orlowski J (1993) Functional properties of the rat Na/H exchanger NHE-2 isoform expressed in Na/H exchanger-deficient Chinese hamster ovary cells. *J Biol Chem* 268: 25536–25541.

Zimmer AM, Brauner CJ, Wood CM (2014) Ammonia transport across the skin of adult rainbow trout (*Oncorhynchus mykiss*) exposed to high environmental ammonia (HEA). *J Comp Physiol B* 184: 77–90.

Zimmer AM, Nawata CM, Wood CM (2010) Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na+ uptake in freshwater rainbow trout. *J Comp Physiol B* 180: 1191–1204.

Zimmer AM, Wright PA, Wood CM (2015) What is the primary function of the early teleost gill? Evidence for Na^+/NH_4^+ exchange in developing rainbow trout (*Oncorhynchus mykiss*). *Proc R Soc B Biol Sci* 281: 20141422–20141422.

APPENDIX A: Investigate the thermo-tolerance of Arctic grayling (*Thymallus arcticus*) via critical thermal maximum

Appendix Preface

SDB, Chris Cahill, and Jessica Reilly (AEP) conceived and designed experiment. Experiment was carried out by SDB, along with data collection, analysis, and writing. This thermotolerance data will be part of a collaborative work with previous mentioned authors as part of the conservation management plan for Arctic grayling, which will include previous salinity tolerance considerations.

Introduction

Concerns over global climate change and its effects on freshwater ecosystems are growing based on evidence of increased atmospheric CO_2 and other greenhouse gasses being linked to a global warming trend (Bolin, 1986). Data indicate a increased global temperature of 0.85°C, over the period 1880–2012, and more recently an increase of 0.72°C over the period 1951–2012 (Hartman et al., 2013). More specifically in the northern hemisphere, data show an increase in air temperatures at an average rate of 0.24°C per decade (Folland et al., 2001). It has been shown that lake and stream temperatures closely follow the same temperature patterns and trends as the air temperature (Mohseni and Sefan, 1999). Increases in air and subsequent water temperature will have direct effects on the aquatic organisms inhabiting these freshwater environments in a variety of ways including direct lethal effects of increased temperature (which will be the focus of this chapter), decreased dissolved oxygen, increased sensitivity to pollutants, and effects on habitat and hydrology (Ficke et al., 2007). As a result of fish being ectotherms, their internal body temperature closely reflects that of the environmental temperature (Fry, 1967). Therefore any changes in water temperature will have quick and direct effects on the fish including enzymatic function, diet and metabolism, growth, swimming performance and lethality (Fry, 1947).

Preferred temperatures for pacific salmon species (Chinook, pink, sockeye, chum) have been documented as 12-14°C (Brett, 1952). However, critical thermal maximums (CTM) have become a common method in order to

indicate temperature tolerance of a species (Becker and Genoway, 1979). Today many fish species CTMs have been demonstrated as the temperature at which the fish experiences a loss of equilibrium (LOE) and can no longer right itself, and salmonids demonstrate the lowest CTMs indicative of their lack of high temperature tolerance (Beitinger et al., 2000). To date only 2 studies have been conducted on Arctic grayling thermotolerance. The first, conducted by LaPerriere and Carlson (1973) measured temperature tolerance of an Alaskan population of Arctic grayling with 96 hr lethal temperature tests and showed that amongst all life stages tested when acclimated to 8°C, the maximum thermal limit range was 20-24.5°C. The second study concerned the isolated Montana populations of Arctic grayling which exhibited CTMs of 26.4°C following 8.4°C acclimation and 29.3 following 20°C acclimation (Lohr et al., 1996). These CTMs are among the lowest of the salmonids tested.

In Alberta, where Arctic grayling are considered a species of special concern as of 2015, populations are in great decline compared to historical ranges (AEP; Walker, 2005). One threat to their population status is habitat decline as a result of increased water temperatures. Along with overfishing, increased water temperature due to logging was a major factor that is blamed for the extirpation and lack of successful reintroductions of the Arctic grayling populations that once inhabited Michigan up to the 1930s (Vincent, 1962). As a salmonid, grayling require habitats with cold clear water. Recent temperature trends in Alberta from 1950-2010 indicate annual average air temperatures increasing 2-4°C in the northern areas as well as lesser snowfall (Kienzle 2016).

The decreased water flow as a result of less snowfall, as well as higher water temperatures will lead to decreased habitat for this species.

In order for proper conservation of the Arctic grayling in Alberta, all factors of population decline must be examined. Increased temperature has been linked to other population declines and range alterations of salmonids, and it should be an aspect of great importance when examining grayling range (Keleher and Rahel, 1996; McCullough et al., 2009). As a result of genetic variation and geographical distance between populations of Arctic grayling of Montana and Alberta populations (Stamford and Taylor, 2004), the Alberta population may have different temperature tolerances compared the Montana population examined by Lohr and colleagues (1996). Therefore the goal of this study was to provide necessary critical thermal maximum data for Arctic grayling acclimated to 13°C and 17°C, based on a 4-year (2011-2014) average summer temperatures for June and July found in two separate boreal streams in Alberta. In collaborative efforts with Alberta Environment and Parks, this vital physiological data will later be used in conjunction with stream temperature modeling data and life history analyses to form a complex management plan for Alberta Arctic grayling taking into past, present, and future stream temperature variations.

Methods

Animal Collection and Housing

Arctic grayling (*Thymallus arcticus*) were collected in collaboration with Alberta Environment and Parks (AEP), via angling (fly-fishing) from the Freeman

River, in central Alberta. Grayling were transported from the catch site to the University of Alberta's bio-secure aquatics facility utilizing an AEP hatchery truck carrying a 1000 L tank containing oxygenated river water (~9.76 mg/L O_2, ~20.4°C), chilled with ice bags. Fish were transferred from the truck into a main holding tank (825 L) with aerated flow-through dechlorinated Edmonton city tap water 10°C. Grayling (22.7 ± 0.4 cm, 69.1 ± 4.2 g, means ± S.E.M.) were maintained and allowed to acclimate in captivity for 6 months prior to experimentation. Grayling were fed to satiation a mixture of *Artemia* and Mysis shrimp every other day.

Critical Thermal Maximum

CTM tests were conducted on Arctic grayling (n=8) for each acclimation temperature. Fish were transferred from the central holding tank (10°C) to an 180L recirculating acclimation system composed of three individual 60 L tanks (header tank, fish acclimation tank, and sump tank). Water was temperature controlled via heat exchange through an external chiller unit, which maintained the set temperature of the system at +/- 0.3°C throughout experiment. Fish were gradually acclimated from 10°C to 13°C over a one-day period and fish acclimated from 10°C to 17°C over a three-day period. Fish were held at their respective acclimation temperatures for 14 days prior to performing CTM tests. On day 10 of the acclimation to 17°C, a single fatality occurred bringing this sample size down to an n=7 for this group. It is unknown whether this lethality was incurred due to the acclimation procedure, as all other Arctic grayling did

survive. Fish were fed during the acclimation period, but fasted 3 days prior to and during testing.

An identical 60 L tank, to that of which the fish had been housed in for the 14-day acclimation period, was used as the testing tank to minimize any environmental change induced stress incurred by the fish. The test tank was supplied with recirculating water from a Lindberg Blue M (Thermo Scientific) heated water bath and attached chiller unit at a flow rate of 10 L/min. A submerged air stone was placed in the test tank to aerate the water and provide constant mixing to avoid thermal stratification in the tank. Dissolved oxygen was constantly measured in the test tank with a handheld oxygen and conductivity probe (YSI Model 85; Yellow Springs, OH, USA) and maintained above 8.7 mg/L. Tank temperature was monitored on opposite sides of the tank with both the handheld O_2 probe as well as a Loligo Witrox system temperature probe (Loligo Systems, Denmark).

Two fish were transferred from the acclimation tank to the experimental tank maintained at the same temperature and allowed to acclimatize for 10 minutes prior to commencement of CTM. Following the 10 min acclimation period, the temperature in the test tank was increased at a rate of 0.2°C /min (R^2=0.999) from the initial acclimation temperature (13°C or 17°C). The temperature at which the fish could no longer right itself and demonstrated a loss of equilibrium (LOE) was recorded as its critical thermal maximum (CTM).

Results

Behaviour

Upon introduction to the experimental tank Arctic grayling quickly became settled within the initial 10min prior to the beginning of the ramp up period. Initial behaviour consisted of slow and constant gill ventilation with very little body movement as the fish stayed near the bottom of the tank. For each of the trials once the temperature reached 18-20°C, fish behaviour shifted from settled to a stressed state. This was indicated by increased ventilation and greatly increased swimming behaviour moving throughout the tank. At a temperature of 20-22°C fish began to swim throughout the entire water column constantly surfacing. Initial signs of buoyancy difficulty began between 24-25°C, with most fish briefly turning over but quickly righting themselves and continued swimming and surfacing. The time period that this final behaviour lasted until complete LOE was reached varied with individual fish.

Critical Thermal Maximum

Arctic grayling acclimated to 13°C demonstrated a significantly lower average CTM than the grayling acclimated to 17°C (Figure 6.1). The 13°C acclimated fish maintained an average CTM of 26.95°C ± 0.82°C compared to 27.80°C ± 0.53°C of the 17°C acclimated fish (mean ± SD, Student's t-test, p value <0.05, $p = 0.0367$, $n = 8$ and 7, respectively). The lowest CTM was of a 13°C acclimated fish at 25.10°C, while the highest CTM was that of a 17°C acclimated fish at 28.41°C (Table 6.1). Additionally, there were no significant differences in

the weight between the two different groups of acclimated fish ($p = 0.234$, t-test). Furthermore, regression analysis yielded no relationship among the combined treatments (all 15 data points) between the individual weight and CTM demonstrated by the Arctic grayling, $R^2 = 0.053$, $p = 0.406$ (Figure 6.2).

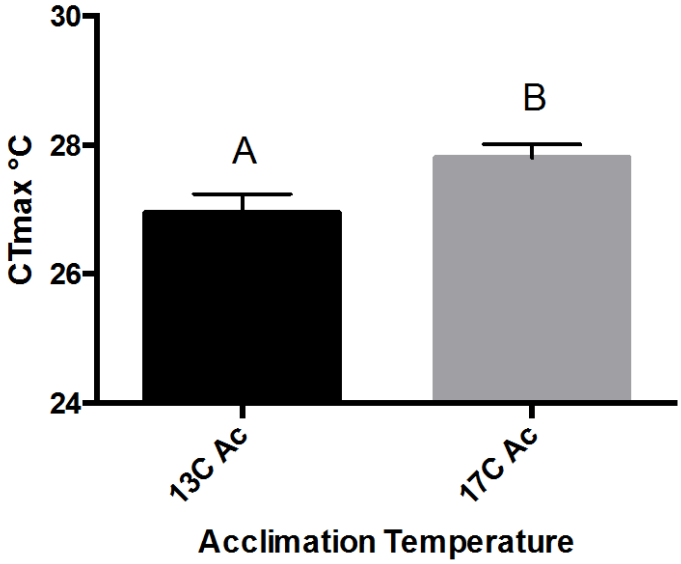


Figure A.1. Mean critical thermal maximums (CTM) for Arctic grayling. Black bar indicates Arctic grayling acclimated to 13°C, while grey bar represents grayling acclimated to 17°C (grey bar). Dissimilar letters indicated significant difference between average CTM (t-test, P value = 0.036).

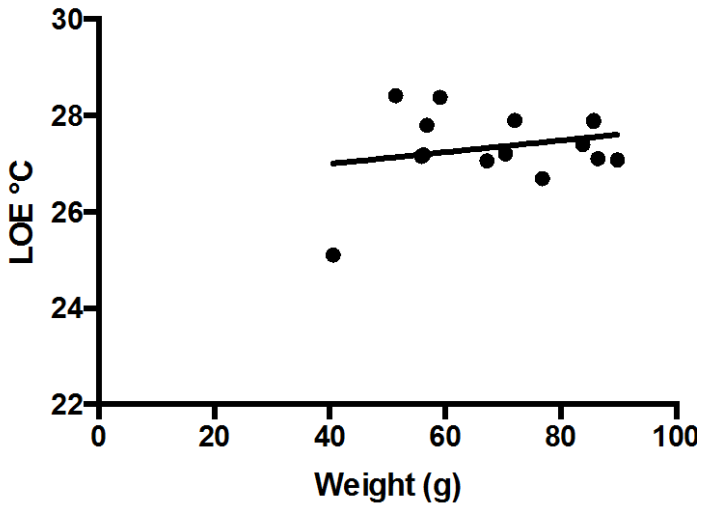


Figure A.2. Regression analysis of LOE temperature and mass of Arctic grayling.

Slope = 0.012 ± 0.014; R^2= 0.053; P value = 0.406

Table A.1 Critical Thermal Maximum Data for Arctic grayling acclimated to 13 and 17°C

Acclimation Temp	Fish	AVG CTmax	Median CTmax	STDEV	SEM	Range
13	8	26.95	27.14	0.82	0.29	25.10 - 27.88
17	7	27.8	27.9	0.53	0.19	27.06 – 28.41

Discussion

Acclimation temperatures of 13°C and 17°C were chosen based on the average summer temperatures in June and July of two rivers in Alberta which are home to Arctic grayling. These streams currently have a population rating of "very low" while historically they maintained "moderate," to "very high" population statuses in throughout various sections of the rivers (AEP 2015). Furthermore, from 1950 – 2010, average summer air temperatures have increased 0.4 to 1.5°C in the areas around these rivers (Kienzle 2016). Grayling acclimated to 13°C and 17°C demonstrated relatively low average CTMs of 26.95°C and 27.80°C, respectively, confirming their cold-water fish designation. Furthermore, statistical analysis (Students t-test, $p<0.05$) showed significant differences in the CTMs between the 13°C and 17°C acclimated fish demonstrating some Arctic grayling ability to increase their upper temperature tolerance limit following an acclimation period, a characteristic shared by most fish (Beitinger et al., 2000). Results did not suggest any correlation between size of fish and temperature, however the smallest fish (40.6g) used in the study did exhibit the lowest CTM of 25.1°C. Additionally, the heat ramping rates were always consistent at 0.18°C/min (~11°C/hr) and linear ($R^2=0.999$) falling within the range of comparable CTM experiments. All fish used were of the same relative size and a final loss of equilibrium or the complete inability of the fish to right itself is analyzed, although initial LOE is also reported. These aspects of the experiment performed here are necessary to mention as they fulfill the

requirements of the amended criteria for a proper CTM test as described in Becker and Genoway (1979).

In comparison to other grayling thermotolerance studies, exact comparisons are difficult to make based on differing acclimation temperatures. However, we argue based on the certain variables used in each study that the Arctic grayling used in this study exhibit a lower thermal tolerance than the Montana population of Arctic grayling reported on by Lohr and colleagues (1997). Fish used in the present study were significantly larger (69.1g compared to 19.5g) than the small juveniles of Montana tested previously. Although in our study there is no correlation between size and thermal tolerance, body temperature is related to body size and can come into play when comparing CTMs (Stevens and Fry, 1974). In contrast to that, specific grayling comparisons between sac fry and fish larger than 20cm revealed no differences in lethal temperature, and the authors noted that young of the year fish that survived the 24°C 96 hr lethality test demonstrated normal behaviour compared to the larger grayling which were in poor condition at the same time point (LaPerriere and Carlson, 1973). Lohr et al. (1997) utilized acclimation temperatures of 8.4, 16 and 20°C, which produced respective average CTMs of 26.4, 28.5, and 29.3°C. This is in comparison to our acclimation temperatures of 13°C and 17°C, which yielded average CTMs of 26.95°C and 27.8°C. Given a linear regression was applied to the Lohr et al. data, with respect to acclimation temperature and acquired CTM, a linear equation with an R^2 of 0.994 can be produced ($y = 0.2535 + 24.316$). If our acclimation temperatures of 13°C and

17°C are plotted against this regression, we can estimate that the Montana fish would possess a CTM of 27.61°C and 28.63°C. Comparing these with our acclimation reveals that the Montana grayling appear to have a higher thermotolerance than the Alberta grayling. However, this analysis is quite theoretical given 1) the limitations of CTM not always maintaining a linear relationship to acclimation temperature 2) the size of fish, and 3) the slight difference in ramping rate (0.2°C/min compared to 0.4°C/min) between the two mentioned studies. The CTM values achieved in this study are consistent with other salmonid CTMs (<30°C) and are lower than many including rainbow trout and some salmon species (Beitinger et al., 2000) acclimated to similar temperatures, confirming these fish have a limited tolerance for warming waters.

With increasing water temperatures in Alberta, these data should be taken into account for future conservation efforts and stream temperature closure designations. The critical thermal maximum again is the temperature-induced point at which the fish loses equilibrium and its ability to right itself. It is incorrect to assume that fish will be unaffected until stream temperatures reach 27°C. CTM should be viewed exactly as described, a critical limit, as in lethality would already have occurred if temperatures reached this point for any length of time. This is made clear in the Lohr et al., (1997) study, which included upper incipient lethal temperatures (UILT) based acclimating fish to the same temperatures as before followed by exposing fish to a number of temperatures close to their CTM for a period of time until death occurred. These data revealed

that only grayling acclimated to 20°C could survive 22°C exposure for more than 2 days. Furthermore, all grayling regardless of acclimation temperature exposed to 26°C or higher could not survive the exposure longer than 4 hrs, with most dying within minutes (Lorh et al., 1997). We would argue that the grayling of Alberta would have even lower UILT based on their experimental CTMs, and thus stream temperatures reaching 24-25°C for periods longer than a few hrs would be quite dangerous to Arctic grayling present in these rivers.

Various examples have been documented in the literature where salmonids have been negatively affected by increasing water temperature, including one example of extirpation of the close relative to the Arctic grayling, the European grayling (*Thymallus thymallus*) from a 30-kilometre stretch of the Rhone River (McCollough et al., 2009; Daufresne et al., 2003). Brown trout populations have shifted to higher elevations in the mountains of Switzerland due to increased water temperatures (Hari et al., 2006). These effects and many sublethal effects likely not yet revealed are the result of just small water temperature changes. Alberta grayling are potentially following the same population patterns as these mentioned affected fish species. Further complex studies, including modeling of stream temperatures according to predicted warming weather trends will be necessary to predict and help slow or stop these negative population changes from occurring, namely those influenced by anthropogenic forces including logging and riparian habitat disruption. The goal will be to recognize hydrological areas which are at high risk and develop management plans to protect these areas and the Arctic grayling that inhabit

them, and additionally attempt to revitalize stream that have succumb to warming temperatures to increase habitat for these native salmonids.