The ontogeny of sodium balance of rainbow trout (Oncorhynchus mykiss) and pink salmon (Oncorhynchus gorbuscha) during post-embryonic development in freshwater

by

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Abstract

This thesis contributes to our general knowledge of ionoregulatory function in developing fish by characterizing the functional ontogeny of sodium (Na\(^+\)) balance in salmonids reared in freshwater during early development.

Chapter 2 investigated the plasticity of Na\(^+\) balance and transport capacity during a critical developmental transition from cutaneous-dominated to gill-dominated ionoregulation in the model teleost fish, the rainbow trout. Fish experienced very high resting unidirectional Na\(^+\) uptake rates early in development which were reduced to values typical of adults following yolk absorption. Maximal uptake rate (\(J_{\text{max}}\)) for Na\(^+\) was high during early development and decreased following yolk absorption while uptake affinity decreased (\(K_m\) increased) following hatch and increased following yolk absorption. It appeared that early in development, high Na\(^+\) uptake rates across cutaneous ionocytes were driven by high maximal uptake rate, while the gill ionocytes that dominate ionoregulation post-yolk absorption had an increased affinity for Na\(^+\).

Following hatch, when ionoregulation occurs predominantly across cutaneous ionocytes, larval fish exhibited little ionoregulatory plasticity in their Na\(^+\) uptake rates and Na\(^+\) uptake kinetics. As ionoregulation shifts to the gill, developing fish exhibited increased uptake affinity for Na\(^+\) in low-[Na\(^+\)] environments as observed in adult fish; however, maximal uptake rate for Na\(^+\) did not increase in low-[Na\(^+\)] environments as seen in adult fish, suggesting that the capacity to overcome Na\(^+\)-poor environments may be limited and still developing at this stage.
Chapter 3 contributed to our understanding of Na\(^+\) transport during early salmonid development and explored Na\(^+\) transport characteristics employed by the early-migrating anadromous salmonid, the pink salmon. It was clear that heightened and increasing whole-body [Na\(^+\)] during yolk absorption in freshwater was not a unique characteristic of developing pink salmon associated with preparation for early ocean entry. This trait was shared by the non-anadromous rainbow trout. Interestingly, the mechanism by which pink salmon and rainbow trout achieved high whole-body [Na\(^+\)] during early development did not appear to be the same. Rainbow trout experienced increasing Na\(^+\) uptake rates during development while pink salmon did not alter Na\(^+\) uptake rates during development, suggesting that pink salmon regulated whole-body [Na\(^+\)] via modulation of Na\(^+\) efflux rates.
Preface

Chapters Two and Three of this thesis are co-authored by Emily J. Gallagher, Jonathan M. Wilson and Colin J. Brauner. I conducted the research for both chapters under the supervision of Dr. Colin J. Brauner, with the exception of the measurements of tissue-specific sodium transporter protein expression in Chapter 2, which were measured by Jonathan M. Wilson at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR), Porto, Portugal. I wrote all four chapters of this thesis and I received editorial feedback from Drs. Colin J. Brauner, Jeffrey G. Richards, and Peter J. Rombough. All procedures involving animals adhered to the UBC’s Animal Care Committee, certificate A11-0235.
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List of abbreviations

°C ................................................................. Degrees Celcius
22Na+ ............................................. Sodium-22 ion
22NaCl ................................................ Sodium-22 chloride
ANOVA ................................................. Analysis of variance
ATPase ................................................ Adenosinetriphosphatase
ATU ....................................................... Accumulated thermal units
[Ca2+] .................................................. Calcium concentration
Ca2+ ........................................................ Calcium ion
CaCO3 ................................................ Calcium carbonate
CBE ........................................................ Chloride-bicarbonate exchanger
CFTR ...................................................... Cystic fibrosis transmembrane regulator
[Cl−] ........................................................ Chloride concentration
Cl− ........................................................ Chloride ion
cpm ........................................................ Counts per minute
°day ......................................................... Degree day
dpf ........................................................ Days post-fertilization
dph ........................................................ Days post-hatch
eKir ......................................................... Inward rectifying K+ channel
ENaC ........................................................ Epithelial sodium channels
H+-ATPase ............................................... Proton-ATPase
[H3]PEG-4000 .......................................... tritium-labeled polyethylene glycol, molecular weight = 4000
HA ........................................................ Proton-ATPase
HCO3− ................................................ Bicarbonate ion
Jin ......................................................... Sodium influx rate
Jnet ........................................................ Sodium net flux rate
Jout ........................................................ Unidirectional sodium efflux rate
Jmax ...................................................... Maximal transport velocity
K+ .......................................................... Potassium ion
Km ........................................................ Michaelis constant (measured inverse of affinity)
Mg2+ ..................................................... Magnesium ion
MRC ........................................................ Mitochondrion-rich cell
mRNA .................................................. Messenger ribonucleic acid
MS-222 ................................................ Tricaine methanesulfonate
MW ........................................................ Molecular weight
[Na+] .................................................. Sodium concentration
Na+ .................................................... Sodium ion
NaC ........................................................ Sodium channel
NaCl .................................................... Sodium chloride
NaHCO$_3$.................................Sodium bicarbonate
[NaCl]$_{\text{ext}}$........................................External NaCl concentrations
NBC..............................................Sodium-bicarbonate cotransporter
NHE..............................................Sodium–hydrogen exchanger
NH$_3$............................................Ammonia
NH$_4^+$.........................................Ammonium
NKA.............................................Sodium-potassium-ATPase
NKCC...........................................Sodium-potassium-2-chloride cotransporter
PEG.............................................Polyethylene glycol
PNA$^-$.........................................Peanut lectin agglutinin-negative
PNA$^+$.........................................Peanut lectin agglutinin-positive
Rhcg2........................................Ammonia transporter, Rhesus protein family, C
glycoprotein 2
T4................................................Thyroxine
UBC............................................University of British Columbia
V-H$^+$-ATPase...............................Vacuolar-type H$^+$-ATPase
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For my parents
Chapter 1 General introduction

1.1 OVERVIEW

The purpose of this thesis was to characterize the functional ontogeny of Na⁺ balance in salmonids reared in freshwater during early development. Both intra- and interspecific comparisons were carried out. The thesis has been divided into two distinct research chapters: I first investigated the effect of environmental [Na⁺] on Na⁺ uptake and balance in rainbow trout (Oncorhynchus mykiss) larvae. I then expanded the work to also include measurements of Na⁺ uptake in pink salmon (Oncorhynchus gorbuscha). This permitted me to compare the developmental trajectories for Na⁺ balance in an early-smolting species, pink salmon, with that of the non-anadromous rainbow trout.

1.2 IONOREGULATION IN TELEOST FISHES

Ionoregulation in adult teleost fishes has been studied extensively since the early-20th century (reviewed in Evans, 1980; Evans et al., 1999; Evans et al., 2005). Teleost fishes require ionic and osmotic homeostasis of their body fluids to ensure maintenance of the electrochemical gradients essential to normal cellular function. Maintaining ionic homeostasis in aquatic environments is challenging because ions passively diffuse down their concentration gradients across semi-permeable membranes. Consequently, teleost fish possess complex ionoregulatory mechanisms to defend against passive ion fluxes and maintain internal ionic balance in both hyper- and hypotonic environments via active ion transport. In adult fish, processes at the gills, kidneys and intestine integrate to balance
diffusional gains and losses with the gills playing the largest role (reviewed by Evans et al., 1999; Evans et al., 2005; Hwang et al., 2011).

Cells involved in active ion exchange are generally referred to as ionocytes, or mitochondrion-rich cells (MRCs; Keys and Willmer, 1932). Mitochondrion-rich cells are large, ovoid-shaped cells which, as their name implies, contain a high density of mitochondria (Philpott, 1980; Pisam and Rambourg, 1991). These cells are highly polarized; the apical and basolateral membranes are morphologically and functionally distinct, as indicated by their dissimilar transport protein expression profiles. The extensive infolding of the basolateral membrane forms a complex tubular system that is tightly linked with the mitochondria (Philpott, 1980; Pisam and Rambourg, 1991). Ionocytes are also characterized by high levels of the ion transport enzyme Na⁺-K⁺-ATPase (NKA), expressed largely in the basolateral membrane (Karnaky et al., 1976).

Sodium (Na⁺) and chloride ions (Cl⁻) are major ionic constituents of extracellular body fluids (reviewed by Evans et al., 2005). Therefore the regulation of both Na⁺ and Cl⁻ levels are critical components of ionoregulation. The bulk of this thesis will focus on Na⁺ regulation but because of the close connection between the two ions I will also provide an overview of Cl⁻ regulation.

**Ionoregulation in freshwater**

Freshwater fishes are hypertonic to their environment and therefore they passively lose ions and gain water. The hydromineral challenge associated with life in freshwater is water load and salt loss. To overcome this challenge freshwater teleosts produce large
amounts of dilute urine while actively taking up ions at the gill (reviewed by Evans et al., 2005; Hwang et al., 2011). The current model for NaCl uptake across the gill epithelium in freshwater rainbow trout (in addition to the different models for Na\(^+\) uptake in freshwater zebrafish and tilapia) has been reviewed by Hwang et al. (2011) and Dymowska et al. (2012) and is illustrated in Figure 1-1. Briefly, in order to achieve Na\(^+\) uptake against its concentration gradient in freshwater, it appears that trout use a combination of two distinct ionocyte types, peanut lectin agglutinin-positive (PNA\(^+\)) and PNA-negative (PNA\(^-\)) ionocytes (Galvez et al., 2002). The PNA\(^-\) cells contain a H\(^+\)-ATPase in the apical membrane (Lin and Randall, 1991) which generates an electrical gradient and drives Na\(^+\) into the cell from the environment through putative Na\(^+\) channels (NaC) in the apical membrane. The PNA\(^+\) cells contain electroneutral Na\(^+\)/H\(^+\) exchangers (NHE2/3) in the apical membrane. To overcome the significant thermodynamic constraints on the function of NHEs at low ion concentrations (i.e. [Na\(^+\)] < 0.1 mM), it has been proposed that an ammonia (NH\(_3\)) transporter (Rhcg2) on the on the apical membrane pumps NH\(_3\) to the outside of the ionocyte where it binds with H\(^+\) to form ammonium (NH\(_4^+\)) in the boundary layer (“ammonia trapping”), forming a favourable H\(^+\) gradient to drive Na\(^+\) uptake from the water through NHE2/3 (Wright and Wood, 2009). In both the PNA\(^-\) and PNA\(^+\) cell types, NKA in the basolateral membrane pumps Na\(^+\) against its concentration gradient from the ionocyte into the extracellular fluids (Avella and Bornancin, 1989; Piermarini and Evans, 2000; Wilson et al., 2000a) along with a putative electrogenic Na\(^+\)-HCO\(_3^-\) cotransporter (NBC) in the basolateral membrane of the PNA\(^-\) cells.
While the molecular mechanism of Cl\textsuperscript{−} uptake in rainbow trout remains to be elucidated, the current model for Cl\textsuperscript{−} uptake occurs across PNA\textsuperscript{+} cells and includes an apical Cl\textsuperscript{−}/HCO\textsubscript{3}\textsuperscript{−} exchanger (CBE) in which Cl\textsuperscript{−} uptake is directly linked to HCO\textsubscript{3}\textsuperscript{−} excretion (Wilson et al., 2000b; Wilson et al., 2002) in a 1:1 equimolar ratio (Goss and Wood, 1990a; Goss and Wood, 1990b; Kerstetter and Kirschner, 1972) and a basolateral Cl\textsuperscript{−} channel (ClC; reviewed by Dymowska et al., 2012; Evans et al., 2005). It has been proposed that a basolateral H\textsuperscript{+}-ATPase drives the apical absorption of Cl\textsuperscript{−} through the CBE against its concentration gradient, alleviating the thermodynamic constraints on Cl\textsuperscript{−} uptake in freshwater (Tresguerres et al., 2006).

**Ionoregulation in seawater**

Marine fishes are hypotonic to their aquatic surroundings and as a result they passively gain ions and lose water across their permeable surfaces. Thus the ionic challenge associated with marine life is salt load and dehydration. To compensate, marine teleosts drink and absorb seawater at the intestine (Smith, 1930) while actively excreting ions at ionocytes located primarily on the gill (Foskett and Machen, 1985; Keys, 1931; Keys and Willmer, 1932). The currently accepted model for NaCl excretion at the gills (Epstein et al., 1967; Silva et al., 1977; Figure 1-2) involves NKA (Karnaky et al., 1976; Lee et al., 1998; McCormick et al., 2003; Piermarini and Evans, 2000; Silva et al., 1977; Wilson et al., 2000a; Wilson et al., 2002) and the Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{−}-cotransporter (NKCC; Marshall et al., 2002; McCormick et al., 2003; Pelis et al., 2001; Wilson et al., 2000a) localized to the basolateral membrane of ionocytes in the gill along with the anion channel, CFTR, situated in the apical
membrane (Marshall et al., 2002; McCormick et al., 2003; Wilson et al., 2000a). Na\textsuperscript{+}, K\textsuperscript{+} and Cl\textsuperscript{−} enter the ionocyte via basolateral NKCC. This process is driven by NKA, through which Na\textsuperscript{+} is actively recycled back into the plasma in exchange for K\textsuperscript{+} (Silva et al., 1977). Potassium is recycled back across the basolateral membrane via an inward rectifying K\textsuperscript{+} channel (eK\textsubscript{ir}; Degnan, 1985; Suzuki et al., 1999). Subsequently Cl\textsuperscript{−} and Na\textsuperscript{+} are excreted down their electrochemical gradients via secondary active transport; Cl\textsuperscript{−} exits through the apical CFTR (Marshall et al., 1995) and Na\textsuperscript{+} is secreted paracellularly through leaky tight junctions across the gill epithelium (Degnan and Zadunaisky, 1980). Overall, this cooperative process results in the excretion of Na\textsuperscript{+} and Cl\textsuperscript{−} against their concentration gradients into seawater (reviewed by Evans et al., 2005; Hwang et al., 2011).

**Ionoregulation in larval fishes and development of the gill**

**Ionoregulation in larval fishes**

Embryos and larvae of teleost fish also face osmotic and ionic challenges. Nonetheless, fewer investigations of ion and osmoregulation in developing fish have been conducted (for reviews see Alderdice, 1988; Kaneko and Hiroi, 2008; Varsamos et al., 2005). During early life stages the gill is not yet fully developed, and as a result, ionoregulatory processes occur largely across extrabranchial epithelia, including that of the yolk sac, head, trunk and fins (Hwang, 1989; Shelbourne, 1957). Extrabranchial exchange is sufficient due to the high surface area-to-mass ratio in these small fish, but as the larvae grow, cutaneous exchange becomes limiting (reviewed in Rombough, 2007). To date, there has been a focus on osmoregulatory ability during embryonic development, including the
morphology of ionoregulatory organs and the specialized cells involved in ion uptake (ionocytes) found within the skin (e.g. Japanese flounder, *Paralichthys olivaceus*, Hiroi et al., 1998; tilapia, *Oreochromis mossambicus*, Hwang, 1990a; killifish, *Fundulus heteroclitus*, Katoh et al., 2000; rainbow trout, *O. mykiss*, Rombough, 1999b; sea bass, *Dicentrarchus labrax*, Varsamos et al., 2002) and gills (e.g. rainbow trout, *O. mykiss*, Gonzalez et al., 1996; Japanese flounder, *P. olivaceus*, Hiroi et al., 1998; ayu, *Plecoglossus altivelis*, Hwang, 1990b; tilapia, *O. mossambicus*, Li et al., 1995; Atlantic salmon, *Salmo salar*, Pisam et al., 2000; sea bass, *D. labrax*, Varsamos et al., 2002) as they develop. As the gills develop, there is a shift in ionocyte distribution from the skin to the gills (Gonzalez et al., 1996; Hiroi et al., 1998; Hwang, 1990b; Li et al., 1995; Pisam et al., 2000; Varsamos et al., 2002). During this time the gills are rapidly proliferating while the surface area of the yolk sac epithelium is declining as the yolk is consumed. As a result, the total number of ionocytes on the yolk sac epithelium peaks at hatch and the total number of ionocytes on the skin reaches a maximum about half-way through yolk absorption and declines afterwards (Rombough, 1999). Conversely, the number of ionocytes on the gills continue to increase rapidly as the gills develop (Rombough, 1999). Consequently, the gills gradually displace the skin and yolk-sac epithelia as the primary site of ion exchange processes. Fu et al. (2010) demonstrated that the gills are already contributing to ion regulation in rainbow trout at 0 days post-hatch (dph), accounting for approximately 10 per cent of total whole-body Na\(^+\) uptake, and by 15 dph the gills account for greater than 50 per cent of Na\(^+\) uptake. These data indicate that the gills play a major role in ionoregulation much earlier in development than was generally believed.
Ion hypothesis

Until recently it was assumed that the first physiological process to transition from extrabranchial to branchial exchange during development was oxygen uptake (Krogh, 1941). However, the data presented by Fu et al. (2010) indicate that in developing rainbow trout ion uptake shifts to the gills about ten days before oxygen uptake transitions to the gills. The developmental stage at which these exchange processes transition from the extrabranchial epithelia to the gills and, more specifically, the relative time at which the various exchange processes transition to the gills in altered and/or extreme environments remains uncertain (i.e. does the transition of one exchange process always precede other exchange processes regardless of external factors?) This topic is of interest as it may provide insight into the initial driving force of gill development. It has been proposed recently that ionoregulation (rather than gas exchange) may represent the initial driving force for gill development, termed the ionoregulatory hypothesis (Brauner and Rombough, 2012; Fu et al., 2010; Li et al., 1995; Rombough, 1999; Rombough, 2002; Rombough, 2007).

It has been demonstrated in adult teleost fishes that ambient ion concentrations strongly influence gill morphology to improve ion uptake capacity, in particular the number and activity of ionocytes in the gill (Greco et al., 1996). However, Fu et al. (2010) reported that soft water rearing did not accelerate the transition of Na\(^+\) uptake to the gills (compared to hard water rearing) and that there were no differences in NKA concentration and activity observed between soft and hard water reared groups. The authors proposed that the timing of transition to the gills for Na\(^+\) uptake is non-plastic; nonetheless the phenotypic plasticity of Na\(^+\) uptake capacity in larval fish has not been directly measured.
Although several research groups have focused on osmoregulation in post-embryonic euryhaline fishes using variations in salinity tolerance as an indicator of capacity to osmoregulate (e.g. Gallagher et al., 2013; Hwang, 1990b; McWilliams and Shephard, 1989; Shen and Leatherland, 1978a; Weisbart, 1968; reviewed in Holliday, 1969; Varsamos et al., 2005), few studies have investigated the effects of water ionic composition on ionoregulatory function of teleost fish during early post-embryonic development within a freshwater context (e.g. effect of \([\text{Ca}^+]\), Genz et al., 2014; Hwang et al., 1996; hard versus soft water, Fu et al., 2010; effect of toxic metals, Brauner and Wood, 2002a; Brauner and Wood, 2002b; Gillis and Wood, 2008; Rombough and Gaside, 1984) and none have investigated the effect of \([\text{Na}^+]\) on ionoregulatory function in post-embryonic larval fish. Chapter 2 aims to fill this gap by investigating the potential plasticity of ionoregulatory development from the eyed stage through to yolk absorption in rainbow trout reared in waters of different \([\text{Na}^+]\) concentrations.

1.3 MODEL SYSTEM: GENUS ONCORHYNCHUS, THE PACIFIC SALMON AND TROUT

Salmonids and the anadromous life history strategy

Most salmon are anadromous; they reside in seawater for most of their juvenile and adult life but return to freshwater for reproduction and early life rearing. This life history strategy is common to a variety of fish species, including lampreys, sturgeons, some species of bass and salmonids. Anadromy is thought to be beneficial because it allows fish to incorporate the advantages of both freshwater and seawater into their lifecycle. The freshwater streams where reproduction occurs offer a space with relatively few predators,
which is advantageous during early life stages when the animal is most vulnerable to predation. Alternatively, marine waters are generally more productive than temperate freshwater streams. This is favourable during maturation as it facilitates rapid growth thereby improving these fishes’ reproductive success (Gross et al., 1988; McDowall, 1997; McDowall, 2008; Stefansson et al., 2008).

The life history of salmonids has been extensively studied (e.g. Crisp, 2000; Pearcy, 1992; Quinn, 2005; Willson, 1997). Salmon spawn within the gravel in pit-like structures called redds. Once the egg hatches the developing salmonid is referred to as an alevin or larva and remains in the gravel, surviving on residual yolk contained within the yolk sac. Once the yolk is nearly exhausted, the alevin emerges from the gravel. Now referred to as a fry, the fish disperses from the gravel and begins to feed exogenously. When the fry disperses from the redd site to feed and defend its territory it is then referred to as a parr. From this stage onward there are a wide variety of strategies exhibited by the different salmonid species. The remainder of this thesis will focus on the genus Oncorhynchus of the Salmonidae family, which includes the Pacific salmon and trout. Within this genus, the amount of time spent in freshwater prior to ocean entry is extremely variable. At one extreme are the pink salmon which enter seawater soon after gravel emergence weighing approximately 0.2 g (Grant et al., 2009; Heard, 1991). Conversely, most other salmon species which spend 1-2 years in freshwater and enter the ocean at sizes 10 to 100 times larger. At the other end of the spectrum are the stream resident forms of O. mykiss, rainbow trout, which are non-anadromous and spend their entire life in freshwater.
Smoltification

Salmonids have the ability to make physiological adjustments to prepare for seawater while residing in freshwater, termed predictive anadromy. This contrasts with other species which adjust to seawater in response to exposure to seawater, termed facultative anadromy. The preparatory changes occurring in freshwater are referred to as smoltification and include a suite of complex behavioural, morphological and physiological transformations crucial for survival in seawater (Bern, 1978; Folmar and Dickhoff, 1980; McCormick and Saunders, 1987). An essential part of smoltification is the development of hypo-osmoregulatory capacity. This involves the transformation of the gill from an ion-absorbing to an ion-secreting epithelium.

Hypo-osmoregulatory strategies employed by salmonids

The development of a hypo-osmoregulatory strategy includes hormonally-controlled major remodelling of ionoregulatory organs, largely at the gill, associated with upregulation of ion excretion activity. It is often characterized by an increase in the size, number and density of ionocytes (Lubin et al., 1989; McCormick, 2001; Pisam et al., 1988), upregulation of the ion transporters NKA, NKCC and CFTR in the gill epithelium (Nilsen et al., 2007; Pelis et al., 2001; Stefansson et al., 2008) and an increase in the expression ratio of the seawater to freshwater NKA isoforms (i.e. upregulation of the NKA α-1a/α-1b expression ratio; Nilsen et al., 2007; Richards et al., 2003).
**Pink salmon, *O. gorbuscha***

Due to their very small size at seawater entry, pink salmon may experience a greater hypo-ionoregulatory challenge than other salmonids because of their higher surface area-to-volume ratio (Houston, 1961). This, compounded with the relative lack of post-emergent time for preparatory adaptation, has led to the idea that perhaps this species makes use of a novel hypo-ionoregulatory strategy as larval alevins in the redd that is not seen in other members of the genus (Sullivan et al., 1983; Weisbart, 1968). Nonetheless the mechanisms involved are largely unstudied.

Developing pink salmon are not seawater tolerant as embryos and alevins (Weisbart, 1968). However, pink salmon fry are able to maintain water balance and increase their gill NKA activity upon transfer to seawater (Sackville et al., 2012) better than other Oncorhynchids at this stage, demonstrating their superior hypo-osmoregulatory capacity (Weisbart, 1968). Additionally, there is evidence that pink salmon undergo osmoregulatory changes associated with smoltification while in freshwater. For instance, pink salmon held in freshwater upregulate their NKA α-1a/α-1b expression ratio at the time of yolk absorption, which corresponds to natural seaward migration (Gallagher et al., 2013; Sackville et al., 2012). Pink salmon also exhibit a window of heightened seawater tolerance during this critical time in freshwater (Gallagher et al., 2013).

Upon transfer to seawater pink salmon initially accumulate very high levels of whole-body ions, which gradually decrease over time as gill NKA activity increases (Grant et al., 2009). Initially it was suggested that this was an indication that pink salmon experience an ionoregulatory disturbance and may not have fully developed their hypo-
ionoregulatory capacity at the time of ocean entry. It was proposed that seawater acted as a stimulus to induce additional smolt-like physiological changes, a strategy which is different from that of other anadromous salmon studied to date and has recently been referred to as “precocious anadromy” (Sackville et al., 2012). However, more recently it was observed that pink salmon held in freshwater achieved whole-body Na⁺ levels similar in magnitude to that observed post-seawater transfer, suggesting that Na⁺ levels are not increasing due to an ionoregulatory disturbance in response to seawater entry but instead may be a controlled process that occurs in freshwater (Gallagher et al., 2013). This trajectory becomes even more remarkable when one considers whole-body Na⁺ levels in adult fish; at yolk absorption pink salmon experience Na⁺ levels greater than twice that observed in adult fish and values continue to rise beyond yolk absorption up to three times that of adults (Gallagher et al., 2013). It is unknown whether this represents a controlled and unique strategy for maintaining water balance for pink salmon entering seawater or is a developmental trend characteristic of all developing fish. Only a few studies have measured whole-body ionoregulatory transport characteristics of teleost fish from hatch beyond yolk absorption (e.g. Atlantic salmon, *S. salar*, McWilliams and Shephard, 1989; rainbow trout, *O. mykiss*, Brauner and Wood, 2002a; Brauner and Wood, 2002b; Fu et al., 2010a; tilapia, *O. mossambicus*, Hwang et al., 1994; zebrafish, *Danio rerio*, Kwong et al., 2013; Shih et al., 2012). Chapter 3 will address this gap by characterizing the Na⁺ transport characteristics of developing pink salmon and comparing their response with that of non-anadromous rainbow trout. Hopefully this will allow us to tease apart ionoregulatory processes
associated with development from those that may be associated with early ocean entry and
smoltification in pink salmon.

**Rainbow trout, *O. mykiss***

Rainbow trout, a member of the family Salmonidae, has been selected as the model
species to achieve the aforementioned thesis objectives because the ionoregulatory
physiology of adult rainbow trout is relatively well understood and the methodologies for
measuring Na\(^+\) uptake and whole-body Na\(^+\) are developed and reliable (Brauner and Wood,
2002a; Brauner and Wood, 2002b; Brauner et al., 2003; Fu et al., 2010). For the purpose of
Chapter 3, rainbow trout, a non-anadromous salmonid belonging to the same genus as
pink salmon, will be used as a model with which the early-migrating anadromous pink
salmon will be compared. The goal is to collect data which will allow us to begin to
distinguish between the physiological processes implicated in seawater preparation or
salmonid development.

1.4 **RESEARCH OBJECTIVES**

**Chapter 2: Effect of environmental [Na\(^+\)] on Na\(^+\) uptake and balance in rainbow trout
(*O. mykiss*) larvae**

The major goal of Chapter 2 was to investigate how developing rainbow trout
modulate Na\(^+\) transport characteristics while ionoregulation is transitioning from a
cutaneous-dominated to a gill-dominated process. This was divided into two research
objectives: (1) Characterize the ontogeny of Na\(^+\) balance and transport capacity in larval
rainbow trout through to yolk absorption and (2) characterize the degree to which
environmental [Na\textsuperscript{+}] influences Na\textsuperscript{+} balance and transport capacity in larval rainbow trout through to yolk absorption. These research objectives were driven by the hypothesis that early in development (when ionoregulation is cutaneous-dominated) ionoregulatory plasticity will be limited while later in development (when ionoregulation is gill-dominated) fish will exhibit greater ionoregulatory plasticity, as seen in juvenile/adult fish.

**Chapter 3: Ion balance during early life stages in salmonids: Insight into a unique pattern of smoltification in pink salmon (O. gorbuscha)**

The aim of Chapter 3 was to investigate how pink salmon (O. gorbuscha) modulate Na\textsuperscript{+} transport characteristics in freshwater during preparation for seawater. The first goal was to characterize the ontogeny of Na\textsuperscript{+} balance in pink salmon and determine how the dramatic increase in whole-body [Na\textsuperscript{+}] is achieved during smoltification (i.e. upregulation of influx or reduction in efflux). The second goal was to determine if the ontogeny of Na\textsuperscript{+} balance in pink salmon was different than that of rainbow trout. To do this, I characterized the ontogeny of Na\textsuperscript{+} balance in the closely related, non-anadromous salmonid, the rainbow trout (O. mykiss), to compare with pink salmon. Parallel characterization of the ontogeny of Na\textsuperscript{+} balance in these two species reared under similar conditions was chosen to address the hypothesis that the early-migrating anadromous salmonid species (pink salmon) would display a different ontogenetic pattern of Na\textsuperscript{+} balance than a non-anadromous salmonid species which is not seawater-tolerant during early development (rainbow trout). In particular, I hypothesized that pink salmon would exhibit a greater increase in whole-body Na\textsuperscript{+} at the time of yolk absorption than rainbow trout.
1.5 FIGURES

Figure 1-1: The current model for NaCl uptake across the gill epithelium in freshwater rainbow trout adapted from Hwang et al. (2011; Figure 1-1) and Dymowska et al. (2012; figure 1C). For details, refer to the text, section 1.2. Peanut lectin agglutinin-positive (PNA+) and PNA-negative (PNA-) cells; CBE, Na+/HCO₃⁻ exchanger; CIC, Cl⁻ channel; HA, H⁺-ATPase; NaC, Na⁺ channel; NBC, Na⁺-HCO₃⁻ cotransporter; NHE, Na⁺/H⁺ exchanger; NKA, Na⁺-K⁺-ATPase; Rhcg2, Rhesus protein family, C glycoprotein 2. Transporters shown in dark gray are putative (i.e. no mRNA or protein localization data).
Figure 1-2: The current model for NaCl secretion across the gill epithelium in seawater teleost fish adapted from Hwang et al. (2011). For details, refer to the text, section 1.2. MR cell, mitochondrion-rich cells; AS cell, accessory cell; CFTR, cystic fibrosis transmembrane conductance regulator; NHE, Na⁺/H⁺ exchanger; eKir, inwardly rectifying K channel; NKCC, Na⁺, K⁺, Cl⁻-cotransporter; NKA, Na⁺-K⁺-ATPase.
Chapter 2 Effect of environmental [Na⁺] on Na⁺ uptake and balance in rainbow trout (Oncorhynchus mykiss) larvae

2.1 INTRODUCTION

Teleost fishes must maintain ionic gradients essential to normal cellular function. Maintaining ionic homeostasis in freshwater environments is challenging because fish face salt loss across their semi-permeable body surfaces, including the gills and skin. Therefore, freshwater fish must compensate via active ion uptake at the gill against a large concentration gradient (reviewed by Evans et al., 1999; Evans et al., 2005; Hwang et al., 2011). Since sodium (Na⁺) is the major cation in the extracellular body fluid, the regulation of Na⁺ balance is critical for ionoregulation and will be the focus of this chapter.

Ionocytes on the gills are the major site of active Na⁺ uptake in freshwater teleost fish (reviewed by Evans et al., 1999; Evans et al., 2005; Hwang et al., 2011). The current model for Na⁺ uptake in freshwater rainbow trout (reviewed by Hwang et al., 2011) occurs at two distinct cell types: peanut lectin agglutinin-positive (PNA⁺) and PNA-negative (PNA⁻) cells (Galvez et al., 2002). The PNA⁺ cells contain a putative H⁺-ATPase in the basolateral membrane which creates a favorable proton gradient to drive Na⁺ uptake through electroneutral Na⁺/H⁺ exchangers (NHE2/3) in the apical membrane. The PNA⁻ cells contain an H⁺-ATPase in the apical membrane (Lin and Randall, 1991) electrically linked to _______________________

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putative Na$^+$ channels (NaC) in the apical membrane. In both cell types, NKA in the basolateral membrane pumps Na$^+$ against its concentration gradient from the ionocyte into the extracellular fluids (Avella and Bornancin, 1989; Piermarini and Evans, 2000; Wilson et al., 2000a) along with a putative electrogenic Na$^+$-HCO$_3^-$ cotransporter (NBC) in the basolateral membrane of the PNA$^-$ cells. The ability of this suite of transporters to actively uptake Na$^+$ from the aquatic environment is determined by a number of factors, including the number of transporters available, the affinity of the Na$^+$ binding sites on the active ion transporters (i.e. not the case for ion channels; e.g. NaC contains no Na$^+$ binding site), and the [Na$^+$] in the external freshwater environment.

Early in development, the gill is not yet fully developed, and as a result ion exchange processes occur largely across the skin and yolk-sac epithelium (Hwang, 1989; Shelbourne, 1957). Following hatch, the number of ionocytes in the integument increases until reaching the juvenile stage; however, the relative contribution of these extrabranchial ionocytes in ionoregulation gradually decreases (Varsamos et al., 2002). As the gills develop, there is a shift in ionocyte distribution from the skin to the gills that corresponds with yolk absorption (Gonzalez et al., 1996; Hiroi et al., 1998; Hwang, 1990b; Li et al., 1995; Pisam et al., 2000; Varsamos et al., 2002) and ion exchange processes transition to the gills (Fu et al., 2010). In rainbow trout, developing ionocytes were observed on the gill approximately –3 dph (stage 24, Gonzalez et al., 1996; stage 23, Rombough, 1999b) although they are not described as mature until the tubular and vesiculotubular systems are well developed, which corresponds to the time of yolk absorption (Gonzalez et al., 1996). Mature branchial ionocytes have been observed at 3 days post-hatch (dph) in the gills of larval tilapia (Li et
al., 1995). Fu et al. (2010) demonstrated that by 0 dph the gills account for approximately 10 per cent of total whole-body Na⁺ uptake in rainbow trout and the majority of Na⁺ uptake has shifted from cutaneous epithelia to the gills by just 15 dph in these fish, about 10 days before the transition of oxygen uptake to the gills (Fu et al., 2010). These data indicate that the gills play a major role in ionoregulation very early in development and it has been proposed that ionoregulation, rather than gas exchange, may represent the initial driving force for larval gill development (Brauner and Rombough, 2012; Fu et al., 2010; Rombough, 1999; Rombough, 2002; Rombough, 2007).

The ability of freshwater juvenile and adult teleost fish to maintain their plasma Na⁺ levels within a narrow range across diverse Na⁺ freshwater environments has been well-documented (e.g. zebrafish, Danio rerio, Boisen et al., 2003; coho salmon, Oncorhynchus kisutch, Miles and Smith, 1968; rainbow trout, Oncorhynchus mykiss, Morgan and Iwama, 1991; tilapia, Tilapia mossambica, Potts et al., 1967). Moreover, in adult fish, exposure to ion-poor environments is associated with remodelling of the ionoregulatory structures at the gill to compensate for low ion concentrations (Greco et al., 1996; Laurent et al., 1985). For example, adult rainbow trout acclimated to ion-poor water experience rapid proliferation in the number, size and density of ionocytes in the gill, presumably to increase ion uptake capacity to the level required to balance the increased passive ion loss fish experience in ion-poor waters (Greco et al., 1996). Indeed, juvenile (2.5 g) and adult (150 – 400 g) rainbow trout acclimated to soft water take up ions at a faster rate than fish acclimated to hard water (Matsuo et al., 2004; McDonald and Rogano, 1986). This is reflected by a 140 per cent increase in maximal transport velocity ($J_{max}$) for Na⁺ and a 20
per cent decrease in the Michaelis constant ($K_m$; measured inverse of $\text{Na}^+$ uptake affinity) in soft water-acclimated fish compared to their hard water counterparts (Matsuo et al., 2004).

Embryos and larvae may also inhabit freshwater environments with a wide range of external $\text{Na}^+$ concentrations. However, remarkably little is known about $\text{Na}^+$ balance and transport capacity of teleost fish during early life stages. To date, several research groups have focused on osmoregulation in post-embryonic euryhaline fishes using variations in salinity tolerance as an indicator of capacity to osmoregulate (e.g. Gallagher et al., 2013; Hwang, 1990b; McWilliams and Shephard, 1989; Shen and Leatherland, 1978a; Weisbart, 1968; reviewed in Holliday, 1969; Varsamos et al., 2005). McWilliams and Shephard (1989) indicate that $J_{\text{max}}$ for $\text{Na}^+$ is higher in developing Atlantic salmon reared in ion-poor water relative to those reared at 0.5‰ salinity, suggesting that developing salmonids may increase ion uptake capacity to balance increased passive ion loss in the same way as juvenile and adult fish. However, their measurements were limited to just 2, 6 and 12 weeks post-hatch. Shen and Leatherland (1978a) demonstrated that rainbow trout reared in different ambient salinities (0, 11 and 13‰ sea water) maintain constant body $\text{Na}^+$ content during the larval and alevin stages, suggesting that these fish are able to regulate their internal ionic environment independent of the external environment. Interestingly, by 14 dph these fish do not exhibit any differences in number or appearance of ionocytes in response to these different ambient salinities (Shen and Leatherland, 1978b). Clearly the available data is limited and very few studies have investigated the effects of water ionic composition on ionoregulatory function within a freshwater context (e.g. effect of $[\text{Ca}^+]$, Genz et al., 2014; Hwang et al., 1996; hard versus soft water, Fu et al., 2010; effect of toxic
metals, Brauner and Wood, 2002a; Brauner and Wood, 2002b; Gillis and Wood, 2008; Rombough and Gaside, 1984). Fu et al. (2010) reported that there are no differences in gill or whole-body NKA α-subunit protein expression or activity level in developing rainbow trout (0 – 18 dph) reared in soft water compared to those reared in hard water. This group also showed that ionoregulation transitions from a cutaneous-dominated to a gill-dominated process at the same time in both soft and hard water reared rainbow trout (Fu et al., 2010), indicating very little plasticity in relation to early ionoregulatory development. Nonetheless, the effect of the environmental [Na\(^+\)] on the capacity for ionoregulatory function during early life stages has not been measured, particularly during the critical transition from cutaneous to branchial ionoregulation.

**Research objectives**

The purpose of the present study was to investigate how developing rainbow trout modulate Na\(^+\) transport characteristics while ionoregulation is transitioning from a cutaneous- (prior to 15 dph at 10\(^\circ\)C) to a gill-dominated (following 15 dph at 10\(^\circ\)C) process. This was divided into two research objectives: (1) Characterize the ontogeny of Na\(^+\) balance and transport capacity in larval rainbow trout through to yolk absorption and (2) characterize the degree to which environmental [Na\(^+\)] influences Na\(^+\) balance and transport capacity in larval rainbow trout through to yolk absorption. These research objectives were driven by the hypothesis that early in development (when ionoregulation is cutaneous-dominated) ionoregulatory plasticity will be limited while later in development (when
ionoregulation is gill-dominated) fish will exhibit greater ionoregulatory plasticity, as seen in juvenile/adult fish.

To characterize the ontogeny of Na\(^+\) balance and transport capacity I have combined measurements of whole-body Na\(^+\) levels with measurements of Na\(^+\) influx \(J_{in}^{Na}\) from the external medium, including estimations of Na\(^+\) uptake kinetic parameters, \(J_{max}\) and \(K_m\). Additionally, tissue-specific expression of Na\(^+\) transport proteins were determined. These measurements began shortly following hatch and continued through to the end of yolk absorption in developing rainbow trout.

### 2.2 MATERIALS AND METHODS

**Animals and rearing**

Rainbow trout, *O. mykiss*, were obtained as embryos from the Vancouver Island Trout Hatchery (Duncan, British Columbia). The embryos had reached 239 accumulated thermal units (ATU; the product of days following fertilization and temperature in °C) before transfer to the University of British Columbia (UBC; Vancouver, Canada) on May 22, 2012. Upon arrival at UBC the embryos were randomly assigned to one of three groups (in triplicate) for rearing: low-, medium- or high-[Na\(^+\)] freshwater. The low-[Na\(^+\)] treatment consisted of Vancouver City dechlorinated tap water (annual average values in mM: Na\(^+\), 0.08; Cl\(^-\), 0.05; Ca\(^{2+}\), 0.03; Mg\(^{2+}\), 0.006; K\(^+\), 0.004; alkalinity, 3.0 mg L\(^{-1}\) as CaCO\(_3\); hardness 3.43 mg L\(^{-1}\) as CaCO\(_3\); pH 6.4 – 6.8; Metro Vancouver, 2012). The medium- and high-[Na\(^+\)] treatments were generated by adding sodium chloride (NaCl; Sigma–Aldrich, USA) to dechlorinated Vancouver City tap water to achieve nominal levels of 450 µM and 1700 µM,
respectively (measured [NaCl] values listed in Table 2-1). Approximately 50 per cent of the embryos had hatched by May 31, 2012 and this was defined as 0 dph. Completion of yolk absorption and subsequent first feeding took place on July 4, 2012. Fish were fed crumbled starter feed (size # 0; Skretting, USA) once or twice daily ad libitum, with the exception of 18 h prior to sampling, at which time feeding was withheld. Embryos and larval fish were held in egg trays submerged in 22 L tanks supplied with 20 L of recirculating low-, medium- or high-[Na\(^+\)] water (each in triplicate) kept in an environmental chamber at 12°C (actual = 11.9 ± 0.1°C) on a 12L:12D photoperiod. Partial water changes (60 – 80% of the total volume) were performed three times per week using the experimental water prepared at least 48 hours in advance to ensure appropriate [NaCl], temperature and oxygen content. Temperature, pH, oxygen saturation, nitrate, nitrite and ammonia levels were monitored and recorded regularly. All procedures involving animals adhered to the UBC's Animal Care Certificate A11-0235.

**Sampling protocol**

Na\(^+\) uptake kinetics were determined on larvae 8, 11, 15, 21, 26 and 32 dph (n=9). Unidirectional Na\(^+\) uptake rates were measured in larvae 9, 12, 16, 22, 27, 33, 46 and 67 dph (n=7). At these times (with the exception of 46 and 67 dph) additional larvae from each treatment were collected, euthanized in 1000 mg L\(^{-1}\) tricaine methanesulfonate (MS-222; Syndel Laboratories, Canada) and (a) immediately frozen in liquid nitrogen and stored at -80°C for measurement of whole-body Na\(^+\) levels (n=6), water content (n=6) and Na\(^+\) transport protein expression (n=6) or (b) fixed in 10% neutral buffered formalin for 24 h at
4°C then transferred to 70% ethanol at 4°C for immunohistochemical analysis (n=6). The latter were shipped to the Ecophysiology Lab at the Interdisciplinary Centre of Marine and Environmental Research (CIIMAR) at the University of Porto in Porto, Portugal prior to analysis for measurement of gill and skin NKA, NHE and V-ATPase protein concentration.

**Wet mass, whole-body Na\(^+\) content and whole-body water content**

Fish were euthanized with MS-222, rinsed with de-ionized water, blotted dry, weighed and wet mass was recorded. Fish were dried in pre-weighed 15 mL polystyrene tubes at 65°C for approximately 4 days, or until no further reduction in mass was observed, at which point dry mass was recorded. Dried fish were digested in 1.5 mL of 1 M nitric acid at 65°C for approximately one week, during which time the tapered end of a metal spatula was also used for mechanical digestion. The supernatant of the digested fish tissue was analyzed for [Na\(^+\)] using flame absorption spectroscopy (Spectra AA-240FS; Varian, Australia) and standardized to both wet and dry mass for total whole-body [Na\(^+\)]. Whole-body water content was calculated as the difference between wet and dry mass and reported as a per cent of wet mass.

**Whole-body Na\(^+\) influx**

Whole-body Na\(^+\) influx rate ($J_{\text{in}}^{\text{Na}}$) was measured in developing larval rainbow trout in the water in which they were reared (low-, medium- or high-[Na\(^+\)] treatment water). For each experiment fish were sampled from each treatment (n=7) and placed individually into flux chambers containing water from their respective treatment group. Until 20 dph, fish were measured in 20 mL polyethylene scintillation vials containing 18.5 mL of the
experimental solution and fish sampled after 21 dph were measured in 60 mL polyethylene Nalgene bottles containing 33.5 mL of the experimental solution to accommodate their larger size and higher metabolic activity. Experimental solutions were aerated continuously throughout the flux experiment and maintained at 12°C in a chilled water bath. Fish were introduced to the chamber 30 minutes prior to the start of each flux to recover from handling. Each flux was initiated by injecting sodium-22 (\(^{22}\text{Na}^+\); as \(^{22}\text{NaCl}\); PerkinElmer, USA) into the chambers to achieve 1 – 27 μCi L\(^{-1}\) (generally decreasing as the fish became larger). At 10 minutes post-injection 250 μL and 1.5 mL water samples were taken in duplicate for measurement of water \(^{22}\text{Na}^+\) and [Na\(^+\)], respectively. Each flux lasted a total of 2 h, after which water samples for measurement of \(^{22}\text{Na}^+\) and [Na\(^+\)] were collected again as described above. The larvae were then immediately removed from the experimental chambers, euthanized with a lethal dose of MS-222, rinsed three times with 5 mM NaCl to displace any surface-bound \(^{22}\text{Na}^+\) and rinsed once with de-ionized water to remove NaCl. Larvae were then blotted dry, weighed and placed individually into vials for measurement of \(^{22}\text{Na}^+\).

**Whole-body Na\(^+\) uptake kinetics**

Na\(^+\) uptake kinetics were characterized in developing larval rainbow trout reared in low-, medium- and high-[Na\(^+\)] treatments. In order to determine \(K_m\) and \(J_{max}\) for each Na\(^+\) treatment at each age, Na\(^+\) uptake rates were measured as previously described in seven different external NaCl concentrations ([NaCl]\(_{ext}\)), where the specific concentrations were adjusted to account for known developmental differences in uptake rates (i.e. early
developmental stages have lower uptake rates and therefore higher $[\text{NaCl}]_{\text{ext}}$ were used; see Table 2-2). Dechlorinated Vancouver City tap water represented the lowest $[\text{NaCl}]_{\text{ext}}$ (approximately 80 µM NaCl) while the higher $[\text{NaCl}]_{\text{ext}}$ were created by adding NaCl (Sigma–Aldrich, USA) to dechlorinated Vancouver City tap water (nominal values of 80, 160, 280, 480, 840, 1600, 2400, 3000, 4500, and 9000 µM NaCl; see Table 2-2 for measured values). Nine larvae were placed into a flux chamber containing one of the experimental solutions and each of the nine fish yielded one uptake rate measurement at one $[\text{NaCl}]_{\text{ext}}$ (n=9). Until 20 dph fish were exposed to 18.5 mL of the experimental solution and fish sampled after 20 dph were exposed to 33.5 mL as described above. Each flux was initiated by adding 2 – 60 µCi L$^{-1}$ $^{22}\text{Na}^+}$ (generally increasing with $[\text{NaCl}]_{\text{ext}}$ and decreasing as the fish became larger) and lasted for a total of 3 h. Water sampling and post-flux procedures were the same as those described in the previous section.

**Tissue-specific $\text{Na}^+$ transport protein expression**

Six larvae from either low- or high-$[\text{Na}^+]$ treatments, ages 9, 12, 16, 22 and 27 dph were euthanized with a lethal dose of MS-222 (1000 mg L$^{-1}$), rinsed with distilled water and immediately frozen intact in liquid nitrogen and stored at -80°C. Larvae were thawed in SEI buffer (150 mM sucrose, 10 mM ethylenediaminetetraacetic acid, 50 mM imidazole, pH 7.3) and the gills and skin were dissected and refrozen separately in 100 µL SEI buffer at -80°C. Tissues were homogenized by sonication on ice (40% intensity, two 2-second pulses; Sonics & Materials Ltd), then centrifuged at 10 000 g at 4°C for 5 min. The supernatant was decanted and serially diluted tenfold in a three step series in 50 mM
imidazole buffer (IB, pH 7.5) and dot blotted using a 96 well vacuum manifold (Convertible™, Life Technologies) onto PVDF membranes (HybondP, GE HealthCare). Wells were rinsed three times with distilled H$_2$O, dried at 37°C and stored at room temperature.

Membranes were rehydrated, and transfer checked by Ponceau-S staining and photographed. Membranes were then blocked with 5% skimmed milk powder in TTBS (0.05% Tween 20, 20 mM Tris-HCl, 500 mM NaCl, pH 7.5) for 4h and probed with a rabbit anti- NKA α-subunit peptide affinity purified polyclonal antibody diluted 1:5000 (Ura et al., 1996; Wilson et al., 2007) in blocking buffer overnight at room temperature on an orbital shaker. Membranes were washed three times with TTBS and incubated with a goat anti-rabbit horse radish peroxidise conjugated secondary antibody diluted 1:50000 (Invitrogen) in TTBS for 1 h at room temperature on an orbital shaker. Membranes were rinsed three more times with TTBS and incubated with ECL solution (Immobilon, Millipore) for 5 min. The signal was detected with a FujiFilm LAS 4000mini imager. Membranes were stripped with 25 mM Glycine-HCl, 1% SDS, pH 2 for 30 min at room temperature and reprobed with rabbit anti- V-ATPaseB subunit peptide affinity purified antibody (gill samples only; Wilson et al., 2007) and rabbit anti- NHE3 peptide affinity purified antibody (Hiroi and McCormick, 2012) diluted 1:5000 in blocking buffer and processed as described above. Spot intensity from NKAα, V-ATPaseB and NHE3b dotblots were measured using MultiGage image analysis software (version 3.1 FujiFilm). Results are presented as chemiluminescence intensity relative to the low-[Na+] treatment group at 9 dph.
Analytical techniques and calculations

Water samples were measured for \([\text{Na}^+]\) using flame atomic absorption spectroscopy (Spectra AA240FS, Varian, Australia). Water samples and fish samples were measured for radioactivity in counts per minute (cpm) using a gamma counter (Wallac 1470 Wizard, PerkinElmer, Finland).

\(j_{\text{in}}^{\text{Na}}\), expressed in \(\mu\text{mol Na}^+ \text{ h}^{-1} \text{ g}^{-1}\) was calculated as described by Lauren and McDonald (1987) using the following equations:

\[
j_{\text{in}}^{\text{Na}} = \frac{a}{(\text{SA} \cdot t \cdot \text{wt})}
\]

where \(a\) is the whole-body activity of \(^{22}\text{Na}^+\) in cpm, \(t\) is the duration of the flux in hours, \(\text{wt}\) is the wet tissue mass of the larvae in grams and \(\text{SA}\) refers to the average specific activity of the \(^{22}\text{Na}^+\) in the water in cpm \(\mu\text{mol}^{-1}\), calculated as:

\[
\text{SA} = \frac{\left[\left(\frac{\text{cpm}_i}{[\text{ion}_i]}\right) + \left(\frac{\text{cpm}_f}{[\text{ion}_f]}\right)\right]}{2}
\]

where \(i\) refers to the initial water samples at 10 minutes post-injection of \(^{22}\text{Na}^+\) and \(f\) refers to the final water samples taken at the end of the flux period (2 h for influx measurements and 3 h for kinetic measurements).

In order to characterize the \(\text{Na}^+\) uptake kinetics, \(K_m\) and \(J_{\text{max}}\) were estimated using SigmaPlot12’s Nonlinear Regression function (Systat Software Inc., USA) to fit curves to the uptake kinetic data by substitution into the Ligand Binding, one site saturation equation, otherwise known as the Michaelis-Menten equation:

\[
j_{\text{in}}^{\text{Na}} = \frac{J_{\text{max}} \cdot [\text{Na}^+]}{K_m + [\text{Na}^+]} \]
where $J_{\text{in}}^{\text{Na}}$ is the Na$^+$ uptake rate at a particular [NaCl]$_{\text{ext}}$, $J_{\text{max}}$ is the maximal uptake rate and $K_m$ is the substrate concentration at which the uptake rate is equal to half of $J_{\text{max}}$ and represents the inverse of affinity. Mean $K_m$ and $J_{\text{max}}$ were determined for rainbow trout reared in the low-, medium- and high-[Na$^+$] treatments at each age.

**Statistical analysis**

Data are expressed as means ± s.e.m. unless otherwise specified. Two-way analysis of variance (ANOVA) was used to compare means. In general, the Holm-Sidak post-hoc test was subsequently applied for pairwise comparisons when effects were found to be significant with the exception of gill NKA protein levels, in which case the Student-Newman-Keuls method was applied. All data passed tests for homogeneity of variance and normality with the exception of the unidirectional Na$^+$ uptake rates. Due to the robustness of ANOVAs (Schmider et al., 2010), the two-way ANOVA was reasoned to be appropriate for this data set as well. All statistical analyses were conducted with SigmaPlot12 unless otherwise specified and a significance level of $P<0.05$ was used throughout.

Kinetic data were observed to fit the Michaelis–Menten function. Estimates of $K_m$ and $J_{\text{max}}$ were determined in GraphPad Prism v5.0 (GraphPad Software Inc., 2007) and SigmaPlot12 and presented as Estimate ± Standard Error of the Estimate, as determined by SigmaPlot12’s Nonlinear Regression function for to the Michaelis-Menten equation. Differences in $K_m$ and $J_{\text{max}}$ estimates between treatments within in each age group were tested using an extra sum-of-squares F-test (Zar, 2010; e.g. Brix and Grosell, 2012). Significant differences in $K_m$ and $J_{\text{max}}$ estimates between age groups within in each
treatment were determined as Estimate ± 2(Standard Error of the Estimate) that did not
overlap.

2.3 RESULTS

To simplify the general trends and emphasize the change in ionoregulatory
machinery that these fish exhibited during development, I have grouped fish into two
categories: (1) those that consist of primarily cutaneous-dominated ionoregulatory
processes (prior to 15 dph) and (2) those that consist of primarily gill-dominated
ionoregulatory processes (following 15 dph).

Growth, development and hydromineral balance

Developmental stage and CO2 level significantly affected P50 (three-way ANOVA,
developmental stage $F_{4,33}=142.01, P<0.001$; CO2 level $F_{2,22}=45.89, P<0.001$; Table 2).
There was a twofold increase in P50 between Stages 27 and 35 ($P<0.001$). Changes in PCO2
from 0.2 to 0.4 kPa significantly increased P50 at Stages 33 and 35 ($P<0.001$), while
changes in PCO2 from 0.2 to 1.2 kPa significantly increased P50 at all developmental
stages ($P<0.001$). P50 was unaffected by hypoxia treatment ($P=0.1$; Table 2).

Wet and dry mass

The effect of [Na+] treatment on wet mass was not statistically significant; however,
there was a statistically significant effect of age on wet mass which increased significantly
through development (two-way ANOVA, age $P<0.001$; [Na+] treatment $P=0.473$; interaction
$P=0.851$; Table 2-3). Similarly, the effect of [Na+] treatment on dry mass was not
statistically significant; however, dry mass decreased significantly through development
(two-way ANOVA, age P<0.001, P=0.653, interaction P=0731; Figure 2-1). The reduction in dry mass through development was matched by a significant gain in per cent whole-body water content, although there were no differences among treatments (two-way ANOVA, age P<0.001, [Na⁺] treatment P=0.113, interaction P=0.749; Figure 2-2).

**Whole-body Na⁺ levels**

Whole-body Na⁺ levels expressed relative to dry mass increased approximately 2-fold from 9 to 32 dph in all treatments and there was a significant [Na⁺] treatment effect, where the low-[Na⁺] treatment was significantly lower than the medium- and high-[Na⁺] treatments (two-way ANOVA, age P<0.001, [Na⁺] treatment P=0.016, interaction P=0.426; Figure 2-3).

**Whole-body Na⁺ influx**

There was a strong effect of age and [Na⁺] treatment on $J_{in}^{Na}$ (two-way ANOVA, age P<0.001, [Na⁺] treatment P<0.001, interaction P=0.018; Figure 2-4). In general, $J_{in}^{Na}$ was highest between 9 and 33 dph and much lower at 46 and 67 dph. Specifically, until 15 dph, when ionoregulation is largely occurring across the skin, $J_{in}^{Na}$ increased. Beyond 15 dph, $J_{in}^{Na}$ continued to increase until the end of yolk-absorption (26 – 32 dph). On average, there was a ~50 – 100 per cent increase in $J_{in}^{Na}$ by the end of yolk-absorption. After yolk absorption was complete, $J_{in}^{Na}$ decreased dramatically by approximately 60 – 80 per cent by 46 and 67 dph. In addition to the strong effect of age on $J_{in}^{Na}$, differences between fish reared in low-, medium- and high-[Na⁺] were observed following 12 dph where $J_{in}^{Na}$ generally increased with rearing [Na⁺]. At 15 dph, the differences between the groups were greatest; the high-
[Na\(^+\)] reared fish exhibited $J_{in}^{Na}$ values 2.5-fold higher than the low-[Na\(^+\)] reared fish. Beyond 15 dph until the end of yolk-absorption (26 – 32 dph), $J_{in}^{Na}$ values remained higher in fish reared in higher [Na\(^+\)] environments but after yolk absorption, there was no effect of rearing [Na\(^+\)] on Na\(^+\) uptake rate.

**Whole-body Na\(^+\) uptake kinetics**

In general, Na\(^+\) uptake rates for rainbow trout reared in low-, medium- and high-[Na\(^+\)] treatments increased with increasing external Na\(^+\) concentrations at different developmental stages and followed a hyperbolic curve that approximated Michaelis–Menten saturation kinetics from which $J_{max}$ and $K_m$ could be estimated (Figure 2-5).

**Changes through development**

In general, fish exhibited a progressive increase in $J_{max}$ until day 26 which then decreased significantly by 3-fold in all [Na\(^+\)] treatments by 32 dph (Table 2-4; Figure 2-7). While the former was most evident in fish reared in the low-[Na\(^+\)] group, all treatments exhibited at least a 1.5-fold increase in $J_{max}$ from 11 to 26 dph.

In general, changes in $K_m$ with development were more variable; however, in the low- and medium-[Na\(^+\)] treatments, $K_m$ was highest at 15 dph and progressively fell and was significantly different at 32 dph (Table 2-4, Figure 2-8). In the high-[Na\(^+\)] treatment, $K_m$ was highly variable with the only statistically significant difference noted between 15 and 26 dph Table 2-4, Figure 2-8).
Effect of rearing [Na⁺]

Differences in Na⁺ uptake kinetics were age-specific (Table 2-4, Figure 2-7, Figure 2-8). Until 15 dph, when ionoregulation is largely occurring across the skin, there were no significant differences in \( J_{\text{max}} \) or \( K_m \) for Na⁺ between fish reared in different [Na⁺] treatments, with the exception of the high-[Na⁺] group which displayed a higher \( K_m \) at 11 dph and lower \( J_{\text{max}} \) at 15 dph relative to the low- and medium-[Na⁺] groups. After 15 dph, when ionoregulation becomes gill-dominated, there are many differences in Na⁺ uptake kinetics between fish reared in low-, medium- or high-[Na⁺] treatments. In general, it appears that the peak in \( J_{\text{max}} \) that was observed in the low- and medium-[Na⁺] fish occurred later in the high-[Na⁺] treatment (Figure 2-7). Shortly after the gills become the dominant site of Na⁺ uptake, at 20 dph, \( J_{\text{max}} \) was lowest in the high-[Na⁺] treatment. By 26 and 32 dph, \( J_{\text{max}} \) in the high-[Na⁺] group was the highest value. Changes in \( K_m \) appear to follow a similar pattern where the peak in \( K_m \) was delayed in the high-[Na⁺] treatment compared to the low- and medium-[Na⁺] fish. \( K_m \) in the high-[Na⁺] treatment was significantly greater than that in the low- and medium-[Na⁺] treatments at 20, 26 and 32 dph (with the exception of 20 dph where the values for the medium- and high-[Na⁺] groups did not differ). Interestingly, \( K_m \) was 3 – 5 times higher in rainbow trout reared in the high-[Na⁺] treatment relative to the low- and medium-[Na⁺] fish at 26 and 32 dph.

Tissue-specific Na⁺ transport protein expression

In general, NKA \( \alpha \)-subunit, NHE3 and V-H⁺-ATPaseB abundance were not statistically different between low- and high-[Na⁺] treatments within tissue type (Figure
There was a statistically significant increase in gill NKA protein with age from 9 to 27 dph (two-way ANOVA, age P=0.01, [Na+] treatment P=0.283, interaction P=0.343) but no statistically significant differences in skin NKA, gill and skin NHE3 or gill V-H+-ATPaseB were observed with age.

2.4 DISCUSSION

This is the first study to rear developing larval rainbow trout under a range of freshwater [Na+] conditions and investigate the plasticity of Na+ transport during gill development, when ionoregulation is transitioning from a cutaneous-dominated to a gill-dominated process. First I characterized the ontogeny of Na+ balance and transport capacity in larval rainbow trout following hatch through to yolk absorption. Rainbow trout exhibited very high \( J_{\text{in}}^{\text{Na}} \) early in development prior to yolk absorption, but immediately following yolk absorption rates were reduced by 60 – 80 per cent to values typical of juvenile and adult rainbow trout in low-[Na+] soft water (Goss and Wood, 1990a; Matsuo et al., 2004). Similarly, \( J_{\text{max}} \) for Na+ increased following hatch and was very high prior to yolk absorption but decreased substantially immediately following yolk absorption, while affinity for Na+ decreased (\( K_m \) increased) following hatch but increased following yolk absorption. Additionally, I investigated the plasticity of Na+ transport during gill development. Before the gills have developed, when ionoregulation is occurring across the extrabranchial epithelia, the relationship between rearing [Na+] and Na+ uptake kinetic parameters was not clear, suggesting that rearing [Na+] has little effect on Na+ uptake kinetics in rainbow trout. Once the gills become the primary site for ionoregulation, Na+
affinity increased ($K_m$ decreased) with decreasing rearing $[Na^+]$, consistent with my hypothesis. Interestingly, $J_{\text{max}}$ for $Na^+$ and $J_{\text{in}}^{Na}$ increased with rearing $[Na^+]$, in contrast with my hypothesis. These findings are discussed below.

It is important to note that $Na^+$ and $Cl^-$ were the only ions manipulated in this experimental series. All other ions and physical properties of the treatment waters were kept constant between treatments. This was done to eliminate the effect of changing $[Ca^{2+}]$ and general water hardness across the treatment waters to specifically investigate the effect of changes in water $[Na^+]$ (and $[Cl^-]$) on ionoregulatory development and plasticity. As a result, the treatment waters did not represent soft and hard water environments, but instead low-, medium- and high-$[Na^+]$ soft water.

**Characterization of $Na^+$ uptake and $Na^+$ uptake kinetics during larval development**

$Na^+$ uptake rates

In general, rainbow trout exhibited very high $J_{\text{in}}^{Na}$ early in development prior to yolk absorption. On average, there was approximately a 50 – 100 per cent increase in $J_{\text{in}}^{Na}$ by the end of yolk absorption. Immediately following yolk absorption $J_{\text{in}}^{Na}$ was reduced by 60 – 80 per cent to values of mass-specific $J_{\text{in}}^{Na}$ typical of juvenile and adult rainbow trout (Goss and Wood, 1990a; Matsuo et al., 2004). It is interesting that these fish experienced such high rates of $J_{\text{in}}^{Na}$ during yolk absorption and also that $J_{\text{in}}^{Na}$ rates decreased to adult levels so early in development. To date, few studies have examined the pattern of $J_{\text{in}}^{Na}$ during embryonic and early larval development. The results presented here were consistent with those reported by Fu et al. (2010); $J_{\text{in}}^{Na}$ increased through development from 0 – 18 dph; however,
this study is the first to characterize the pattern following hatch until the end of yolk absorption in a larval freshwater teleost fish.

The observed high and increasing Na\(^+\) uptake rates early in development (prior to yolk absorption) relative to later in development (post yolk absorption, juvenile and adult rates) may be associated with an increased demand for Na\(^+\) in developing tissues, as seen in the doubling in whole-body Na\(^+\) content in rainbow trout between 9 and 33 dph. Additionally, greater ion uptake rates may be required to balance increased diffusive ion loss that may be associated with development of the respiratory surface area at the gill (Evans et al., 2005; Sardella and Brauner, 2007). In fact, Fu et al. (2010) estimated that 75 – 85 per cent of \(J_{in}^{Na}\) was balancing Na\(^+\) efflux in larval rainbow trout.

**Na\(^+\) uptake kinetics**

This is the first study to characterize Na\(^+\) uptake kinetics in a larval freshwater teleost fish from hatch through to the end of yolk absorption and major changes were observed. I showed that rainbow trout reared in low- or medium-[Na\(^+\)] water displayed a clear developmental pattern for Na\(^+\) uptake kinetics. \(J_{max}\) for Na\(^+\) increased following hatch and was very high prior to yolk absorption but decreased substantially immediately following yolk absorption. Alternatively, Na\(^+\) uptake affinity decreased (ie. \(K_m\) increased) following hatch but increased following yolk absorption. Remarkably, by this time (32 dph) these groups displayed Na\(^+\) uptake kinetics similar to those seen in juvenile and adult rainbow trout held in freshwater (Matsuo et al., 2004; Wood, 1991).
An increase in $J_{\text{max}}$ is generally attributed to an increase in the number of transporters and the proliferation of ionocytes, for instance the number, size or density of ionocytes. The high and/or increasing $J_{\text{max}}$ values observed following hatch in the present study was likely due to the rapid proliferation of branchial ionocytes occurring during this time (Li et al., 1995; Rombough, 1999). McWilliams and Shephard (1989) showed that $J_{\text{max}}$ increases during early development in Atlantic salmon held in moderately soft water ([Na$^+$] = 0.22 mM). However, following hatch, $J_{\text{max}}$ in the Atlantic salmon is substantially lower (5 – 10-fold) than that of the rainbow trout used in this study. This may be associated with their exposure to higher Ca$^{2+}$ levels which are associated with the tightening of leaky junctions and reduced diffusive Na$^+$ loss. However, $J_{\text{max}}$ at 12 weeks post-hatch is similar to those measured at 32 dph in the present study. The mechanism underlying the decrease in $J_{\text{max}}$ at the end of yolk absorption is not well-understood. It is possible that it may be associated with changes in mass-specific metabolic rate, decreasing surface area-to-mass ratios (Rombough, 1999), and/or decreasing whole-body Na$^+$ efflux rates. The tissue-specific relative protein expression data for a variety of Na$^+$ transporters presented here do not exhibit rapid increases in expression throughout development, with the exception of a 2-fold increase in gill NKA α subunit prior to yolk absorption. However, these data do not take into account the many different isoforms of NKA that are known to function in teleost fish. Fu et al. (2010) also measured tissue-specific relative protein expression for the NKA α-subunit in rainbow trout during development from 0 – 18 dph and observed a 3-fold increase in NKA activity which is associated with a 2-fold increase in relative protein content.
Changes in the affinity of transporters (i.e. $K_m$) are generally associated with changes in transporter type. Whole-body Na$^+$ uptake affinity is a product of the affinity of all the Na$^+$ transporter types involved in Na$^+$ uptake. Until 15 dph, larval rainbow trout exhibited a pattern of decreasing Na$^+$ affinity (i.e. increase in $K_m$), followed by a gradual increase in Na$^+$ affinity until 32 dph. McWilliams and Shephard (1989) showed that Na$^+$ affinity does not vary during development up to 12 weeks after hatching in Atlantic salmon in fresh water ($K_m \sim 200 \mu M$), however, their measurements were limited to just 2, 6 and 12 weeks post-hatch. The time of complete yolk absorption (26 – 32 dph) appeared to be a time when Na$^+$ transporters and mechanisms changed the most, suggesting that the functional mechanism of ionocytes early in larval development may be different than those at the time of yolk absorption. The tissue-specific Na$^+$ transporter expression data presented here did not indicate that the types of transporters were changing through development. Relative NKA protein levels in the gill increased through development, as was expected in the developing gill, but changes in gill and skin NHE3 or gill V-ATPase were not seen between 9 and 27 dph. However, it is possible that changes in NKA protein abundance along with changes and NKA isoform abundance (not tested here) were contributing to the changing Na$^+$ affinity observed throughout development. Additionally, the change in whole-body Na$^+$ affinity during early life stages in rainbow trout may be a reflection of a changing pool of ionocytes and their affinities for Na$^+$ with development. During this time, there is a transition from ionoregulation occurring predominantly at mature ionocytes on the skin and yolk sac epithelium following hatch, to a mixture of declining mature ionocytes on the skin and yolk sac epithelium and increasing immature
and mature ionocytes on the gill mid-way through yolk absorption, to finally ionoregulation occurring at mature branchial ionocytes at yolk absorption.

The developmental pattern for Na\(^+\) uptake kinetics was less clear in the high-[Na\(^+\)] reared fish. It appeared that the increase and subsequent decrease in \(J_{\text{max}}\) and \(K_m\) observed in the low- and medium-[Na\(^+\)] treatments was somewhat delayed in the high-[Na\(^+\)] reared fish, the basis for which is unknown. It is possible that this was a result of the high [Na\(^+\)] and [Cl\(^-\)] in the treatment waters not being accompanied with increased concentrations of other ionic constituents that exist in natural high-[Na\(^+\)] waters as it has been shown that increased external [Ca\(^{2+}\)] causes a tightening of leaky junctions which may reduce passive ion loss in freshwater fish.

**Characterization of the effect of freshwater rearing [Na\(^+\)] on Na\(^+\) balance and transport capacity**

**Na\(^+\) uptake rates**

\(J_{\text{in}}^{\text{Na}}\) increased with rearing [Na\(^+\)] during larval development in rainbow trout. This is most likely due to the fact that Na\(^+\) uptake rate is dependent on water [Na\(^+\)] and the high-[Na\(^+\)] reared fish had access to increased levels of Na\(^+\) relative to the low- and medium-[Na\(^+\)] reared fish. Although no previous studies have investigated the effect of freshwater [Na\(^+\)] on \(J_{\text{in}}^{\text{Na}}\) at any life stage, previous work has been done on fish acclimated to ion-poor soft water and ion-rich hard water. It is well established that juvenile and adult fish acclimated to ion-poor soft water have a higher \(J_{\text{in}}^{\text{Na}}\) than fish acclimated to hard water (Matsuo et al., 2004; McDonald and Rogano, 1986). In juvenile and adult fish, increased \(J_{\text{in}}^{\text{Na}}\)
observed in soft water is correlated with proliferation of branchial ionocytes (Greco et al., 1996) and is thought to balance the increased passive ion loss that occurs in low external ion levels (Laurent et al., 1985). It is possible that the differences in $j_{in}^{Na}$ between studies may be due to changes in other important ions, particularly $Ca^{2+}$ and $Mg^{2+}$, between the soft and hard water treatments used in other studies, in addition to changes in NaCl content. However, Fu et al. (2010) reported that larval rainbow trout acclimated to ion-poor soft water have a higher $j_{in}^{Na}$ than fish acclimated to hard water. Perhaps the differences in Na$^+$ uptake between studies are due to differences between developing larval fish and juvenile/adult ionoregulatory systems. Future studies using the same acclimation conditions for many life stages may help to address this gap.

*Na$^+$ uptake kinetics*

During early larval development, prior to yolk absorption, the relationship between rearing [Na$^+$] and Na$^+$ uptake kinetic parameters was not clear, suggesting that rearing [Na$^+$] has little to no effect on Na$^+$ uptake kinetics in rainbow trout. Until 15 dph, when the skin is playing a large role in active ion uptake, Na$^+$ uptake kinetics appeared to be non-plastic. There were few differences between $J_{max}$ or Na$^+$ affinity of fish reared in low-, medium- or high-[Na$^+$] treatments at this time. At this developmental stage ionoregulation occurs across a mixture of declining mature ionocytes on the skin and yolk sac epithelium and increasing immature and mature ionocytes on the gill. The lack of ionoregulatory plasticity observed early in development was likely a reflection of this transition and the
fact that the proliferation of ionocytes at the gills was occurring maximally at this time, with limited capacity for upregulation.

Beyond 15 dph, when ionoregulation is gill-dominated, Na\(^+\) uptake affinity increased (i.e. \(K_m\) decreased) with decreasing rearing [Na\(^+\)] in rainbow trout and \(J_{\text{max}}\) increased. This was somewhat unexpected due to the well-documented effects of soft water acclimation (compared to hard water) on \(K_m\) and \(J_{\text{max}}\) in juvenile and adult fish which are quite different. Juvenile and adult rainbow trout acclimated to low-[Na\(^+\)] soft water exhibit a higher Na\(^+\) uptake affinity (lower \(K_m\)) compared to high-[Na\(^+\)] hard water acclimated fish (Boisen et al., 2003; Brix and Grosell, 2012; Matsuo et al., 2004; McWilliams, 1982), which is similar to what was seen in low-[Na\(^+\)] reared fish compared to the high-[Na\(^+\)] reared fish in the present study. On the other hand, juvenile and adult rainbow trout acclimated to low-[Na\(^+\)] soft water exhibit an increased Na\(^+\) uptake capacity compared to high-[Na\(^+\)] hard water acclimated fish (Greco et al., 1996; Matsuo et al., 2004), which is different than what was seen in low-[Na\(^+\)] reared fish compared to the high-[Na\(^+\)] reared fish in the present study. These differences are likely due to changes in other important ions, particularly Ca\(^{2+}\) and Mg\(^{2+}\), between the soft and hard water treatments used in other studies. Na\(^+\) uptake capacity may be affected by low [Ca\(^{2+}\)] and [Mg\(^{2+}\)] which dramatically increases diffusive loss, thereby increasing the need to compensate via increased uptake. Alternatively, Na\(^+\) uptake affinity may be less affected by these water-hardening ions. It is also possible that differences in Na\(^+\) uptake kinetics between studies were due to differences between developing larval fish and juvenile/adult ionoregulatory systems. The only other study to measure \(K_m\) and \(J_{\text{max}}\) during early development in salmonids showed
that $J_{\text{max}}$ was significantly higher in developing Atlantic salmon fry held in moderately soft water ($[\text{Na}^+] = 0.22 \text{ mM}$) relative to those in 0.5% salinity ($[\text{Na}^+] = 11.7 \text{ mM}$) at 2 and 12 weeks post-hatch and $K_m$ did not differ between treatment groups (McWilliams and Shephard, 1989). Other studies suggest that the kinetics of $\text{Na}^+$ transporters in embryos and fry of Atlantic salmon depends on the timing of exposure to low-[Na$^+$] soft water; transfer from high-[Na$^+$] hard water to low-[Na$^+$] soft water at 250 ATU (early embryonic development) results in a lower $K_m$ for Na$^+$ while a similar transfer at 400 ATU results in no change in $K_m$ when Na$^+$ uptake characteristics are measured at 550 ATU (75 ATU post hatch; McWilliams, 1993). These data emphasize the importance of considering all aspects of water quality in water, especially ionic constituents such as $[\text{Ca}^{2+}]$ and $[\text{Mg}^{2+}]$, when investigating ionoregulation in freshwater fishes and the caution that must be taken when making comparisons across studies. Future studies should focus on identifying the effects of soft versus hard waters and low-[Na$^+$] versus high-[Na$^+$] waters on Na$^+$ uptake kinetics in developing fish.

*Hydromineral balance*

Despite increased $J_{\text{in}}^{\text{Na}}$ in fish reared in high-[Na$^+$] water compared to those reared in low-[Na$^+$] water prior to yolk absorption, there was no difference in whole-body Na$^+$ or water content between the treatments, indicating that rainbow trout were able to maintain hydromineral balance across a range of freshwater [Na$^+$] environments. Apparently, the increased $J_{\text{in}}^{\text{Na}}$ observed in fish reared in high-[Na$^+$] water did not contribute to a greater amount of biologically incorporated Na$^+$ levels. Assuming that active $J_{\text{in}}^{\text{Na}}$ in developing fish
is either balancing diffusive ion loss across permeable surfaces or providing Na\(^+\) that is incorporated into growing animals, it appeared that the differences between low-, medium- and high-[Na\(^+\)] reared fish in terms of Na\(^+\) fluxes may be at the level of efflux. The specific mechanisms underlying reducing ion loss in low ion content environments are largely unknown, but may be the result of passive diffusion through paracellular pathways. In adult fish, a reduction in ion efflux appears to be an important response to defend against ion loss in ion-poor environments (McDonald and Rogano, 1986; Perry and Laurent, 1989).

It is possible that developing fish are modulating Na\(^+\) balance via regulation of efflux similar to adult fish. However, developing fish appeared to modulate uptake to a much lesser extent than adult and juvenile fish to favor Na\(^+\) balance in a Na\(^+\)-poor environment.

**The ionoregulatory hypothesis**

Until recently it was assumed that the first physiological process to transition from extrabranchial to branchial exchange during larval development was oxygen uptake (Krogh, 1941); however, recent data indicates that it may be ionoregulation (Li et al., 1995). Based on morphological (Gonzalez et al., 1996; Hiroi et al., 1998; Hwang, 1990b; Pisam et al., 2000; Varsamos et al., 2002) and functional evidence (Fu et al., 2010; Rombough, 2002) it appears that the transition of ionoregulation to the gills precedes that of gas exchange during larval development which may have implications for the ‘primary function’ of the gill during early development in fish (Brauner and Rombough, 2012; Fu et al., 2010; Rombough, 2007).
Based on the work presented here, it appears that the mechanisms of modulating Na$^+$ balance in response to the low-[Na$^+$] environments is different in larval fishes than in adult fish where acclimation of ion-poor environments induces changes in gill morphology (i.e. increased number, size and density of ionocytes in the gill), presumably to improve ion uptake capacity (Greco et al., 1996). Fu et al. (2010) measured Na$^+$ uptake across the skin and gills of developing rainbow trout following hatch and determined the age at which the gills accounted for more than 50 per cent of total uptake. The timing at which the gills contributed to more than half of Na$^+$ uptake was determined to be the same (15 dph) in fish reared in both soft and hard water, suggesting that ionoregulatory development was not plastic. This study has provided additional quantification of the plasticity of ionoregulatory development in larval fish in freshwater. Prior to 15 dph, when ionocytes are beginning to mature at the gills, larval rainbow trout exhibited little ionoregulatory plasticity. Beyond 15 dph, however, as ionoregulation shifts to mature ionocytes located on the gill, developing fish exhibited increased ionoregulatory plasticity associated with strategies observed in juvenile and adult fish exposed to ion-poor waters. This suggests that the ontogeny of active ion uptake is relatively fixed very early in development and that the first physiological function of the developing gill will continue to be some aspect of ionoregulation even when the organism is faced with an environmental stressor. Of course, this needs to be verified with more direct functional experiments.
2.5 SUMMARY

Overall, developing fish appeared to modulate Na\(^+\) balance differently than juvenile and adult fish when faced with a range of [Na\(^+\)]. This is likely due to the changing distribution of ionocytes during development. Following hatch ionoregulation occurs predominantly at mature ionocytes on the skin and yolk sac epithelium while immature ionocytes are proliferating at the gills. During this time, it appears that developing fish exhibited little plasticity in their Na\(^+\) uptake kinetics or Na\(^+\) uptake rates. As the yolk is consumed and the number of mature ionocytes on the skin and yolk sac epithelium declines and the number of immature and mature ionocytes on the gill increase, developing fish appeared to have increased plasticity in their affinity for Na\(^+\), as seen in adult fish.
2.6 TABLES

Table 2-1: [Na⁺] for each of the three rearing treatments for rainbow trout larvae.

<table>
<thead>
<tr>
<th>Treatment Name</th>
<th>Measured [Na⁺] in Rearing Tanks (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-[Na⁺]</td>
<td>60.0 ± 0.7</td>
</tr>
<tr>
<td>Medium-[Na⁺]</td>
<td>427.1 ± 3.4</td>
</tr>
<tr>
<td>High-[Na⁺]</td>
<td>1695.5 ± 13.7</td>
</tr>
</tbody>
</table>
Table 2-2: $[\text{Na}^+]$ in the experimental solutions used for kinetics experiments. Seven of the ten solutions listed were used to determine the Na$^+$ uptake kinetic parameters for rainbow trout larvae at each age; the solutions used varied with age as indicated. Values are mean ± s.e.m.

<table>
<thead>
<tr>
<th>Solution #</th>
<th>Nominal [NaCl] (µM)</th>
<th>Actual $[\text{Na}^+]$ (µM)</th>
<th>Ages Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>80</td>
<td>115 ± 11</td>
<td>8, 11, 15, 20, 26, 32 dph</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>179 ± 11</td>
<td>8 dph</td>
</tr>
<tr>
<td>3</td>
<td>280</td>
<td>250 ± 13</td>
<td>8, 32 dph</td>
</tr>
<tr>
<td>4</td>
<td>480</td>
<td>431 ± 11</td>
<td>8, 11, 15, 20, 26, 32 dph</td>
</tr>
<tr>
<td>5</td>
<td>840</td>
<td>806 ± 19</td>
<td>8, 11, 15, 20, 26, 32 dph</td>
</tr>
<tr>
<td>6</td>
<td>1600</td>
<td>1484 ± 34</td>
<td>8, 11, 15, 20, 26, 32 dph</td>
</tr>
<tr>
<td>7</td>
<td>2400</td>
<td>2339 ± 81</td>
<td>11, 15, 20, 26, 32 dph</td>
</tr>
<tr>
<td>8</td>
<td>3000</td>
<td>2943 ± 61</td>
<td>8, 11, 15, 20, 26, 32 dph</td>
</tr>
<tr>
<td>9</td>
<td>4500</td>
<td>4170 ± 75</td>
<td>11, 15, 20 dph</td>
</tr>
<tr>
<td>10</td>
<td>9000</td>
<td>9037 ± 447</td>
<td>26 dph</td>
</tr>
</tbody>
</table>
Table 2-3: Whole-body wet body mass (including yolk) of rainbow trout larvae reared in low-, medium- and high-[Na⁺] treatments from 8 to 33 days post-hatch. Letters that differ indicate statistical differences between age groups (P<0.05). There were no statistically significant differences between low-, medium- and high-[Na⁺] treatments. Data are mean ± s.e.m.

<table>
<thead>
<tr>
<th>Age (dph)</th>
<th>Low-[Na⁺] Treatment</th>
<th>Medium-[Na⁺] Treatment</th>
<th>High-[Na⁺] Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wet Mass</td>
<td>Wet Mass</td>
<td>Wet Mass</td>
</tr>
<tr>
<td>n</td>
<td>Mean</td>
<td>n</td>
<td>Mean</td>
</tr>
<tr>
<td>8</td>
<td>63 0.063 ± 0.001</td>
<td>63 0.063 ± 0.001</td>
<td>63 0.063 ± 0.001</td>
</tr>
<tr>
<td>9</td>
<td>13 0.063 ± 0.001</td>
<td>12 0.060 ± 0.003</td>
<td>13 0.062 ± 0.002</td>
</tr>
<tr>
<td>11</td>
<td>63 0.065 ± 0.001</td>
<td>63 0.066 ± 0.001</td>
<td>63 0.065 ± 0.001</td>
</tr>
<tr>
<td>12</td>
<td>42 0.063 ± 0.001</td>
<td>43 0.069 ± 0.001</td>
<td>43 0.066 ± 0.001</td>
</tr>
<tr>
<td>15</td>
<td>63 0.069 ± 0.001</td>
<td>63 0.071 ± 0.001</td>
<td>63 0.070 ± 0.001</td>
</tr>
<tr>
<td>16</td>
<td>43 0.068 ± 0.001</td>
<td>43 0.071 ± 0.001</td>
<td>43 0.072 ± 0.001</td>
</tr>
<tr>
<td>20</td>
<td>63 0.070 ± 0.001</td>
<td>62 0.069 ± 0.001</td>
<td>63 0.070 ± 0.001</td>
</tr>
<tr>
<td>21</td>
<td>43 0.071 ± 0.001</td>
<td>43 0.071 ± 0.001</td>
<td>42 0.069 ± 0.002</td>
</tr>
<tr>
<td>26</td>
<td>63 0.070 ± 0.001</td>
<td>63 0.069 ± 0.001</td>
<td>63 0.069 ± 0.001</td>
</tr>
<tr>
<td>27</td>
<td>31 0.070 ± 0.002</td>
<td>31 0.068 ± 0.001</td>
<td>31 0.069 ± 0.001</td>
</tr>
<tr>
<td>32</td>
<td>63 0.082 ± 0.002</td>
<td>63 0.076 ± 0.002</td>
<td>63 0.080 ± 0.002</td>
</tr>
<tr>
<td>33</td>
<td>31 0.080 ± 0.003</td>
<td>31 0.077 ± 0.003</td>
<td>31 0.074 ± 0.003</td>
</tr>
</tbody>
</table>
Table 2-4: Kinetic parameters for Na\(^+\) uptake in rainbow trout larvae reared in low-, medium- and high-[Na\(^+\)] treatments from 8 to 32 dph. \(J_{\text{max}}\) and \(K_m\) values are shown as Estimates ± Standard Error of the Estimate, as determined by SigmaPlot12’s Nonlinear Regression function for the Michaelis-Menten equation. Letters that differ indicate significant differences between treatments within in each age group (lower case; determined by an extra sum-of-squares F-test), or between age groups within each treatment (upper case; determined as values Estimate ± 2[Standard Error of the Estimate] that did not overlap).

<table>
<thead>
<tr>
<th>Age (dph)</th>
<th>Rearing [Na(^+)]</th>
<th>(J_{\text{max}}) (µmol g(^{-1}) h(^{-1}))</th>
<th>(K_m) (µM Na(^+))</th>
<th>(R^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Low</td>
<td>1.4 ± 0.1 (\text{A})</td>
<td>171.2 ± 54.4 (\text{A})</td>
<td>0.39</td>
</tr>
<tr>
<td>11</td>
<td>Low</td>
<td>2.0 ± 0.1 (\text{a B})</td>
<td>264.9 ± 76.5 (\text{a ABC})</td>
<td>0.50</td>
</tr>
<tr>
<td>11</td>
<td>Medium</td>
<td>2.4 ± 0.2 (\text{a A})</td>
<td>879.2 ± 213.8 (\text{a A})</td>
<td>0.69</td>
</tr>
<tr>
<td>11</td>
<td>High</td>
<td>2.6 ± 0.6 (\text{a AB})</td>
<td>3266.5 ± 1349.1 (\text{b AB})</td>
<td>0.59</td>
</tr>
<tr>
<td>15</td>
<td>Low</td>
<td>2.2 ± 0.2 (\text{a B})</td>
<td>649.3 ± 174.2 (\text{a C})</td>
<td>0.60</td>
</tr>
<tr>
<td>15</td>
<td>Medium</td>
<td>2.6 ± 0.3 (\text{a A})</td>
<td>1213.5 ± 329.1 (\text{a A})</td>
<td>0.64</td>
</tr>
<tr>
<td>15</td>
<td>High</td>
<td>1.2 ± 0.1 (\text{b A})</td>
<td>442.6 ± 179.8 (\text{a A})</td>
<td>0.22</td>
</tr>
<tr>
<td>20</td>
<td>Low</td>
<td>3.5 ± 0.2 (\text{ab C})</td>
<td>431.0 ± 76.0 (\text{a BC})</td>
<td>0.79</td>
</tr>
<tr>
<td>20</td>
<td>Medium</td>
<td>4.0 ± 0.3 (\text{a B})</td>
<td>1079.2 ± 253.6 (\text{b A})</td>
<td>0.73</td>
</tr>
<tr>
<td>20</td>
<td>High</td>
<td>3.0 ± 0.2 (\text{b B})</td>
<td>1027.9 ± 197.4 (\text{b AB})</td>
<td>0.78</td>
</tr>
<tr>
<td>26</td>
<td>Low</td>
<td>3.0 ± 0.1 (\text{a C})</td>
<td>345.1 ± 62.0 (\text{a ABC})</td>
<td>0.74</td>
</tr>
<tr>
<td>26</td>
<td>Medium</td>
<td>4.1 ± 0.2 (\text{b B})</td>
<td>604.6 ± 97.1 (\text{b A})</td>
<td>0.80</td>
</tr>
<tr>
<td>26</td>
<td>High</td>
<td>4.8 ± 0.3 (\text{c C})</td>
<td>1586.9 ± 244.7 (\text{c B})</td>
<td>0.86</td>
</tr>
<tr>
<td>32</td>
<td>Low</td>
<td>1.1 ± 0.1 (\text{a A})</td>
<td>168.4 ± 62.5 (\text{a AB})</td>
<td>0.36</td>
</tr>
<tr>
<td>32</td>
<td>Medium</td>
<td>0.9 ± 0.1 (\text{b C})</td>
<td>102.7 ± 57.2 (\text{b B})</td>
<td>0.16</td>
</tr>
<tr>
<td>32</td>
<td>High</td>
<td>1.6 ± 0.3 (\text{c A})</td>
<td>1296.9 ± 515.5 (\text{c AB})</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Figure 2-1: Dry mass for developing rainbow trout (including yolk) reared in low- (open circles), medium- (grey-filled circles) and high-[Na⁺] treatments (black-filled circles) from 9 to 33 dph. Data are means ± s.e.m; n = 6. Letters that differ indicate statistically significant differences (P<0.05) between ages with treatments combined. No statistically significant effect of treatment was observed.
Figure 2-2: Per cent body water content for developing rainbow trout (including yolk) reared in low- (open circles), medium- (grey-filled circles) and high-[Na⁺] treatments (black-filled circles) from 9 to 33 dph. Data are means ± s.e.m; n = 6. Letters that differ indicate statistically significant differences between ages with treatments combined (P<0.05). No statistically significant effect of treatment was observed.
Figure 2-3: Whole-body Na⁺ content for developing rainbow trout reared in low- (open circles), medium- (grey-filled circles) and high-[Na⁺] treatments (black-filled circles) from 9 to 33 dph. Data are means ± s.e.m; n = 6. Letters that differ indicate statistically significant differences between ages with treatments combined (lower case; P<0.05) or between treatments at that respective age (upper case; P<0.05). There is a statistically significant effect of [Na⁺] treatment.
Figure 2-4: Unidirectional Na\(^+\) uptake rates ($J_{in}^{Na}$) for developing rainbow trout reared in low- (open circles), medium- (grey-filled circles) and high-[Na\(^+\)] treatments (black-filled circles) from 9 to 33 dph, measured in the water in which they were reared. Data are means ± s.e.m; n = 6. Letters that differ indicate statistically significant differences between ages with treatments combined (lower case; P<0.05) or between treatments at that respective age (upper case; P<0.05).
Figure 2-5: Na⁺ uptake rates ($J_{in}^{Na⁺}$) as a function of external Na⁺ concentrations (µM) for rainbow trout larvae reared in low- (open circles with dotted regression line), medium- (grey-filled circles with dashed regression line), or high-[Na⁺] treatments (black-filled circles with solid regression line) from 8 to 32 dph. (A) 8 days post-hatch (dph), (B) 11 dph, (C) 15 dph, (D) 20 dph, (E) 26 dph and (F) 32 dph. Data are means ± s.e.m; n = 7 – 9. See Table 2-4 for estimates of $K_m$ and $J_{max}$ for each age and treatment.
**Figure 2-6:** Tissue specific relative protein content of Na\(^+\) transporter proteins in the gill and skin of rainbow trout larvae reared in low- (white bars) and high-[Na\(^+\)] treatments (black bars) from 9 to 27 dph. (A) Gill NKA α-subunit, (B) skin NKA α-subunit, (C) gill NHE3, (D) skin NHE3 and (E) gill V-ATPase. All values presented as means ± s.e.m.; n = 6.
Letters that differ indicate statistically significant differences between ages with treatments combined (P<0.05).
Figure 2-7: Estimated $J_{\text{max}}$ for Na$^+$ uptake in developing rainbow trout larvae reared in low- (open bars), medium- (grey-filled bars), or high-[Na$^+$] treatments (black-filled bars) from 8 to 32 dph. Values are Estimates ± Standard Error of the Estimate, as determined by SigmaPlot12’s Nonlinear Regression function for to the Michaelis-Menten equation. Letters that differ indicate significant differences between treatments within in each age group (lower case; determined by an extra sum-of-squares F-test), or between age groups within each treatment (upper case; determined as values Estimate ± 2(Standard Error of the Estimate) that did not overlap).
Figure 2-8: Estimated $K_m$ for Na$^+$ in developing rainbow trout larvae reared in low- (open bars), medium- (grey-filled bars), or high-[Na$^+$] treatments (black-filled bars) from 8 to 32 dph. Values are Estimates ± Standard Error of the Estimate, as determined by SigmaPlot12’s Nonlinear Regression function for to the Michaelis-Menten equation. Letters that differ indicate significant differences between treatments within in each age group (lower case; determined by an extra sum-of-squares F-test), or between age groups within each treatment (upper case; determined as values Estimate ± 2(Standard Error of the Estimate) that did not overlap).
Chapter 3 Ion balance during early life stages in salmonids: Insight into a unique pattern of smoltification in pink salmon (*Oncorhynchus gorbuscha*)?²

3.1 INTRODUCTION

Most salmon are anadromous; they reside in seawater for a high growth period of their lifecycle but rely on freshwater for reproduction and early life rearing. Unique to salmonids is the ability to prepare for seawater entry while residing in freshwater, termed predictive anadromy. The preparatory adaptations occurring in freshwater are collectively referred to as smoltification and include coordinated yet independent behavioural, morphological and physiological transformations which are crucial for survival in seawater (reviewed by Bern, 1978; Folmar and Dickhoff, 1980; McCormick and Saunders, 1987).

An essential part of smoltification is the development of a hypo-osmoregulatory strategy (Clarke and Hirano, 1995) which involves the transformation of the gill from an ion-absorbing to an ion-secreting epithelium (McCormick, 2001) and allows the smolt to control body water and electrolyte levels following seawater entry. Before the smolt stage, salmonids possess a limited capacity for hypo-osmoregulation (Clarke and Hirano, 1995). For instance, Atlantic salmon parr transferred to seawater show evidence of ionic and osmotic perturbation, decreased growth and increased mortality (McCormick et al., 1987; ²)

² A version of this chapter will be submitted for publication: Gallagher, E. J., and Brauner, C. J. Ion balance during early life stages of salmonids: Insight into a unique pattern of smoltification in pink salmon (*Oncorhynchus gorbuscha*)?
Stefansson et al., 1991). Smoltification includes hormonally-controlled major remodelling of ionoregulatory organs, largely at the gill, associated with upregulation of ion excretion activity often characterized by an increase in number and size of ionocytes, upregulation of the Na\(^+\)-K\(^+\)-2-Cl\(^-\) co-transporter (NKCC) mRNA (Nilsen et al., 2007) and protein expression (Pelis et al., 2001), upregulation of gill Na\(^+\)-K\(^+\)-ATPase (NKA) activity (Boeuf and Harache, 1982; McCormick et al., 1987; Nielsen et al., 1999; Nilsen et al., 2003), and an increase in the relative NKA \(\alpha 1b/NKA\alpha 1a\) mRNA expression ratio (i.e. the seawater and freshwater NKA isoforms, respectively; (Bystriansky et al., 2006; Gallagher et al., 2013; Nilsen et al., 2007; Richards et al., 2003; Sackville et al., 2012). Together, these changes occurring in freshwater greatly improve seawater adaptability in salmonids.

Pink salmon, *Oncorhynchus gorbuscha*, are the early-migrating anadromous salmonid species, migrating to seawater from freshwater streams as alevins (0.2 g), immediately following emergence from the gravel (Grant et al., 2009; Heard, 1991). This contrasts sharply with most other anadromous salmonids which enter seawater after 1 – 2 years of freshwater residency at a size orders of magnitude larger (i.e. a mass of 2 – 30 g; Clarke, 1982; Quinn and Myers, 2004; Rounsefell, 1958). Due to their very small size (and associated high surface area-to-mass ratio) at seawater entry, pink salmon may experience a greater hypo-osmoregulatory challenge than other salmonids (Houston, 1961). This, compounded with the relative lack of post-emergent time for preparatory adaptation, has led previous researchers to ask the question: Do pink salmon smolt? (Gallagher et al., 2013; Sackville et al., 2012; Weisbart, 1968)
Developing pink salmon embryos and alevin are not seawater tolerant immediately following hatch (Gallagher et al., 2013; Sackville et al., 2012; Weisbart, 1968). However, by the time they are fry, pink salmon are able to maintain water balance and regulate Na\(^+\) and Cl\(^-\) levels to a greater extent than other species of Pacific salmon upon transfer to seawater (Weisbart, 1968). Like other salmonid species, pink salmon increase their gill NKA activity (Grant et al., 2009; Sackville et al., 2012), increase their NKA 1b/1a mRNA expression ratio and increase the frequency of NKCC immunoreactive cells in the branchial epithelium (Sackville et al., 2012) upon transfer to seawater during the fry stage, weighing approximately 0.2 g.

Additionally, there is evidence that pink salmon undergo hypo-osmoregulatory changes while residing in freshwater, characteristic of smoltification. Gallagher et al. (2013) demonstrated that pink salmon undergo changes during development in freshwater that result in seawater tolerance, which corresponds with complete yolk absorption and seaward migration in nature. This development of salinity tolerance was accompanied by physiological changes, including increased gill NKA activity and gill NKA α1b/α1a mRNA expression ratio that have been used to characterize smoltification in other salmonid species.

Despite the fact that pink salmon undergo hypo-osmoregulatory changes associated with smoltification while in freshwater, it does not appear that pink salmon are fully prepared for life in seawater prior to seawater entry. Exposure to seawater acts as a strong environmental stimulus to induce additional smolt-like physiological changes in pink salmon, even in those fish that are the most prepared and enter seawater at the time of
gravel emergence when yolk absorption is complete. Indeed, pink salmon smolt transferred to seawater upon the completion of yolk absorption experience an eight-fold increase in NKA α-1b/α-1a mRNA expression during the first 2 weeks in seawater (Gallagher et al., 2013). Additionally, upon seawater entry, juvenile pink salmon undergo a doubling of whole-body ion levels which do not return to pre-transfer levels until gill NKA activity peaks nearly 8 weeks post-transfer (e.g. Gallagher et al., 2013; Nendick et al., 2011; Sackville et al., 2011; Sackville et al., 2012), which is considerably longer than the response time of hours to days observed in other salmonids (Oncorhynchus keta, Black, 1951; Oncorhynchus mykiss, Leray et al., 1981; Oncorhynchus kisutch, Miles, Harry and Smith, Lynwood, 1968; Salmo salar, Prunet and Boeuf, 1985). This suggests that pink salmon experience an ionoregulatory disturbance and may not have fully developed their hypo-ionoregulatory capacity at the time of ocean entry (Grant et al., 2009). In summary, it is clear that preparation for seawater in pink salmon is different from that of other anadromous salmon studied to date.

Surprisingly, it was recently observed that whole-body $[Na^+]$ increased considerably in freshwater-held juvenile pink salmon at the time of natural seawater entry. Between hatch and yolk absorption there was a five-fold increase in whole-body $[Na^+]$ which included an almost doubling around the end of yolk absorption (Gallagher et al., 2013). It is unknown whether this large increase in whole-body ion levels is a normal developmental trend in salmonids or is a trait specifically associated with seawater preparation unique to pink salmon. It has been proposed that increased Na$^+$ levels may be associated with the maintenance of water balance at the time of seawater entry by reducing the osmotic
gradient between the fish and its environment (Gallagher et al., 2013; Sackville et al., 2012). It is clear that more information about ionoregulation during salmonid development is required in order to elucidate the hypo-osmoregulatory strategy employed by pink salmon.

**Research objectives**

The purpose of the present study was to investigate how pink salmon modulate Na\(^+\) in freshwater during preparation for seawater. The first goal was to characterize the ontogeny of Na\(^+\) balance in pink salmon and determine how the dramatic increase in whole-body [Na\(^+\)] is achieved during smoltification (i.e. upregulation of influx or reduction in efflux). To do this, I characterized whole-body water and Na\(^+\) balance, unidirectional Na\(^+\) flux rates, and influx of a paracellular permeability marker ([H\(^3\)PEG-4000) in freshwater-held pink salmon starting immediately following hatch and weekly through until beyond complete yolk absorption. The second goal was to determine if the ontogeny of Na\(^+\) balance in pink salmon was similar to that of other salmonids. To do this, I characterized the ontogeny of Na\(^+\) balance as described above, in the closely related, non-anadromous salmonid, the rainbow trout (*O. mykiss*). Parallel characterization of the ontogeny of Na\(^+\) balance in these two species reared under similar conditions, using the same techniques was chosen to address the hypothesis that the early-migrating anadromous salmonid species (pink salmon) would display a different ontogenetic pattern of Na\(^+\) balance than a non-anadromous salmonid species which is not seawater-tolerant during early
development (rainbow trout). In particular, I hypothesized that pink salmon will exhibit a greater increase in whole-body Na\(^+\) at the time of yolk absorption than rainbow trout.

### 3.2 MATERIALS AND METHODS

**Animals and rearing**

Pink salmon, *O. gorbuscha*, were obtained as eyed embryos from Quinsam River Hatchery (Campbell River, British Columbia) on November 8, 2012 at which time they had 303 accumulated thermal units (ATU; the product of days following fertilization and temperature in °C). Rainbow trout, *O. mykiss*, were obtained as eyed embryos from the Vancouver Island Trout Hatchery (Duncan, British Columbia) on January 9, 2013, at 248 ATU. Both species were transported to the University of British Columbia (UBC; in Vancouver, Canada) where they were held for the duration of the study.

Upon arrival at UBC, pink salmon were held at 4 °C (actual = 3.7 ± 0.1 °C) in a Heath try egg incubation system supplied with flow-through dechlorinated Vancouver City tap water (annual average values in mM: Na\(^+\), 0.08; Cl\(^-\), 0.05; Ca\(^{2+}\), 0.03; Mg\(^{2+}\), 0.006; K\(^+\), 0.004; HCO\(_3\)\(^-\) unknown; alkalinity, 3.0 mg L\(^{-1}\) as CaCO\(_3\); hardness 3.43 mg L\(^{-1}\) as CaCO\(_3\); pH 6.4 – 6.8; Metro Vancouver, 2012) on a 12L:12D photoperiod. When the rainbow trout arrived, they were added to additional Heath trays within the same system and temperature was increased and maintained at ambient groundwater temperatures for the remainder of the study period (average = 6.5 ± 0.1 °C; minimum = 4.6 °C; maximum = 8.4 °C). Fifty per cent of the embryos had hatched by January 7, 2013 (532 ATU) and January 28, 2013 (338 ATU) for pink salmon and rainbow trout, respectively. At the time of yolk absorption, both
species were transferred to separate 45 L plastic tanks (in triplicate for each species) supplied with flow-through dechlorinated Vancouver City tap water where they were kept until the last sampling date, April 26, 2013. Completion of yolk absorption and subsequent first feeding took place on March 17, 2013 which corresponded to 932 ATU and 650 ATU in pink salmon and rainbow trout, respectively. Both species were fed crumbled starter feed (size #0; Skretting, USA) once or twice daily \textit{ad libitum}, with the exception of 18 h prior to sampling, at which time feeding was withheld. Temperature, pH, oxygen saturation, nitrate, nitrite and ammonia levels were monitored and recorded regularly. All procedures involving animals adhered to the UBC’s Animal Care Certificate A11-0235.

**Experimental protocol**

Fish were held as described above and sampled weekly (actually every 6 – 8 days) for 14 weeks, from January 26 until April 26, 2013 for measurement of unidirectional Na\(^+\) flux (n=8) and \[^3\text{H}\]PEG-4000 uptake rates (n=12). At these times additional larvae from each treatment were collected, euthanized in 1000 mg L\(^{-1}\) tricaine methanesulfonate (MS-222; Syndel Laboratories, Canada) and immediately frozen in liquid nitrogen and stored at –80\(^\circ\)C for measurement of whole-body Na\(^+\) levels (n=8) and water content (n=8).

**Wet mass, dry mass, whole-body Na\(^+\) content and whole-body water content**

Fish were euthanized with 1000 mg L\(^{-1}\) MS-222, rinsed with de-ionized water, blotted dry, weighed, and wet mass was documented. Fish were dried in pre-weighed 15 mL polystyrene tubes at 65\(^\circ\)C to constant mass (approximately 4 days) and dry mass was recorded. Dried fish were digested in 1 M nitric acid (approximately 1 mL per 0.1 g fish
mass) at 65°C for approximately one week and the tapered end of a metal spatula was used to assist digestion mechanically. The supernatant of the fish tissue digest was analyzed for [Na\(^+\)] using flame absorption spectroscopy (Spectra AA-240FS; Varian, Australia) and standardized to both wet and dry mass for total whole-body [Na\(^+\)]. Whole-body water content was reported as a per cent of wet mass and calculated as the difference between wet and dry mass.

**Whole-body unidirectional Na\(^+\) flux rates**

Unidirectional Na\(^+\) flux rates (influx, \(J_{in}^{Na}\); efflux, \(J_{out}^{Na}\); net flux, \(J_{net}^{Na}\)) were determined in rainbow trout and pink salmon weekly for 14 weeks post-hatch. For each species at each age, fish were sampled from the replicate tanks (n=7 – 8) and placed individually into flux chambers containing the water in which they were reared (dechlorinated Vancouver City tap water). Chamber size and water volume increased as the fish became larger to accommodate their increasing size and metabolic activity, ranging from 20 mL polyethylene scintillation vials containing 12 mL of water (for fish immediately following hatch) to 60 mL polyethylene Nalgene bottles containing 22 mL of water (for fish beyond yolk absorption). The flux chambers were aerated continuously throughout the experiment and maintained at 7 °C in a chilled water bath. Fish were placed in the chambers 30 minutes before the start of each flux measurement to allow for recovery from handling. To initiate the flux measurement, radioisotope sodium-22 (\(^{22}\)Na\(^+\); as \(^{22}\)NaCl; PerkinElmer, USA) was added to each chamber to achieve approximately 8.3 µCi L\(^{-1}\). At 10 minutes post-injection a water sample (1 mL) was taken in duplicate for measurement of \(^{22}\)Na specific
activity and [Na⁺]. The total flux exposure period was 6 h. At the end of the flux experiment, water was sampled for water radioactivity and [Na⁺]. The fish were then immediately removed from flux chambers, euthanized with a lethal dose of MS-222 (1000 mg L⁻¹) buffered with NaHCO₃, rinsed three times with 5mM NaCl to displace surface bound ²²Na and rinsed once with de-ionized water to remove NaCl. Fish were then blotted dry, weighed and placed individually into vials for measurement of radioactivity.

**Whole-body PEG-4000 uptake**

The whole-body [H³]PEG-4000 uptake was measured in pink salmon and rainbow trout weekly for 14 weeks post-hatch under the same laboratory conditions described for the Jₜ Na experiment using the method described by Kumai et al. (2011), with minor variations. For each species at each age, fish were randomly sampled from the replicate tanks (n=12) and placed in groups of four or six into flux chambers containing the water in which they were reared and each of the 4 – 6 fish yielded one measurement of permeability. As in the Jₜ Na experiments, chamber size and water volume increased as the fish became larger, ranging from 20 mL polyethylene scintillation vials containing 12 mL of experimental water to 60 mL polyethylene Nalgene bottles containing 27 mL of water. To initiate the flux experiment, fish were exposed to 0.88 – 1.25 µCi mL⁻¹ of [³H]PEG-4000 (MW: 4000; American Radiolabeled Chemicals, Inc., USA). At 5 minutes post-injection a water sample (1 mL) was collected in duplicate for determination of radioactivity. Each flux lasted a total of 6 hours. At the end of the flux experiments, sampling for water
radioactivity was repeated and the fish were euthanized and weighed as described above and placed individually into vials for measurement of radioactivity.

**Analytical techniques and calculations**

**Na⁺ flux experiment**

Water samples were measured for [Na⁺] using a flame atomic absorption spectrometer (Spectra AA240FS, Varian, Australia). Water samples and fish samples were measured for radioactivity using a gamma counter (Wallac 1470 Wizard, PerkinElmer, Finland) in counts per minute (cpm).

Unidirectional Na⁺ influx ($J_{in}^{Na}$), efflux ($J_{out}^{Na}$) and net flux ($J_{net}^{Na}$), expressed in µmol Na⁺ h⁻¹ g⁻¹ were calculated as described by (Lauren and McDonald, 1987):

$$J_{in}^{Na} = \frac{a}{(SA \cdot t \cdot wt)}$$

where $a$ is the whole-body activity of $^{22}$Na in cpm, $t$ is the duration of the flux in hours, $wt$ is the wet tissue mass of the fish in grams and $SA$ refers to the specific activity of the $^{22}$Na in the water, calculated as:

$$SA = \frac{[\text{cpm}_i + \text{cpm}_f]}{2}$$

where $i$ refers to the initial water samples at 10 minutes post-injection of $^{22}$Na and $f$ refers to the final water samples taken at the end of the flux period. $J_{net}$ is calculated as:

$$J_{net}^{Na} = \frac{([\text{ion}]_i - [\text{ion}]_f) \cdot v}{t \cdot wt}$$
where \( v \) refers to the volume of water the fish is exposed to between 10 minutes post-injection of \( ^{22}\text{Na} \) and the end of the flux. A positive \( J_{\text{Na}}^{\text{net}} \) value indicates a net gain of \( \text{Na}^+ \), whereas a negative \( J_{\text{Na}}^{\text{net}} \) value indicates a net loss of \( \text{Na}^+ \). \( J_{\text{Na}}^{\text{net}} \) is calculated as:

\[
J_{\text{Na}}^{\text{net}} = J_{\text{Na}}^{\text{in}} - J_{\text{Na}}^{\text{out}}
\]

where a negative \( J_{\text{out}} \) value indicates that efflux has occurred.

**Whole-body PEG-4000 uptake**

Fish were digested in an aqueous tissue solubilizer (Solvable\textsuperscript{TM}; Perkin Elmer, USA) then neutralized using glacial acetic acid. Subsequently, liquid scintillation cocktail (BioSafe-II; RPI co. Mt. Prospect, IL, USA) was added to both the fish digests and water samples and radioactivity of each was determined using a liquid scintillation counter (Beckman LS 6500 Scintillation Counter, Beckman Coulter Co., Canada).

The rate of \([^3\text{H}]\text{PEG-4000}\) influx \( (J_{\text{in}}^{\text{PEG}}) \) was calculated as:

\[
J_{\text{in}}^{\text{PEG}} = \frac{a}{(\text{SA} \cdot t \cdot wt)}
\]

where \( a \) is the whole-body activity of \([^3\text{H}]\text{PEG-4000}\) in cpm, \( t \) is the duration of the flux in hours, \( wt \) is the wet tissue mass of the fish in grams and \( \text{SA} \) refers to the specific activity of the \([^3\text{H}]\text{PEG-4000}\) in the water, calculated as:

\[
\text{SA} = \left( \frac{\text{cpm}_i + \text{cpm}_f}{2} \right) / \left( \left[^{3}\text{H}\right] \text{PEG - 4000} \right)
\]

where \( i \) refers to the initial water samples at 10 minutes post-injection of \( ^{22}\text{NaCl} \) and \( f \) refers to the final water samples taken at the end of the flux period.
**Statistical analysis**

All data are expressed as means ± s.e.m. unless otherwise specified. Within each species, one-way analysis of variance (ANOVA) was used to compare means if the data satisfied the assumptions of normality and equal variance. In general, the Holm-Sidak post-hoc test was subsequently applied for pairwise comparisons when effects were found to be significant. In some cases, the assumptions of normality and/or equal variance were not met. In such cases, an ANOVA on Ranks was used to compare the medians (data sets include per cent body water content, whole-body [Na+] relative to wet mass, and whole-body PEG uptake rates). The Turkey test (equal sample sizes) or Dunn's method (unequal samples sizes) post-hoc tests were subsequently applied for pairwise comparisons when effects were found to be significant. All statistical analyses were conducted with SigmaPlot12 and a significance level of P<0.05 was used throughout.

Development was reported as ATU throughout this study for both rainbow trout and pink salmon. ATU describes the cumulative effect of temperature and time and is calculated as the product of days and temperature in °C. ATUs were used to define the development of the fish because fish were exposed to ambient water temperature for most of the study and therefore temperature was not held constant.
3.3 RESULTS

Pink salmon

Wet mass, dry mass and per cent whole-body water

In general, there was a statistically significant effect of age on total wet mass, dry mass and whole-body water in developing pink salmon (P≤0.001; Figure 3-1). Wet mass (Figure 3-1a) increased approximately 25 per cent from 621 ATU until the end of yolk absorption (=932 ATU) and an additional 1.5-fold following yolk absorption and the beginning of exogenous feeding. However, the apparent growth was not achieved through an increase in dry mass (Figure 3-1b). Instead, dry mass decreased by 40% from 0.050 g at 621 ATU until the end of yolk absorption, reaching a minimum of 0.035 g at 877 ATU. This loss of dry mass was compensated by a gain in water content (Figure 3-1c), which increased significantly from 69 per cent at 621 ATU to approximately 82 per cent by the end of yolk absorption. Following yolk absorption and the beginning of exogenous feeding, dry mass increased 2-fold, reaching approximately 0.070 g by 1232 ATU, during which time water content was maintained around 80 per cent.

Whole-body [Na\(^+\)] levels

In general, there was a statistically significant effect of age on whole-body [Na\(^+\)] relative to wet and dry mass in developing pink salmon (P≤0.001; Figure 3-2). Whole-body [Na\(^+\)], expressed relative to wet mass (Figure 3-2a), increased about 1.5-fold from approximately 35 µmol g wet mass\(^{-1}\) at 621 ATU to 52 µmol·(g wet mass\(^{-1}\)) by the time of complete yolk absorption. Following yolk absorption, whole-body Na\(^+\) relative to wet mass
did not change. Whole-body Na\(^+\), expressed relative to dry mass (Figure 3-2b), increased approximately 3-fold from 115 \(\mu\text{mol g}^{-1}\) (dry mass) at 621 ATU to 295 \(\mu\text{mol g}^{-1}\) (dry mass) by the time of complete yolk absorption. In general, whole-body [Na\(^+\)] relative to dry mass did not change following yolk absorption.

**Whole-body unidirectional Na\(^+\) flux rates**

In general, there was a statistically significant effect of age on mass-specific whole-body \(J_{\text{in}}^{\text{Na}}, J_{\text{out}}^{\text{Na}}\) and \(J_{\text{net}}^{\text{Na}}\) in developing pink salmon (P\(\leq\)0.001; Figure 3-3); however, there did not appear to be a clear increasing or decreasing trend following hatch until beyond the time of complete yolk absorption, approximately 1232 ATU. There was a temporary 2-fold increase in \(J_{\text{in}}^{\text{Na}}\) beyond yolk absorption at 921 and 966 ATU, but this increase was not maintained beyond 966 ATU. There was also a 2-fold increase in \(J_{\text{out}}^{\text{Na}}\) at the end of yolk absorption, from 966 to 1121 ATU but beyond 1121 ATU, \(J_{\text{out}}^{\text{Na}}\) returned to values not significantly different from most of those seen prior to yolk absorption. In general, \(J_{\text{net}}^{\text{Na}}\) was consistently negative (net Na\(^+\) loss from the fish) and there was a significant age effect (P=0.001); however, \(J_{\text{net}}^{\text{Na}}\) did not exhibit any specific significant differences throughout the duration of the study, with the exception of 966 and 1014 ATU, which were statistically more negative (net Na\(^+\) loss from the fish increased) than the other time points measured.

**Whole-body PEG-4000 uptake**

In general, there was a statistically significant effect of age on \(J_{\text{in}}^{\text{PEG}}\) in developing pink salmon (P\(\leq\)0.001; Figure 3-4). Mass-specific whole-body \(J_{\text{in}}^{\text{PEG}}\) was variable in pink salmon following hatch until beyond the time of complete yolk absorption, approximately...
1232 ATU. Following hatch, \( J_{\text{PEG}}^{\text{in}} \) was low from 621 to 744 ATU at which point there was a 2-fold increase in \( J_{\text{PEG}}^{\text{in}} \) that was maintained, with some variation, until 1232 ATU.

**Rainbow trout**

*Wet mass, dry mass and per cent whole-body water*

In general, there was a statistically significant effect of age on wet mass, dry mass and whole-body water in developing rainbow trout (\( P \leq 0.001 \); Figure 3-5). Wet mass (Figure 3-5a) increased approximately 2-fold from 338 ATU until the end of yolk absorption (=650 ATU) and an additional 2.5-fold following yolk absorption, which coincided with the beginning of exogenous feeding. However, the increased wet mass was not achieved through increased dry mass (Figure 3-5b) as dry mass did not change significantly from 0.015 g at 338 ATU until the end of yolk absorption. Instead, increased wet mass was achieved through a gain in water content (Figure 3-5c), which increased significantly from 69 per cent at 427 ATU to approximately 82 per cent by the end of yolk absorption. Following yolk absorption and the beginning of exogenous feeding dry mass increased 3-fold, reaching 0.057 g by 950 ATU, during which time water content was maintained around 85 per cent.

*Whole-body \([\text{Na}^+]\) levels*

In general, there was a statistically significant effect of age on whole-body \([\text{Na}^+]\) relative to wet and dry mass in developing rainbow trout (\( P \leq 0.001 \); Figure 3-6). Whole-body \([\text{Na}^+]\), expressed relative to wet mass (Figure 3-6a), increased approximately 3-fold
from 23 µmol·(g wet mass)⁻¹ at 338 ATU to 55 µmol·(g wet mass)⁻¹ by the time of complete yolk absorption. Whole-body [Na⁺], expressed relative to dry mass (Figure 3-6b), increased approximately 4-fold from 80 µmol·(g dry mass)⁻¹ at 338 ATU to 360 µmol·(g dry mass)⁻¹ by the time of complete yolk absorption. In general, whole-body [Na⁺] did not change following yolk absorption when expressed relative to either wet mass or dry mass.

*Whole-body unidirectional Na⁺ flux rates*

In general, there was a statistically significant effect of age on mass-specific whole-body $J_{in}^{Na}$, $J_{out}^{Na}$ and $J_{net}^{Na}$ in developing rainbow trout ($P \leq 0.001$; Figure 3-7). Mass-specific whole-body $J_{in}^{Na}$ and $J_{out}^{Na}$ increased progressively following hatch until the end of complete yolk absorption and then decreased until 1038 ATU. $J_{in}^{Na}$ increased 7-fold from 338 to 730 ATU, a trend which was mirrored in $J_{out}^{Na}$. Beyond yolk absorption, $J_{in}^{Na}$ and $J_{out}^{Na}$ decreased approximately 70 per cent in rainbow trout fry. In general, $J_{net}^{Na}$ was quite stable throughout larval development and beyond yolk absorption. With the exception of 372 and 638 ATU, at which times $J_{net}^{Na}$ was reduced and very close to zero, $J_{net}^{Na}$ was consistently slightly negative (net Na⁺ loss from the fish).

*Whole-body PEG-4000 uptake*

In general, there was a statistically significant effect of age on $J_{in}^{PEG}$ in developing rainbow trout ($P \leq 0.001$; Figure 3-8). Mass-specific whole-body $J_{in}^{PEG}$ in rainbow trout was variable throughout development, and no clear trend was observed except for a temporary 50 per cent reduction in $J_{in}^{PEG}$ just prior to complete yolk absorption.
3.4 DISCUSSION

This chapter represents the first comparative characterization of Na\(^+\) balance in developing salmonids while in freshwater as well as the first documentation of Na\(^+\) flux measurements in pink salmon. We demonstrate for the first time that two salmonid species, the early-migrating anadromous salmonid, pink salmon, and the non-anadromous salmonid, rainbow trout, exhibit similar heightened and increasing whole-body [Na\(^+\)] during early development in freshwater. Therefore, the trend of increasing whole-body [Na\(^+\)] in freshwater pink salmon does not appear to be a unique strategy for maintaining hydromineral balance upon early ocean entry, as previously suggested (Gallagher et al., 2013; Sackville et al., 2012). However, the heightened and increasing whole-body [Na\(^+\)] appears to be achieved via different mechanisms in pink salmon and rainbow trout. Rainbow trout experience increasing Na\(^+\) uptake rates during development which peak around yolk absorption before dropping to adult levels and paracellular permeability is maintained throughout this time-frame. In contrast, pink salmon do not alter Na\(^+\) uptake rates during development and paracellular permeability appears to increase suddenly just prior to yolk absorption.

Whole-body [Na\(^+\)] during post-embryonic development

This is the first direct comparison of Na\(^+\) balance in freshwater reared pink salmon (early-migrating anadromous salmonid) and rainbow trout (a non-anadromous salmonid) from hatch until beyond complete yolk absorption reared under the same freshwater conditions and using the same techniques. As expected (based on Gallagher et al., 2013),
pink salmon increased whole-body [Na⁺] levels dramatically following hatch until completion of yolk absorption. Relative to wet mass, whole-body [Na⁺] levels increased 1.5-fold during this time (Figure 3-2a). Since pink salmon experience catabolic metabolism during yolk absorption, dry mass decreases substantially while the increasing water content compensates during development and consequently wet mass does not increase until the onset of exogenous feeding. To account for these changes in body water, whole-body [Na⁺] levels were also expressed relative to dry mass where a 3-fold increase was observed over the same developmental period. This difference presumably indicates the amount of Na⁺ taken up from the environment and incorporated into the animal. These results are consistent with those reported by Gallagher et al. (2013), with the exception that they observed a 5-fold increase in whole-body [Na⁺] relative to dry mass over a similar developmental period. The basis for this difference between studies is unknown, however, it is worth noting that from hatch until approximately 688 ATU the values for whole-body [Na⁺] relative to dry mass are nearly identical in the two studies.

Interestingly, in rainbow trout, a non-anadromous salmonid, whole-body [Na⁺] levels also increased dramatically following hatch until completion of yolk absorption where whole-body [Na⁺] relative to wet mass increased 3-fold consistent with that of Brauner et al. (2003). Through to yolk absorption, wet mass progressively increased while dry mass remained relatively constant. Whole-body [Na⁺] levels relative to dry mass increased 4-fold increase during that period, similar to, over even greater than, the increase observed in pink salmon.
There is only one other study that has measured Na\(^+\) levels in salmonids throughout yolk absorption. Atlantic salmon (S. salar), an anadromous salmonid which typically spends 1 – 5 years in freshwater before migrating to sea (Hansen and Quinn, 1998). It appears that Atlantic salmon also increase whole-body Na\(^+\) content during development in freshwater but not nearly to the same extent as pink salmon (Rombough and Garside, 1984). Whole-body [Na\(^+\)] gradually increases through development, reaching adult levels near the end of yolk absorption. Based on these data, it appears that both pink salmon and rainbow trout experience comparatively high Na\(^+\) levels during early life stages in freshwater and that these whole-body [Na\(^+\)] levels early in development appear consistently higher than those in adult fish (Figure 3-2 and Figure 3-6). Unfortunately, there are no data following yolk absorption for Atlantic salmon and data for other salmonids is limited.

Limited data is available for whole-body [Na\(^+\)] in juvenile and adult salmonids. Juvenile rainbow trout (average mass 2.4 g) held in freshwater have a whole-body [Na\(^+\)] level of 57 µmol·(g wet mass\(^{-1}\)), similar to the values observed at 950 ATU in the present study (Lauren and McDonald, 1987). In adult rainbow trout (average mass 169 g), whole-body [Na\(^+\)] is approximately 38 mmol·(kg wet mass\(^{-1}\)), nearly 50 per cent less than the whole-body [Na\(^+\)] measured at 950 ATU (13 weeks post-hatch) in the present study (Prodocimo et al., 2007). To date, there is no equivalent whole-body [Na\(^+\)] data for juvenile or adult pink salmon in freshwater; however, levels of approximately 45 – 50 mmol·(kg wet mass\(^{-1}\)) have been reported for juvenile pink salmon (2 – 4 g) in seawater, approximately
25 – 30 per cent lower than values reported here. Overall, it appears that whole-body Na⁺ is very high during larval development compared to juvenile and adult fish.

It is not known whether the increased whole-body [Na⁺] in developing fish is representative of high plasma Na⁺ levels as has been shown in adult rainbow trout (Prodocimo et al., 2007). It has been demonstrated in several vertebrate species that extracellular volume decreases during periods of rapid growth associated with development (reviewed by Holmes and Donaldson, 1969). Additionally, during early development, the yolk is an important store for Na⁺ (Brauner, 2008). Likely, the relationship between whole-body [Na⁺] and plasma [Na⁺] is different in larval fish and the increased [Na⁺] represents a combination of increased plasma and tissue sodium levels but this has yet to be investigated.

It is important to emphasize that developing salmonids investigated in this study did in fact increase Na⁺ content in their bodies during development (as opposed to changing the proportions low Na⁺ and high Na⁺ tissues/stores). Indeed, in the present study, the average alevin contained 5.6 µmol Na⁺ (pink salmon) and 1.2 µmol Na⁺ (rainbow trout) shortly following hatch, and near the end of yolk absorption, before the onset of exogenous feeding, Na⁺ content had increased to 9.5 and 6.7 µmol Na⁺ per fish, respectively. Prior to exogenous feeding, there are only two possible sources of the Na⁺ available to the fish: the water and the contents of the fish itself. Therefore, this increase in Na⁺ content (equal to 3.8 and 5.5 µmol in pink salmon and rainbow trout, respectively) reflects the biological incorporation of Na⁺ that has be actively taken up from the freshwater environment.
How do developing fish achieve such high Na\(^+\) levels?

Interestingly, despite the fact that the early-migrating anadromous salmonid, pink salmon, and the non-anadromous salmonid, rainbow trout, displayed similar patterns of increasing whole-body [Na\(^+\)] levels in freshwater during early development, the two species exhibited very different patterns of Na\(^+\) uptake and efflux during the stages investigated. Therefore, perhaps pink salmon regulate Na\(^+\) influx and efflux as a unique strategy for maintaining hydromineral balance upon transition to sea water as opposed to increasing whole-body [Na\(^+\)] levels (as previously described); to date the former has not been previously investigated in pink salmon.

Active uptake of Na\(^+\) from the water is very low in salmonid embryos prior to hatch (rainbow trout, Brauner et al., 2003; Atlantic salmon, McWilliams and Shephard, 1989). During yolk absorption, \(J_{\text{in}}^{\text{Na}}\) levels in rainbow trout increase rapidly (Figure 3-7; Brauner et al., 2003; Fu et al., 2010) – see also Figure 2-4 of this thesis), to levels far greater than those observed in juveniles and adults (Goss and Wood, 1990), which correlates with increasing whole-body NKA activity (Brauner et al., 2003; Fu et al., 2010). This trend was verified for rainbow trout in the present study; \(J_{\text{in}}^{\text{Na}}\) at yolk absorption increased 5-fold in rainbow trout following hatch. \(J_{\text{in}}^{\text{Na}}\) rates in rainbow trout would cause a significant increase in net uptake from the environment which is consistent with the progressive increase in whole-body [Na\(^+\)] observed in these fish during early development. Interestingly, immediately following yolk absorption \(J_{\text{in}}^{\text{Na}}\) rates drop about 60 per cent to levels seen in juveniles and adults. \(J_{\text{in}}^{\text{Na}}\) rates in larval fish have been described as much higher than in juveniles and adults (i.e.
Brauner, 2008) but evidence that uptake rates are reduced to those seen in juveniles and adults so rapidly immediately following yolk absorption has not previously been shown (but see also Figure 2-4 in this thesis). Surprisingly, this trend does not appear to hold true for all salmonids. Pink salmon did not alter $J_{\text{Na}}$ rates during larval development; from shortly following hatch until beyond yolk absorption and the onset of exogenous feeding, only a few very minor differences in $J_{\text{Na}}$ were measured (Figure 3-3). Therefore, the large accumulation of Na\textsuperscript{+} in freshwater observed in pink salmon during yolk absorption was not the result of increased uptake from the environment. Instead, pink salmon must be reducing their diffusive loss to the environment. The efflux data presented here does not reflect this hypothesis, however, it is important to note that efflux values reported here were not measured directly, but instead using a less sensitive, indirect method. A direct measurement of Na\textsuperscript{+} efflux, as used by Kwong et al., (2013) would be useful in future studies.

Based on changes in whole-body [Na\textsuperscript{+}] measured here, we estimate that between hatch and yolk absorption (prior to feeding), approximately 85 per cent (pink salmon) and 80 per cent (rainbow trout) of Na\textsuperscript{+} uptake balances diffusive Na\textsuperscript{+} loss, while only 20 and 25 per cent of Na\textsuperscript{+} uptake, respectively, is incorporated into the body between hatch and complete yolk absorption. Similar values for per cent biological incorporation of $J_{\text{in}}^{\text{Na}}$ during early life stages have been estimated in rainbow trout (Fu et al., 2010) but no such estimates have previously been made in pink salmon. The differences in biological incorporation of Na\textsuperscript{+} between the two species is reflected in the relative increases in [Na\textsuperscript{+}].
observed at this stage; rainbow trout increase their whole-body [Na⁺] to a greater extent (4-fold) than pink salmon (3-fold) during yolk absorption. It is not known why diffusive efflux is so high in developing salmonids or why it represents such a large proportion of influx, especially pink salmon, but given that acid-base regulation is tightly linked to ionoregulation it may be associated with the need to modulate net Na⁺/H⁺ exchange; however this remains to be investigated. Clearly more work is required to understand this phenomenon in developing salmonids.

The mechanism underlying Na⁺ efflux in freshwater fish remains largely unknown, however, the loss of Na⁺, at least partially, via passive diffusion through paracellular pathways, particularly across branchial epithelia is commonly accepted (e.g. Chasiotis and Kelly, 2011; Kumai et al., 2011; Kwong et al., 2013). The findings of the present study suggest that the early-migrating anadromous salmonid, pink salmon, and the non-anadromous salmonid, rainbow trout, display different patterns of paracellular permeability in freshwater following hatch until the time of complete yolk absorption. In general, pink salmon mass-specific whole-body $J_{in}^{PEG}$ (Figure 3-4) was low immediately following hatch, but gradually increased 2-fold following yolk absorption, with some variation. On the other hand, rainbow trout mass-specific whole-body $J_{in}^{PEG}$ (Figure 3-8) is more variable throughout development, although no clear trend was observed except for a large temporary reduction prior to yolk absorption. Using PEG uptake as a marker of paracellular permeability, these data suggest that permeability is relatively unchanged in post-emergent rainbow trout while permeability increases in pink salmon prior to yolk absorption. Generally, tight junctions become more leaky upon entry to seawater,
presumably to facilitate paracellular Na\(^+\) extrusion, compared to the less leaky tight junctions found in the gills of freshwater fish. Since pink salmon are preparing for seawater in freshwater during this time (Gallagher et al., 2013), this could represent a novel component of smoltification in pink salmon.

While PEG is a well-established marker of paracellular transport (Pappenheimer and Reiss, 1987), recent zebrafish studies (Chasiotis and Kelly, 2011; Jönsson et al., 2006; Kumai et al., 2011; Kwong et al., 2013b) reported conflicting relationships between Na\(^+\) fluxes and gill permeability as indicated by PEG uptake. In the present study, the correlation between mass-specific PEG uptake and Na\(^+\) efflux was species specific; pink salmon exhibited a positive relationship between mass-specific PEG uptake and Na\(^+\) efflux while rainbow trout showed no correlation between the two measured variables (data not shown).

It is important to note that PEG-4000 is also a classic marker of drinking rate (e.g. Shehadeh and Gordon, 1969) which can confound gill permeability measurements. Robertson and Wood, (2014) showed that when measuring permeability as [3H]PEG-4000 influx in adult freshwater rainbow trout, 75\% of the recovered PEG is contained within the body, while 25\% ended up in the gut contents as a result of drinking. The authors also demonstrate that correction for drinking rate is effectively achieved via terminal removal of the gut, including all contents. Due to the small size of the fish used in the present study (0.05 – 3.5 g) collection of the gut, including its contents, was not possible.

Nonetheless, the PEG uptake measurements reported here represent PEG that entered the fish through either paracellular routes, via drinking, or some combination of
the two. Drinking rate in developing rainbow trout in freshwater increases from 0.22 on
day 4 to 3.2 nl mg-1 h-1 on day 40, which is much higher than for adult fish in freshwater,
which drink very little water (Tytler et al., 1990). PEG uptake rate measured here in
rainbow trout fluctuates between 0.8 to 1.4 nL·(mg·h)-1, indicating that at least two-thirds
of the PEG uptake measured here is representative of paracellular transport. This could be
further investigated by measuring drinking rate separately using a very large MW marker
that cannot move into the fish paracellularly, such as radiolabeled dextran (MW > 60,000)
as has been done previously in larval fish species (rainbow trout, Miyazaki et al., 1998;
tilapia, Tytler et al., 1990) and subtracting it from PEG uptake rate. Nonetheless,
paracellular permeability has not been previously measured in larval or developing
salmonids. This study represents an important starting point when considering passive ion
fluxes and the movement of water in the context of osmoregulation in post-embryonic
salmonids.

3.5 SUMMARY

It is now clear that heightened and increasing whole-body [Na+] during yolk
absorption in freshwater is not unique to pink salmon, suggesting that it is not associated
with preparation for early ocean entry. Interestingly, the mechanism by which pink salmon
and rainbow trout achieve high whole-body [Na+] during early development appears to be
different. Rainbow trout increase Na+ uptake rates during development until the time of
complete yolk absorption and paracellular permeability rates are maintained throughout
this time-frame, suggesting that rainbow trout increase whole-body [Na+] via increased Na+
uptake. In contrast, pink salmon do not alter Na\(^+\) uptake rates during development and paracellular permeability appears to increase suddenly just prior to yolk absorption, suggesting that these fish regulate whole-body [Na\(^+\)] via modulation of Na\(^+\) efflux rates. Perhaps altered patterns of Na\(^+\) influx and efflux (compared to trout) are employed by pink salmon as a strategy for maintaining hydromineral balance upon transition to saltwater. The simultaneous increase in permeability observed in pink salmon suggests that these fish are preparing for Na\(^+\) secretion associated with life in seawater via paracellular pathways by increasing leakiness of the tight junctions while in freshwater.
3.6 FIGURES
**Figure 3-1**: Changes in (a) wet mass, (b) dry mass, and (c) per cent body water content in pink salmon alevin and fry, with development expressed as accumulated temperature units (ATUs) following hatch (0 ATU). The downward-facing arrow corresponds to hatch; the hatched bar represents yolk absorption and the final measurement corresponds to approximately 14 weeks post-hatch. Letters that differ indicate statistically significant differences (p<0.05). Symbols indicate mean values ± s.e.m. (n=8 – 10).
Chapter 4 Summary of findings and conclusions

This thesis makes several contributions to our general knowledge of ionoregulatory function in developing fish by characterizing the functional ontogeny of sodium (Na⁺) balance in salmonids reared in freshwater during early development. Chapter 2 furthers our understanding of the ontogeny of Na⁺ balance and transport capacity while assessing the plasticity of Na⁺ balance and transport capacity during a critical developmental transition from cutaneous-dominated to gill-dominated ionoregulation using an intra-specific comparison in the model teleost species, the rainbow trout. Chapter 3 also contributes to our understanding of Na⁺ transport during early salmonid development while advancing our knowledge of Na⁺ transport characteristics employed by the early-migrating anadromous salmonid, the pink salmon, using an inter-specific comparison between the closely related pink salmon and the non-anadromous rainbow trout.

4.1 CHAPTER 2 SUMMARY

Thesis objectives: Revisited

As outlined in previous chapters, the research objectives for Chapter 2 were: (1) Characterize the ontogeny of Na⁺ balance and transport capacity in larval rainbow trout through to yolk absorption and (2) Characterize the degree to which environmental [Na⁺] influences Na⁺ balance and transport capacity in larval rainbow trout through to yolk absorption. This work was guided by the hypothesis that early in development, when ionoregulation is cutaneous-dominated, ionoregulatory plasticity will be limited while later
in development, when ionoregulation is gill-dominated, fish will exhibit greater ionoregulatory plasticity, as seen in juvenile and adult fish.

**Conclusions from Objective 1**

This investigation confirmed previous findings that rainbow trout experience very high resting unidirectional Na$^+$ uptake rates early in development when ionoregulation is cutaneous-dominated. Additionally, we revealed for the first time that resting unidirectional Na$^+$ uptake rates are reduced to values typical of juvenile and adult rainbow trout immediately following yolk absorption, when ionoregulatory function first transitions to the gills. This study also represents the first kinetic analysis of Na$^+$ uptake in larval rainbow trout. Maximal uptake rate ($J_{max}$) for Na$^+$ increased following hatch and was very high during early development when ionoregulation is primarily cutaneous, but decreased substantially immediately following yolk absorption while uptake affinity ($K_m$) decreased following hatch and increased following yolk absorption. This suggests that early in development, high Na$^+$ uptake rates across cutaneous ionocytes are driven by a high maximal uptake rate, while the rapidly proliferating gill ionocytes that dominate ionoregulation post-yolk absorption have an increased affinity for Na$^+$.

**Conclusions from Objective 2**

This was the first investigation of the effect of freshwater [Na$^+$] on Na$^+$ balance and transport capacity in larval fish. Following hatch, when ionoregulation occurs predominantly at ionocytes on the skin and yolk sac epithelium (while ionocytes are beginning to mature at the gills), larval fish exhibited little plasticity in their Na$^+$ uptake
rates and Na\textsuperscript{+} uptake kinetics. As the yolk is consumed and ionoregulation shifts to ionocytes located on the gill, developing fish exhibited increased uptake affinity for Na\textsuperscript{+} in low-[Na\textsuperscript{+}] environments, similar to the effect observed in juvenile and adult fish, indicating increased capacity to overcome the increased gradient for diffusive Na\textsuperscript{+} loss. However, maximal uptake rate for Na\textsuperscript{+} did not increase in low-[Na\textsuperscript{+}] environments, as observed in adult fish, suggesting that this ‘improved’ capacity to overcome the increased diffusive Na\textsuperscript{+} loss associated with an ion-poor environments may be limited and/or still developing at this stage. This is likely due to the changing distribution of ionocytes and the fact that the capacity for upregulation of mature ionocytes gills is limited early in development. Nonetheless, these fish were able to maintain hydromineral balance despite differences in rearing [Na\textsuperscript{+}], suggesting that developing fish modulate Na\textsuperscript{+} balance differently than juvenile and adult fish when faced with a range of [Na\textsuperscript{+}].

4.2 CHAPTER 3 SUMMARY

Thesis objectives: Revisited

As outlined in previous chapters, the research objectives for Chapter 3 were: (1) Characterize the ontogeny of Na\textsuperscript{+} balance in pink salmon and determine how the dramatic increase in whole-body [Na\textsuperscript{+}] is achieved during smoltification (i.e. upregulation of influx or reduction in efflux) and (2) Determine if the ontogeny of Na\textsuperscript{+} balance in pink salmon was characteristic of salmonids. This research was driven by the hypothesis that the early-migrating anadromous salmonid species (pink salmon) would display a different ontogenetic pattern of Na\textsuperscript{+} balance than a non-anadromous salmonid species which is not
seawater-tolerant during early development (rainbow trout). In particular, I hypothesized that pink salmon will exhibit a greater increase in whole-body Na⁺ at the time of yolk absorption than rainbow trout.

**Conclusions from Objectives 1 & 2**

Although surprising, it is now clear that heightened and increasing whole-body [Na⁺] during yolk absorption in freshwater is not unique to pink salmon, suggesting that it is not a unique characteristic of developing pink salmon associated with preparation for early ocean entry. This trait is shared by at least two fish species of the genus *Oncorhynchus* with differing life history strategies: the early-migrating anadromous pink salmon and the non-anadromous rainbow trout. Nonetheless, the observation of increasing and high levels of whole-body Na⁺ common to these fish species during early development is novel and has not been previously characterized in this context.

Interestingly, the mechanism by which pink salmon and rainbow trout achieve such high whole-body [Na⁺] during early development does not appear to be the same. Rainbow trout experience increasing Na⁺ uptake rates during development which peak around yolk absorption before dropping to adult levels. Paracellular permeability rates are maintained throughout this time-frame, suggesting that rainbow trout increase whole-body [Na⁺] via increased Na⁺ uptake. In contrast, pink salmon do not alter Na⁺ uptake rates during development and paracellular permeability appears to increase suddenly just prior to yolk absorption, suggesting that these fish regulate whole-body [Na⁺] via modulation of Na⁺ efflux rates. Perhaps regulation of Na⁺ influx and efflux is a strategy for maintaining
hydromineral balance upon transition to saltwater in pink salmon. The simultaneous increase in permeability observed in pink salmon suggests that these fish are preparing for Na⁺ secretion associated with life in seawater via paracellular pathways by increasing leakiness of the tight junctions while in freshwater.

4.3 LIMITATIONS OF RESEARCH

Fish exposure prior to the eyed stage (Chapter 2)

Both species used throughout the two data chapters were obtained as eyed embryos to minimize the mortality that is commonly associated with transportation of fish earlier in development. Rainbow trout used for Chapter 2 were obtained at 239 ATU post-fertilization from the Fraser Valley Trout Hatchery (Abbotsford, BC; annual average values in mM: Na⁺, 0.3 – 0.4; Cl⁻, 0.3 – 0.8; Ca²⁺, 1.0 – 1.2; Mg²⁺, 0.3 – 0.4; K⁺, 0.03 – 0.05; HCO₃⁻ unknown; alkalinity, 75 - 100 mg L⁻¹ as CaCO₃; hardness unknown; pH 7.1 – 8.1; Sara Northrup, personal communication, January 2014). Following arrival to UBC’s research facilities the embryos were subjected to their respective freshwater rearing [Na⁺] made by adding NaCl to Vancouver City dechlorinated tap water (annual average values in mM: Na⁺, 0.08; Cl⁻, 0.05; Ca²⁺, 0.03; Mg²⁺, 0.006; K⁺, 0.004; HCO₃⁻ unknown; alkalinity, 3.0 mg L⁻¹ as CaCO₃; hardness 3.43 mg L⁻¹ as CaCO₃; pH 6.4 – 6.8; Metro Vancouver, 2012) to achieve levels of 0.08, 0.45 and 1.7 mM for the low-, medium- and high-[Na⁺] treatments, respectively. It is not known whether timing of exposure to [Na⁺] treatment waters is critical in freshwater reared rainbow trout; however, there is one report indicating that timing of dilute seawater-reared (Na⁺, 11.7 mM) Atlantic salmon to low-[Na⁺] water (Na⁺,
0.22 mM) influenced long-term Na\(^+\) uptake kinetic characteristics (McWilliams, 1993). Thus, changes in water ion composition during embryonic development in freshwater rainbow trout may have an influence on the ultimate phenotype and is worthy of further investigation.

**Factors affecting measured Na\(^+\) flux rates (Chapters 2 and 3)**

It is likely that the measurement of Na\(^+\) uptake rates in resting, normoxic conditions underestimate uptake rates in the natural environment. Additionally, the nature of the Na\(^+\) uptake experiments was likely stressful. Relatively low water to fish mass ratios were used to ensure the sensitivity of the experiment was not compromised; nonetheless, a ratio of at least 1 mL of water per 0.1 g fish mass was achieved in all cases. Additionally, the flux chambers were continuously aerated and ammonia and pH measurements were taken throughout to ensure water quality was maintained.

**The use of whole-body [Na\(^+\)] as a proxy for plasma [Na\(^+\)] (Chapters 2 and 3)**

Typically, plasma ion levels are used as an indicator of ionoregulatory status in large fish, but in small fish, such as larval and juvenile salmonids (<5 g), plasma samples are difficult to obtain and whole-body levels are used as a proxy (e.g. Brauner et al., 2003; Gallagher et al., 2013; Grant et al., 2009; Kumai et al., 2011; Sackville et al., 2011; Sackville et al., 2012). For the purpose of Chapter 2, whole-body [Na\(^+\)] and body water content were used as an indicator of hydromineral balance and I concluded that there was no evidence of hydromineral disturbance in any of the groups. For the purpose of Chapter 3, whole-body [Na\(^+\)] was compared between species and I concluded that the developmental trend in both
species was similar. It is possible that plasma $[\text{Na}^+]$ measurements may have led to a different conclusion, unfortunately this information was not available.

In adult rainbow trout (average mass 169 g), whole-body $[\text{Na}^+]$ correlates with plasma $[\text{Na}^+]$ following short-term exposure to progressive increases in salinity; plasma $[\text{Na}^+]$, reported as mmol L$^{-1}$, is consistently approximately 3.5 – 4 times higher than whole-body $[\text{Na}^+]$, reported as mmol·(kg wet mass)$^{-1}$ (Prodocimo et al., 2007). It is not known whether the relationship between whole-body $[\text{Na}^+]$ correlates with plasma $[\text{Na}^+]$ in a similar manner in larval fish and adult fish or if the increased whole-body $[\text{Na}^+]$ in developing fish is representative of high plasma Na$^+$ levels. Likely, the relationship between whole-body $[\text{Na}^+]$ and plasma $[\text{Na}^+]$ is different in larval fish and the increased $[\text{Na}^+]$ represents a combination of increased plasma and tissue Na$^+$ levels but this has yet to be investigated. Measurement of whole-body Cl$^-$ may have provided a more complete picture of environmental $[\text{Na}^+]$ on hydromineral balance. Likely, the relationship between whole-body $[\text{Na}^+]$ and plasma $[\text{Na}^+]$ is different in larval fish and the increased $[\text{Na}^+]$ represents a combination of increased plasma and tissue Na$^+$ levels but this has yet to be investigated. This will be further discussed in sections 4.4.

4.4 POTENTIAL APPLICATIONS OF THE RESEARCH FINDINGS

The ionoregulatory hypothesis

Until recently it has generally been accepted that gas exchange, particularly oxygen uptake, is the primary selective pressure driving gill development (Krogh, 1941). In recent years, however, a body of research has emerged which challenges this idea, supporting the
hypothesis that the first physiological function to transition to at the gill during
development is ionoregulation (Li et al., 1995), termed the ionoregulatory hypothesis.
Morphological evidence suggests that ionocytes appear on the gills significantly earlier
than gas exchange structures in every teleost species examined to date (e.g. rainbow trout,
*O. mykiss*, Gonzalez et al., 1996; Japanese flounder, *Paralichthys olivaceus*, Hiroi et al., 1998;
bass, *D. labrax*, Varsamos et al., 2002). Further biochemical and microscopic structural
analysis has suggested that these ionocytes are not only present, but are functional as early
as three days post hatch in tilapia (Li et al., 1995).

Despite the growing body of evidence supporting the ionoregulatory hypothesis the
functional significance of the morphological and biochemical studies is not well
understood; more physiological data is required. The first physiological study used a
functional ablation technique to determine that the role of the gill becomes critical earlier
in development in terms of ionoregulation compared to that of oxygen uptake (Rombough,
2002). More recently, flux experiments designed to partition the fish into anterior and
posterior compartments demonstrated that ion uptake was a gill-dominated process
significantly earlier than gas exchange (Fu et al., 2010).

However, it has been demonstrated in adult teleost fishes that ambient ion
concentrations strongly influence gill morphology, particularly of ionocytes in the gill to
improve ion uptake capacity (Greco et al., 1996). Although some evidence exists to suggest
this may not be the case in larval fish (Fu et al., 2010; Shen and Leatherland, 1978a), the
phenotypic plasticity of ion uptake in larval fish has not been directly measured. Chapter 2 addressed this gap in the literature.

Chapter 2 provided the first quantification of the plasticity of ionoregulatory development in larval fish in freshwater. First, it appears that the mechanisms of modulating Na\(^+\) balance in response to the low-[Na\(^+\)] environments is different in larval fishes than in adults. Second, the ability of larval fish to modulate Na\(^+\) uptake transport in response to environmental [Na\(^+\)] depends on the age of the fish. Early in development, when ionocytes are beginning to mature at the gills, larval rainbow trout exhibited little plasticity. As ionoregulation shifts to mature ionocytes located on the gill, developing fish exhibited increased plasticity associated with strategies observed in juvenile and adult fish exposed to ion-poor waters. This is likely due to the changing distribution of ionocytes and the fact that the proliferation of ionocytes at the gills is hard-wired early in development and capacity for upregulation of mature ionocytes is limited. This suggests that the ontogeny of active ion uptake is relatively fixed in the developing rainbow trout and that the first physiological function of the developing gill will continue to be some aspect of ion regulation, providing support for the ionoregulatory hypothesis.

Lastly, data from Chapter 3 has shed light on a novel idea concerning the ionoregulatory hypothesis. Early in development, at least two species of teleost fish, rainbow trout and pink salmon, exhibit very high and increasing levels of whole-body [Na\(^+\)] in freshwater. This trend becomes even more remarkable when compared to Na\(^+\) content observed in adult fish; developing fish experience [Na\(^+\)] greater than twice that observed in adult fish. Although not measured in the present study, it is likely that the majority of this
measured Na$^+$ is aqueously available and represents extracellular Na$^+$ stores. This suggests that extracellular fluid volume is likely far greater than intracellular fluid volume in larval fish. It has been demonstrated in several vertebrate species that extracellular volume decreases during periods of rapid growth (reviewed by Holmes and Donaldson, 1969). Indeed, the opposite is true of adult teleost fish; intracellular fluid makes up approximately 55% of total body mass whereas extracellular fluid makes up about 15% of body mass (reviewed in Holmes and Donaldson, 1969). It is possible that the observed increase in whole-body [Na$^+$] represents a disproportionate increase in the extracellular space relative to intracellular space in developing fish. The extracellular fluid includes the tissue fluid, coelomic fluid, and blood plasma. Perhaps developing fish require a more developed circulatory system in place before the rapid increase in tissue growth can occur. If this is the case, this would be consistent with the ionoregulatory hypothesis where an increase in extracellular ions are a pre-requisite of an expansion in extracellular volume and cardiovascular system development. While largely speculative, this is clearly an area worthy of future investigation.

4.5 DIRECTION FOR FUTURE RESEARCH

Future research should focus on characterizing ionoregulatory development in additional fish species, especially non-model teleosts. Much of our current knowledge of ionoregulation in larval (and adult) fishes is based upon work in a few model teleost species (i.e. zebrafish, tilapia and to a lesser extent, rainbow trout). This thesis, through examination of a non-model species, pink salmon, has illustrated the hazards of
generalizing physiological phenomena based on data from a limited number of species. For instance, it was previously thought that all developing fish experience high levels of ion uptake early in development before falling to values seen in juvenile and adult fish (i.e. Brauner, 2008); this does not appear to be the case in developing pink salmon.

**Based on Chapter 2**

Future studies should consider the effects of other environmental stressors on the ontogeny of Na\(^+\) balance and transport capacity in larval fish during the transition from cutaneous-dominated to gill-dominated ionoregulation. For instance, what effect will hypoxia have on ionoregulatory development? McDonald and McMahon (1977) reported that differences in lamellar surface area were not observed in the gill of Arctic char exposed to chronic hypoxia during development until 47 dph, suggesting that gas exchange may not be altered in response to hypoxia as has been described in adult fish. Increased water flux during hypoxia associated with the osmoregulatory compromise may result in ionoregulatory challenges that would need to be compensated. The characterization of ion uptake during development in hypoxia- versus normoxia-reared fish is important to consider when determining the major role of the gills during development.

**Based on Chapter 3**

It is not yet clear whether the heightened and increasing whole-body Na\(^+\) levels observed in rainbow trout and pink salmon is common to all developing Pacific salmon and trout, all salmonids or even all fish. Future work should focus on increasing the number of species for which we have whole-body [Na\(^+\)] data for developing fish. Similarly, it would be
interesting to measure levels of other ions, in particular those that are more specific to intracellular fluids, such as potassium, to help confirm that the high levels of body Na⁺ observed here are in fact contained within the extracellular space.

Lastly, more direct measurements of Na⁺ efflux rate would be beneficial to our understanding of Na⁺ balance in developing fish to help elucidate the specific mechanisms by which developing fish are increasing whole-body Na⁺ levels. Measurements in pink salmon would be especially interesting, since they are undergoing smoltification during early life stages and a reduction of Na⁺ loss in freshwater may represent a novel component of smoltification in this unique fish species.
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