THE DEVELOPMENT OF SALINITY TOLERANCE IN JUVENILE PINK SALMON (*ONCORHYNCHUS GORBUSCHA*)

by

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B.Sc., Queen's University, 2008

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Zoology)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)

November 2011

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Abstract

Following yolk-sac absorption and gravel emergence pink salmon (*Oncorhynchus gorbuscha*) migrate into seawater (SW) at as small as 0.2 g. This life-history strategy is in contrast with most anadromous salmonid species that generally spend 1-2 years growing in fresh water (FW) and physiologically preparing for life in SW before they migrate to SW as smolts. This study characterized for the first time the ontogeny of SW tolerance in pink salmon around the period of yolk-sac absorption. Post-hatch juvenile pink salmon were either held in FW for 26 weeks or transferred to SW every two weeks for 20 weeks to follow % survival, whole body (WB) Na⁺ and water content, as well as changes in wet and dry mass, gill Na⁺K⁺ATPase (NKA) activity and α1a and α1b mRNA isoform expression. An increase in gill NKA activity and the ratio of the α1b/α1a isoform expression, a plateau in WB water and Na⁺ levels, and the switch from catabolic to anabolic growth were all observed at the time of yolk-sac absorption in fish retained in freshwater. At this time, morbidity following subsequent SW transfer fell to 0% from a high of 100% for newly hatched alevins, but then rose to 25% in older fry, suggesting that a window of increased salinity tolerance exists for pink salmon at the time of yolk-sac absorption. This proposed window of SW tolerance is similar to the smolt window that has been identified for other salmonids; but in pink salmon appears to be endogenously mediated, as fish were reared under constant (12L:12D) photoperiod and at 5°C throughout the study. Moreover, smoltification is incomplete since transfer to SW further elevated gill NKA activity and increased gill NKA α1b/α1α isoform expression
ratio 8-fold at yolk-sac absorption. Thus, even the most SW-tolerant fish were not fully prepared for SW before entry, but responded directly to SW by further increasing hypo-osmoregulatory ability. This study filled the previously existing void of knowledge regarding the acquisition of salinity tolerance in juvenile pink salmon.
Preface

The UBC research ethics board that granted the permission to do this research was the UBC Animal Care Committee. The certificate that was approved for this work was A07-055 entitled the impact of sea-lice on pink salmon smolts.
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<table>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<tr>
<td>ATU</td>
<td>Accumulated Temperature Units</td>
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<tr>
<td>cDNA</td>
<td>Complimentary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Conductance Regulator</td>
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<tr>
<td>CIC-3</td>
<td>Chloride Channel</td>
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<tr>
<td>EF1-α</td>
<td>Elongation Factor</td>
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<tr>
<td>ENaC</td>
<td>Epithelial Sodium Channel</td>
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<tr>
<td>GH</td>
<td>Growth Hormone</td>
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<tr>
<td>FW</td>
<td>Freshwater</td>
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<tr>
<td>IGF-1</td>
<td>Insulin Like Growth Factor</td>
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<tr>
<td>LBP</td>
<td>Light Brain Pituitary Axis</td>
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<tr>
<td>MRCs</td>
<td>Mitochondrial Rich Cells</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MS-222</td>
<td>Tricaine Methanesulfonate</td>
</tr>
<tr>
<td>N</td>
<td>Sample Size</td>
</tr>
<tr>
<td>NHE</td>
<td>Sodium Proton Exchanger</td>
</tr>
<tr>
<td>NKA</td>
<td>Na⁺K⁺ ATPase</td>
</tr>
<tr>
<td>NKCC</td>
<td>Na⁺K⁺Cl⁻ Cotranspoter</td>
</tr>
<tr>
<td>POA</td>
<td>Pre-optic Area</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
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<tr>
<td>SW</td>
<td>Seawater</td>
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<tr>
<td>TH</td>
<td>Thyroid Hormone</td>
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<td>T4</td>
<td>Thyroxine</td>
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<tr>
<td>WBI</td>
<td>Whole Body Ions</td>
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<td>WB</td>
<td>Whole Body</td>
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<tr>
<td>W</td>
<td>Weeks Post-Hatch</td>
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Acknowledgements

I would like to acknowledge the entire comparative physiology group for making my time at UBC enjoyable, and for the large amount of knowledge COMPHY members imparted on me ranging from topics as diverse as plumbing and fish cardio-respiratory physiology.

I would especially like to thank my supervisors, Tony Farrell and Colin Brauner for the support and guidance they have provided over the last 3 years. I am proud of my completed thesis, and I know the quality would be lesser had my supervisors not taken the time to carefully edit and provide feedback on thesis drafts numerous times. You are both fantastic supervisors and I have grown as a scientist and learned a great deal under your tutelage, thank you for providing me with such an engaging topic, it has been a tremendous experience.

To my Mom, thank-you so much for always being enthusiastic and always encouraging me in the endeavors I undertake, and for allowing me to follow my interests, as peculiar as they sometimes may seem. I would also like to thank both my parents for raising me near the beach, where I developed a strong bond and love for all things connected to the ocean. Thank you to my father for instilling a love of learning and feeding my curiosity from a young age.

To Ted, thank-you for moving to Vancouver, I can't imagine what the last three years would have been like without you here. You never ceased to have faith in my ability to complete this degree, and for that I am grateful, because there were many times when my own confidence was severely lacking. You have been a tremendous boyfr
and I am excited (relieved) you have developed an appreciation for salmon both alive and in sushi form! Lastly, you are hilarious, so thank-you for all of the great laughs.
Chapter 1: General Introduction

Anadromous salmonids migrate from freshwater (FW) to seawater (SW) as juveniles but a great deal of interspecific variation exists in the timing and distance of this migration. Smoltification is a general term for the process that prepares some salmon species (Atlantic salmon, coho salmon, masu salmon, stream-chinook, sockeye) for SW while they are still in FW. Smolting generally occurs after the first 1 or 2 years of development in FW and is an environmentally mediated co-ordination of behavioral, morphological and physiological changes (Figure 1.1). Pink salmon are unusual compared with other salmons in that they migrate to SW immediately following the emergence from their gravel redds, where they hatched not long before (Figure 1.1). Gravel emergence occurs around the time that all the yolk has been absorbed, and the young fry must start to feed to survive. Thus, pink salmon can be as small as 0.2 g at SW entry (Heard 1991).

This almost immediate entry into SW after hatch represents the most extreme form of anadromy seen in salmonids, one that is shared by chum salmon (but chum fry emerge with a larger size due to their larger egg size compared with pink salmon). Despite the uniqueness of the pink salmon life strategy, little is known about the development of salinity tolerance in this species, which must occur and is the focus of this thesis. The remainder of this introduction will provide background information on the mechanisms of osmoregulation in both FW and SW, and the associated physiological challenges with diadromy (a migration of fish from FW to SW or vice versa).
Information regarding typical salmonid smolts, and the environmental influence on smoltification will be provided for comparison with pink salmon. Lastly, background information on pink salmon as well as the specific objectives of this thesis will be presented.

**The Importance of Ionoregulation and Osmoregulation**

The ability to control the concentration of electrolytes and volume of extracellular fluid is crucial to all vertebrate life. Fish in SW face desiccation while fish in FW face over-hydration due to differing osmotic gradients between the extracellular fluid and the ambient water across the gill and skin, which are permeable to both ions and water. Among aquatic vertebrates the *Osteichthyes*, or bony fishes, represent an extremely diverse and abundant group consisting of over 29,000 fish species. These fish occupy almost every aquatic and semi-aquatic niche, and can be found in waters of vastly varying salinities. Teleosts, a class of *Osteichthyes*, typically maintain a relatively narrow range of osmolality in their extracellular fluid (260-360 mOsm kg\(^{-1}\)) (Evans 2008, Krogh 1939, Smith 1932, Stefansson et al., 2008), with only slightly higher values in SW vs. FW species. However, with FW typically at 1-10 mOsm kg\(^{-1}\) and SW at about 1000 mOsm kg\(^{-1}\), maintaining osmotic homeostasis has opposite challenges in FW vs. SW. Thus, FW fish are subjected to the constant gain of water and loss of ions, while in SW fish are subjected to passive water loss and salt loads.
**Osmo and Ionoregulation in FW**

The kidneys of fish in FW produce a large amount of dilute urine to counteract the constant influx of water from the environment (Stefansson et al., 2008). The very low ionic content of FW requires fish in this medium to utilize food as a source of ions and to actively absorb ions from the hypotonic freshwater (Stefansson et al., 2008) to compensate for ions lost passively across the gill, skin and with the urine. The gill ionoregulatory mechanisms have yet to be completely elucidated, but the current consensus will be summarized here. The cells involved in ionoregulation at the fish gill are typically referred to as mitochondrion rich cells or MRCs (Hwang and Lee 2007). Na⁺ is transported across the apical membrane of MRCs in exchange with H⁺ either through a Na⁺,H⁺ exchanger (NHE) or via an epithelial Na⁺ channel (ENaC) energized by an H⁺-ATPase. Na⁺ is then transported across the basolateral membrane into the blood via Na⁺,K⁺ ATPase (NKA) (Hwang and Lee 2007, Evans et al., 2005, Kaneko et al., 2008). Cl⁻ uptake from the water is thought to occur via Cl⁻/HCO₃⁻ exchange on the apical membrane of chloride cells (Evans et al., 2005, Hwang and Lee 2007) and it has been hypothesized that a cystic fibrosis transmembrane conductance regulator (CFTR) transports Cl⁻ across the basolateral membrane, but this has yet to be characterized (Figure 1.2a). A ClC3 chloride channel has also been implicated in basolateral membrane Cl⁻ transport (Hwang and Lee 2007, Tang et al., 2009), but there are likely species-specific differences.
Osmo and Ionoregulation in SW

SW fish avoid dehydration by drinking SW and actively take up monovalent ions (Na\(^+\) and Cl\(^-\)) at the gut, which then draws water osmotically into the extracellular fluid. The excess Na\(^+\) and Cl\(^-\) in the fish are then excreted at the gills. The divalent ions in SW remain in the gut (they are precipitated to render them osmotically inactive) (Grosell et al., 2006) and any taken up by the gut are excreted in the small amount of urine that SW fish produce (Smith 1930, Kaneko and Hiroi 2008). The mechanisms by which Na\(^+\) and Cl\(^-\) are excreted at the gill in SW fish are better understood than that of uptake in FW. On the basolateral membrane of the MRCs, NKA generates an electrochemical gradient providing the driving force for Na\(^+\) and Cl\(^-\) excretion. Since NKA exchanges Na\(^+\) and K\(^+\) in a ratio of 3:2, respectively, the intracellular environment becomes electronegative (approximately -100mV) and extracellular Na\(^+\) is elevated. Basolateral Na\(^+\),K\(^+\),2Cl\(^-\) channels (NKCC) transport Cl\(^-\) from the blood into the negatively charged cell, and Cl\(^-\) exits the cell through an apical CFTR driven electrochemically. Na\(^+\) exits via a paracellular pathway between the leaky tight junction of the chloride cell and an accessory cell, also down its electrochemical gradient into the environment (Figure 1.2b) (Evans et al., 2005, Evans 2008, Stefansson et al., 2008, Keys and Willmer, 1932, Potts, 1984).

While the gills of FW and SW fish both possess MRCs, they differ in their morphology. The MRCs of fish in SW possess larger, more numerous MRCs that have an extensive tubular system that is continuous with the basolateral membrane and they possess a large apical crypt. Such a crypt does not exist in the MRCs of FW fish; instead the apical surface is broad and contains many microvilli (Stefansson et al., 2008). For a
more detailed description of ionoregulatory cells see reviews by Evans et al., (2005), Hwang and Lee (2007), and Kaneko et al., (2008).

Most fish species are stenohaline, meaning they can only tolerate a narrow range of salinities, however; approximately 1 % of fish species can tolerate and maintain osmotic homeostasis in a wide range of salinities, termed euryhaline, despite the reciprocal challenges posed by FW and SW (Moyle and Cech 1996). In order to migrate between environments that differ dramatically in salinity, fish MRCs must possess the plasticity to undergo both functional alterations and molecular and cellular remodeling (Hwang and Lee 2007, Kaneko et al., 2008).

**Diadromy as a Strategy**

One form of euryhalinity is diadromy, a term that refers to fish that migrate between FW and SW, or vice versa, and generally occurs at a specific life-history stage (Meyers 1949, McDowall 1997). There are three forms of diadromy: anadromy, catadromy and amphidromy. Anadromous fish such as salmonidae and clupeidae (shads and herrings) spend most of their time feeding and growing in SW and migrate to FW to reproduce. Catadromous fish such as anguillidae (eels) and galaxiidae feed and grow in FW and migrate to SW to reproduce. Aphidromous fish, such as some galaxiidae and aplochitonidae (South American peladillos), migrate to SW soon after hatching with early growth occurring in SW, juvenile fish then migrate back to FW where they continue to grow and mature before reproducing in FW (Meyers 1949, McDowell 1997). There are 227 known diadromous species (110 are anadromous, 56 catadromous and 61 amphidromous) although this is likely an underestimate (McDowell 1988, McDowall
1997). On a global scale anadromy is much more common in temperate and sub-arctic regions; and is therefore more prevalent in the northern hemisphere, whereas catadromy is more widespread in the tropics (McDowell 1987, Gross et al., 1988, Stefansson et al., 2008), likely because the marine environment is more productive than the limnic environment in the northern hemisphere while the opposite is true for the southern hemisphere (Gross et al., 1988, Dodson et al., 2009). Anadromy allows fish to take advantage of beneficial aspects of both FW and SW eco-systems within the lifespan of an individual. Such migrations presumably evolved to allow species to exploit conditions that would be beneficial to both growth and reproduction (Gross et al., 1988).

A well-known anadromous family is *Salmonidae*, but anadromy is not unique to salmonids. Other anadromous fish include, lamprey, sturgeons, osmerids, clupeid (shads) and basses (striped bass) (Reynolds 2002, Stefansson et al., 2008). A unique characteristic of salmonids is the possession of a ‘smolt stage’ in which they become physiologically prepared for life in SW while still residing in FW (Hoar 1988, Stefansson et al., 2008). This differs from the usual strategy of diadromy in which acclimation to FW/SW is initiated following exposure to the new environment (Stefansson et al., 2008, McCormick 2009).

**Smoltification**

The term smoltification encompasses a wide range of changes to a juvenile salmon's morphology, physiology, biochemistry and behaviour. The term ‘smolt’ was first given to Atlantic salmon (*salmo salar*) to describe the silver stage (caused by hypoxanthine and guanine deposits) observed at the time that the fish migrated from
rivers to SW (Hoar 1976, Hoar 1988). An analogous stage has been identified in anadromous Pacific salmon that have juveniles that possess extended FW residency (amago, coho, masu, sockeye and stream-type chinook salmon). Prior to smolting, juveniles, (referred to as parr) from these species cannot ionoregulate in hyper-osmotic environments. The increased salinity tolerance required for SW entry is acquired only after a period of growth in FW, and generally occurs during a specific season (spring) where photoperiod and temperature are strong cues for smoltification (Clarke and Hirano 1995).

**Non-Ionoregulatory Changes Associated with Smoltification**

Only brief mention will be made to the numerous behavioral, morphological and physiological changes occurring during smoltification that are not directly related to the acquisition of SW tolerance, which is the focus of this thesis (for a more complete description see the review by Hoar 1988). These changes include the loss of parr marks, silvering, the darkening of fin margins, altered retinal pigmentation, olfactory sensitivity, and buoyancy and an increased metabolic rate (Hoar 1988, McCormick 2009). Behavioral changes include the loss of positive rheotaxis and the development of schooling behaviors as well as salinity preference (Hoar 1988, McCormick 2009). Typically fish need to reach a certain size threshold before smolting. Smolts have a more streamlined body and lower condition factor than parr, (the FW precursor to smolts). The growth and increased metabolism (Atlantic salmon smolts have a metabolic rate that is 50% higher than that measured in parr) likely prepare smolts for the change of prey composition they will experience upon SW entry (Stefansson et al., 2003a).
Development of Hypo-osmoregulatory Ability

Arguably the most critical physiological change that occurs during smoltification is the development of a hypo-osmoregulatory ability. The morphological features of SW MRCs are absent in parr but appear in smolts in FW during the spring, before they embark on their SW migration (Pisam et al., 1988, Lubin et al., 1989, Stefansson et al., 2008). Also the number and size of MRCs have been found to increase in Atlantic salmon smolts, as is characteristic of SW relative to FW adapted species (McCormick 2001).

Many of the transporters involved in ion excretion outlined above have been found to play a role in the development of euryhalinity in salmon smolts. One transporter, gill NKA has become a reliable indicator of SW readiness, and both NKA activity and protein abundance become elevated during smoltification in FW prior to SW migration (Stefansson et al., 2008).

Gill NKA consists of three subunits (α, β, γ) and several isoforms of each exist. Richards et al. (2003) found that α1a levels decrease while α1b levels increase during the SW acclimation of *Oncorhynchus mykiss* (rainbow trout). Smolting Atlantic salmon have been shown to increase the mRNA expression of the α1b isoform 6 fold and decrease α 1a expression by 75% during smoltification (prior to SW entry) (Nilsen et al., 2007, Stefansson et al., 2007), a pattern that is dampened in landlocked Atlantic salmon (Nilsen et al., 2007). There is likely a differential role of the two isoforms in FW and SW, but this remains to be determined (Stefansson et al., 2008). Thus, the dramatic shift in the ratio of α1b/α1a NKA isoform has become a new indicator of smoltification.
Increases in gill NKCC expression and protein abundance can also be indicative of smoltification. Gill NKCC protein levels increased 3.3 fold in Atlantic salmon from February to May (Pelis et al., 2001), which corresponded with an increase in gill NKA activity, and salinity tolerance associated with smoltification. Furthermore, the abundance and mRNA levels of NKCC have been found to be higher in anadromous Atlantic salmon compared with landlocked populations (Nilsen et al., 2007). Nilsen et al. (2007) also found increased CFTR mRNA levels during smoltification.

The intestine must also prepare its uptake and transport mechanisms for SW, prey availability (of a different type), and increased scope for growth (Stefansson et al., 2008). Intestinal fluid absorption rate increases significantly during parr-smolt development in Atlantic and coho salmon (Collie and Bern 1982, Veillette et al., 1993, Sundell et al., 2003). This appears to be linked to cortisol-mediated increases in intestinal (and gill) NKA activity and an increase in intestinal epithelial paracellular permeability during smoltification (Veillette et al., 1993, Sundell et al., 2003). Upon SW entry further increases in gill and intestinal NKA activity as well as decreased intestinal paracellular permeability have been observed in Atlantic salmon by Sundell et al., (2003), who suggested water flow at the intestine may occur through a transcellular pathway, driven by NKA activity, once fish are in SW (Sundell et al., 2003).

**Environmental Control of Smolting**

All of the smolt related changes are typically synchronously induced in the spring following 1 or 2 years of growth in FW. They appear to be strongly influenced by environmental cues, especially the increasing day length associated with spring.
Environmental control seems to be via the stimulation of the light-brain-pituitary axis (LBP) and involves hormonal control mechanisms (for review see Stefansson et al., 2008). The main environmental factors that have been identified as smolt initiation signals are photoperiod and temperature, the roles of which will be discussed below.

**Photoperiod**

Classic smolting species such as Atlantic, coho and stream-type chinook salmon depend upon photoperiod cues to coordinate the physiological changes related to smoltification (e.g. increased SW tolerance). Photoperiod phase at the time of emergence from gravel greatly influences smolt development. For example, smolt development in coho fry that initially emerge in the spring is blocked by the long photoperiod until they are exposed to a short photoperiod the following winter. Lengthening photoperiod in the second spring then triggers smoltification (Clarke and Hirano 1995). Smoltification can be artificially advanced if fry emerge under a short winter photoperiod; the lengthening photoperiod in the first spring provides sufficient stimulation for fish to smolt a year early (Clarke and Hirano 1995). Similarly, Zaugg et al. (1986) observed that spring-chinook that hatched 4-5 weeks early under a short photoperiod developed smolt characteristics within 6-7 months as opposed to the usual 18 months.

Clarke et al. (1989) investigated the importance of initial day length on the development of smolt characteristics in chum, coho, ocean-type chinook, and stream type chinook. The fish were subjected to short (9.5 h) or long (14.5 h) day length for 2 months from first feeding and then were subsequently reared on a natural photoperiod
for 4 months. The coho and stream-type chinook initially exposed to the long day length exhibited poor growth and SW-adaptability compared with those exposed to the short day length. Chum and ocean-type chinook (both of which employ early ocean-entry) displayed similar growth and SW-adaptability regardless of the photoperiod treatment indicating that early ocean-entry salmonids are much less affected by photoperiod manipulation.

Thorarensen and Clarke (1989) investigated how Pacific salmon measure day length by rearing coho under a short photoperiod (6L:18D or 10L:14D) for 2 months and then exposing them to a long-day photoperiod (16L:8D) the equivalent skeleton photoperiod (9L:6D:1L:8D) or a short photoperiod (same total light as the skeleton) (10L:14D). They found the skeleton photoperiod (and long photoperiod) was effective in producing coho smolts while the short photoperiod was not, suggesting that the time of day fish experience direct light exposure and not accumulated light exposure stimulates smolting (Thorarensen and Clarke 1989).

Since 1989 the mechanisms through which photoperiod triggers smoltification has been further elucidated. The light-brain-pituitary (LBP) axis is thought to convey photoperiod information to the endocrine system (Stefansson et al., 2008). The retina and pineal organ detect light and send the information to central brain regions including the preoptic area (POA). The POA incorporates the light information with other neural inputs before sending it to the pituitary, which regulates hormonal release into the circulation (blood) (Stefansson et al., 2008, Ebbesson et al., 2007). Cortisol, growth hormone (GH) and thyroid hormone (T4) levels all increase in blood plasma during smoltification and play transformational roles in the process (Hoar 1988, McCormick et
al., 1998). In particular, cortisol is involved in the preparatory changes that occur to increase salinity tolerance including, increases in the number and size of chloride cells, and gill NKA and NKCC protein levels. This is achieved via interactions of cortisol with GH, IGF-1 and corticosteroid receptors on the gills (Björnsson et al., 1995, Björnsson 1997, Seidelin et al., 1999, McCormick 2001). In contrast, thyroid hormones mediate olfactory imprinting, body silvering, behavioral changes, and changes in visual sensitivity, all of which are related to smoltification (Hoar 1988, Stefansson et al., 2008, Iwata 1995, Hutchinson and Iwata 1998, Lema and Nevitt 2004).

A period of structural neural plasticity has been identified in coho smolts and involves increased innervation of retinal and pineal fibers into the preoptic area and other brain regions (Ebbesson et al., 2003). These changes were found to occur prior to the major increases in circulating TH and GH, supporting the hypothesis that increased retinal innervation of the POA is involved in the subsequent photoperiod triggered endocrine response (Ebbesson et al., 2003). Exposure of Atlantic salmon parr to continuous light was found to hinder both the proliferation of new, and the extension of existing retinal fibers (Ebbesson et al., 2007). Atlantic salmon parr subjected to a natural photoperiod displayed these changes in a manner similar to the coho mentioned above (Ebbesson et al., 2007). In a separate experiment exposure of Atlantic salmon to continuous light resulted in less circulating GH, cortisol and TH, as well as a dampened hypo-osmoregulatory response (Stefansson et al., 2007). Continuous light exposure appears to inhibit the parr-smolt transformation, but exactly what the short photoperiod provides smolting coho or Atlantic salmon that initiates brain development has yet to be fully understood (Ebbesson et al., 2007).
Temperature

Temperature is not the zeitgeber (environmental cue) for smoltification (McCormick et al., 2002), like photoperiod. Instead, temperature controls growth rate, and therefore impacts the time at which the size threshold for smoltification is reached (Clarke and Hirano, 1995). This means that salmon populations spawning at more northern latitudes generally have a longer FW residency period than more southerly populations. For example, in Europe the age of Atlantic salmon smolts increases from 1+ years in northern Spain to 4+ or 5+ years in northern Norway and Russia (Stefansson et al., 2008).

Fish that enter SW towards the end of the smolt window possess lower feed intake and growth, higher plasma ion levels and reduced growth hormone levels (Arnesen et al 2003). If smolts remain in FW beyond the smolt window, (when fish display peak smolt characteristics) their physiology, behavior, and metabolism will revert back to the state more suited for life in FW (desmoltification, or parr-reversion) (Hoar 1988). The effect of temperature on development has been simplified for fish by using accumulated temperature units (ATU, calculated by multiplying days post-fertilization by the temperature (°C)). The duration and timing of the smolt window has been found to be largely influenced by ATU (and therefore temperature) (McCormick 1999, Zydelewski et al., 2004).

Seasonal temperature cycles can also influence the timing and success of smolting (Clarke and Hirano, 1995). For example, steelhead trout raised on simulated seasonal temperature regimes (6.9°C to 18.6°C) showed greater migratory behaviour and greater
elevation of gill NKA activity, than those raised at constant 12°C (Zaugg and Wagner 1973; Wagner 1974)

Temperature can also combine with photoperiod to affect smoltification. If embryos experience higher temperatures then they will hatch earlier, and this will dictate the photoperiod they experience at hatch and first feeding, the importance of which was discussed above (Clarke and Hirano 1995). Temperature can control the rate of response to photoperiod and it has been found that a very low temperature exposure can prevent Atlantic salmon from responding to photoperiod (McCormick et al., 2000). There is evidence that upper and lower temperature thresholds may exist, above or below which various aspects of smoltification can be impaired and it is possible species or population specific temperature limits exist (Stefansson et al., 2008).

**Other Forms of Anadromy Within the Salmonid Family**

Not all salmonid species undergo the classic smoltification process described above. Some species are not even anadromous, or have non-anadromous populations (Quinn 2005). Also, considerable variation exists in the age and size at which salmonids embark on SW migrations. Rounsefell (1958) ranked the degree of “anadromy” among salmonid species based on characteristics of the anadromous life cycle (geographical length of migration, duration at sea, state of maturity at sea, spawning habits, mortality after spawning, and occurrence of freshwater forms). He concluded that anadromy amongst salmonids was least developed in the char (*Salvelinus* sp.), more developed in *Salmo* (e.g. Atlantic salmon), and most developed in Pacific salmon (*Oncorhynchus* sp.) (cited from McCormick 1994). A large factor affecting this variation in anadromy is the
age at which salmon migrate to SW, and three general patterns have been identified. Brown trout (Salmo trutta), Arctic char (Salvelinus alpinus), and brook trout (Salvelinus fontinalis) generally spend 3 or more years in FW, not migrating to SW until they are larger than 17 cm and only spend 2-5 months exploiting the coastal environment (McCormick 1994). Coho (O. kisutch) masu (O. masou), steelhead (anadromous rainbow trout, O. mykiss), and Atlantic salmon (S. salar) spend at least 1 year in FW before migrating in the spring as smolts, to SW where they will spend at least 1 year (McCormick 1994). The third pattern is exhibited by pink (O. gorbuscha) and chum (O. keta) salmon, which leave FW as fry around the time of gravel emergence (Hoar 1988, McCormick 1994, Heard 1991).

The timing of salinity tolerance varies in a genus-dependent manner, with Oncorhynchus developing SW tolerance at the earliest age followed by Salmo and then Salvelinus (McCormick 1994). Of the Oncorhynchus spp., pink salmon have the shortest FW residency and smallest size as SW-migrants, even compared to the closely related chum salmon (0.2g vs. 0.4g for chum Kojima et al., 1993). Since salmonids are generally thought to have a FW origin (Hoar 1976, Clarke and Hirano 1995, McCormick 1994) pink salmon have been deemed the most derived member of the salmonid family.

**Pink Salmon Background**

Pink salmon are the most abundant of the Pacific salmon and are widely distributed across the North Pacific. Their range spans from the coast of central California to Alaska in North America, and from Japan to the Russian arctic on the Asian side of the Pacific (Heard 1991). It was reported in 1967 that pink salmon made up 60%
in numbers of all salmon caught commercially in the North Pacific Ocean (Heard 1991). They remain important to fisheries today, and like other salmon species play a critical role in the eco-systems they inhabit, contributing to both FW and SW systems.

Pink salmon are unique for a number of reasons: they possess a fixed two-year life cycle, spending only 18 months in the ocean, and attain the smallest size at maturation. Furthermore, pink salmon migrate immediately following emergence from gravel to SW and emerge silver, without displaying parr marks (Heard 1991, Quinn 2005)

**What is Known About SW Tolerance in Juvenile Pink Salmon**

It is impressive that pink salmon are able to tolerate SW when so small because a very high surface area to body size ratio confounds osmotic and ionic homeostasis (Houston 1961, Conte and Wagner 1965, McCormick and Naiman 1984). Pink salmon embryos and newly hatched alevins are stenohaline (Honma 1982, Weisbart 1968) and so some gill transformation must occur before SW entry. Grant et al. (2009) observed a doubling of whole body ion (WBI) levels following SW entry in juvenile pink salmon, which took over 8 weeks to recover, a recovery period that corresponded with an increase in gill NKA activity. This result suggests that pink salmon may up-regulate hypo-osmoregulatory ability upon SW entry, and therefore do not possess a typical smolt-like phase. However, Sullivan et al. (1983) observed increases in plasma T4 levels, as well as elevated gill NKA activity in FW-held developing pink salmon fry, traits that are commonly seen during parr-smolt transformation. All observations were made in
FW-held pink salmon leading to uncertainty as to whether these changes would aid in SW tolerance or were solely developmental in nature (Sullivan et al., 1983). Also, pink salmon fry when placed in SW possess a superior ability to regulate plasma osmolality, Na⁺ and Cl⁻ levels, when compared to most other Oncorhynchus fry (Weisbart 1968). Thus, these results raise the possibility of a partial preparation for SW while in FW. Nevertheless, whether pink salmon, prepare prior to, at or following SW entry is currently unclear, and is the topic of my thesis.

**Thesis Design and Objectives**

This thesis investigated the ontogeny of SW tolerance in pink salmon, and the time at which they are physiologically prepared for SW entry. This was achieved by transferring developing fish from FW to SW every two weeks for 20 weeks following hatch, while following survival and sampling tissues for up to 8 weeks in SW for comparison with fish maintained in FW. The tissues were then used to assay a number of physiological indicators of hypo-osmoregulatory ability including whole body (WB) water content, WB Na⁺ levels, gill NKA activity and mRNA α1b/α1a isoform expression in both FW-held and SW-transferred fish. The two main objectives of my thesis were:

1. To characterize the ontogeny of salinity tolerance in juvenile pink salmon using SW-survival and physiological indices, and determine if pink salmon, like other smolting species, possess a physiological smolt window, or window of salinity tolerance, during which they are most ready for SW-entry

2. To determine whether increased size at SW entry confers increased hypo-osmoregulatory ability
Figure 1.1: A depiction of the generic anadromous salmonid life cycle. Mature adult salmon generally spawn in the summer or fall and lay their eggs in the gravel redd of FW streams and tributaries. Eyed embryos hatch in the winter as alevins and remain in the gravel redd, feeding endogenously on their yolk until emerging from the gravel as fry in the spring. Fish begin feeding exogenously at this stage, and spend at least one year as parr growing in FW. The following spring, salmon will become smolts which are physiologically prepared for SW. Smolts migrate to SW, where they will live and feed until returning to FW 1-6 years later as mature adults to spawn. Pink salmon differ from many anadromous salmonids because they migrate to SW as fry immediately after emerging from gravel redds.
Figure 1.2: A depiction of NaCl uptake mechanisms in a) Fresh water (FW) and NaCl extrusion in b) seawater (SW) modified from Evans et al., 2005. a) The current model for NaCl uptake is depicted here, as the mechanisms have yet to be fully elucidated and species-specific differences likely exist. Na⁺ ions are thought to enter the cell via either an ENaC like channel powered by a V⁺H⁺ATPase, or via an NHE, and likely enter the blood by a basolateral Na⁺K⁺ATPase. Cl⁻ ions enter the cell in exchange with HCO₃⁻ and exit the cell and
enter the blood possibly via a Cystic fibrosis transmembrane conductance regulator (CFTR) (see text for more details). b) The model for NaCl extrusion in SW is better understood for teleosts than that of NaCl uptake in FW. Na⁺, K⁺, and Cl⁻ ions enter the MRC via a basolateral Na⁺K⁺2Cl⁻ co-transporter (NKCC); Na⁺ is pumped back into the plasma via Na⁺K⁺ATPase; Cl⁻ exits the cell across the apical membrane via a CFTR. Na⁺ exits across leaky tight junctions between the MRC and AC (see text for more details).
Chapter 2: The Ontogeny of Salinity Tolerance in Juvenile Pink Salmon

Introduction

Many salmon species such as Atlantic salmon (Salmo salar) and coho salmon (Oncorhynchus kisutch) are known to undergo smoltification, a physiological condition that prepares fish to successfully osmoregulate in seawater while still residing in freshwater. Although increased salinity tolerance is one of the most important aspects of smoltification (Stefansson et al. 2008, McCormick 2009), the term ‘smolt’ was originally applied to Atlantic salmon, which lose their ‘parr’ marks and develop a silvery colour (due to guanine and hypoxanthine deposits). Smolts also have a more streamlined body type and lower condition factor than parr (for reviews see Hoar 1988, McCormick and Saunders 1987, Stefansson et al., 2008). In addition, a classic smolting species spends 1-5 years in FW before reaching a critical size threshold (for Atlantics more than 12 cm) that triggers smolting (Stefansson et al., 2008, McCormick 2009). Environmental factors also influence the timing of smoltification, with photoperiod being the main environmental cue (Hoar 1988, Stefansson et al., 2008). Since smolting species typically overwinter in freshwater and begin their seaward migration in the spring, the lengthening photoperiod triggers increases in circulating hormones such as, thyroxine (T4), growth hormone (GH), insulin like growth factor (IGF-I) and cortisol, which all correlate with increased salinity tolerance (Stefansson et al., 2008, McCormick 2009). The associated increase in size and quantity of chloride cells at the gill and up-regulation of ion transporter proteins, such as the Na⁺K⁺Cl⁻ cotransporter (NKCC), Na⁺K⁺ ATPase
(NKA) and cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels, allow for a greater gill ion excreting capacity and increase salmon survival in SW (Pisam et al., 1988, Hoar 1988, McCormick 2009).

Three subunits of gill NKA exist (a, b, g), with multiple isoforms of each subunit. In rainbow trout, Richards et al. (2003) found that the gill α-1a and α-1b subunit mRNA levels decreased and increased during SW acclimation, respectively. This pattern has also been observed in Atlantic salmon smolts (Nilsen et al., 2007, Stefansson et al., 2007). Thus, the differential roles of the two NKA isoforms in FW and SW may be diagnostic of the smoltification process.

Salmonid species have a smolt window during which time SW entry is favourable and salinity tolerance is elevated. Prior to and following this window salmonids are less able to successfully hypo-osmoregulate in SW (Boeuf and Harache 1982, Duston et al., 1991, Duston et al., 2011, Hoar 1988, McCormick et al., 1999, Stefansson et al., 2008, Handleand et al., 2004, Zydlewski et al., 2005, McCormick 2009), which can result in an excessive, perhaps lethal, elevation in plasma ions (Conte and Wagner 1965, Boeuf and Harache 1982, Arnesen et al., 2003). McCormick et al. (1999) found that a decrease in gill NKA activity (associated with the loss of salinity tolerance) following this window was directly related to accumulated temperature units (ATU; calculated by multiplying days post fertilization by °C) and that the length of the smolt-window was approximately 300-450 ATU (Stefansson et al., 1998, McCormick et al., 1999, Stefansson et al., 2008).

Pink salmon possess a unique life history relative to other salmonids. Their FW residency period is very short because they migrate to seawater immediately upon gravel emergence. Thus, they have little time to either grow or prepare for life in
seawater prior to seawater entry. In fact, their large surface area to volume ratio may be particularly disadvantageous for osmoregulation in SW. Indeed, previous work has suggested that pink salmon may not be fully prepared for SW entry at this time, which is suggested by the large initial increase in whole body ions (WBI) that occurred following SW transfer (Grant et al., 2009). WBI levels did decline progressively over time with residence in SW and in association with an increase in gill NKA that peaked at 8 weeks post-SW transfer. This suggests that pink salmon may respond to SW exposure itself rather than undergoing a smolt-like phase. Countering this suggestion is the observation that plasma thyroxine (T4) and gill NKA activity increase in juvenile pink salmon that remain in FW at the normal time for out-migration (Sullivan et al., 1983). But, in the absence of SW transfers by Sullivan et al. (1983), it is unknown whether or not these changes increased salinity tolerance.

Regardless of whether pink salmon become ready for SW entry prior to, during, or following entry, their ability to withstand SW as alevins and fry is much greater compared to most other Oncorhynchus species. Weisbart (1968) found a greater ability to regulate blood sodium and chloride levels following SW transfer compared with other salmon species. Thus, while pink salmon may not display some of the classic morphological smolt characteristics, it is possible that there is a time during which SW entry is favoured and that pink salmon do in fact possess a preparatory phase prior to SW entry like other salmon species.

This study examined the ontogeny of salinity tolerance in juvenile pink salmon from hatch through to 20 weeks post-hatch (568-1269 ATU), by holding fish in FW and conducting bi-weekly SW transfers over this duration. Pink salmon from Quinsam River
(the source for fish used in this study) naturally emerge and begin SW migration at
approximately 1000 ATU (personal comm. Pauline Scott and Dan Babcock), therefore SW
transfers in this study were conducted prior to, at, and following normal SW entry. A
suite of indicators of SW readiness were examined, which included survival in SW, whole
body (WB) water content, WB [Na⁺], gill NKA enzyme activity and mRNA expression of
the α-1a and α-1 b isoforms. These measurements will elucidate whether: 1. Pink
salmon, like other smolting species, possess a window of SW tolerance; or 2. Increased
body size improves SW tolerance.

Methods
Fish Sources/Husbandry

The Quinsam River Hatchery, Campbell River, British Columbia generously
donated approximately 5,000 pink salmon eyed embryos for these experiments. The
embryos had accumulated 487 temperature units (ATU; days following fertilization x
temperature (°C)) before transfer to the University of British Columbia on November 27,
2008. Embryos were then maintained in an environmentally controlled room and reared
in the dark in Heath trays supplied with re-circulating dechlorinated Vancouver city tap
water (5°C) that was replaced daily. Oxygen levels were maintained at > 95% of air-
saturation.

Embryos started hatching on Dec 11, 2008 and hatching was completed by
December 14, 2008. On January 11, alevins were transferred into two 50 L glass aquaria
containing charcoal-filtered, re-circulating dechlorinated FW that was changed 3 times
each week. The environmental chamber room maintained in these aquaria at 5°C and a
12L:12D photoperiod for the remainder of the experiment, which consisted of regularly transferring them to SW and sub-sampling over time, or sampling them as FW controls. Fish developmental stage is described by both the number of weeks post-hatch and ATUs (i.e., W0; 568 refers to 0 weeks post-hatch and 568 ATUs). Fish still possessing yolk are referred to as alevins and those with a fully absorbed yolk sac are referred to as fry (Figure 1.1). The first feeding occurred on February 23, 2009 when fish had absorbed approximately 90% of their yolk (W10; 924 ATU; Figure 2.1). Fish were fed powdered trout chow (Bio-vita Bio-Oregon, Longview, Washington) once or twice daily ad libitum. Feeding was withheld for approximately 18 h prior to sampling.

**Experimental Protocol and Sampling**

**FW Controls**

A total of 20-30 fish were terminally sampled from the FW holding tank every two weeks from December 14, 2008 through to June 14, 2009 (W0-W26; 568-1479 ATU; Figure 2.1) for measurement of whole body wet and dry weight (used to calculate % body water content) and whole body [Na+] (N=10), and gill NKA α1a and b mRNA isoform expression and gill NKA enzyme activity (N=10, fish aged W0-W4 (568-709 ATU), or N=20, fish aged W6-W26 (779-1479 ATU)). Fish were not sampled from FW on May 17, 2009 (W22, 1408 ATU) and fish aged W0 and W2 (568 and 639 ATU, respectively) were too small to successfully extract gills; consequently data are lacking from these groups.
SW Transfers

At two week intervals from December 14, 2008 until May 3, 2009 (Figure 2.1), 195 (W0-W4, 568-709 ATU; Dec 14-Jan 11) or 245 (W6-W20, 779-1269 ATU; Jan 25-May 3) fish randomly selected every two weeks from the FW holding tank were transferred to 100% SW (Instant Ocean, 32.5± 2.5 ppt) in a 50 L glass aquaria equipped with a charcoal filter. To assess salinity tolerance, fish were monitored daily and any moribund fish were euthanized immediately. Terminal sampling of live fish occurred at regular intervals after each SW transfer: 24 h, 5 days (d), 2 weeks (wks), 4 wks and 8 wks. The fish samples were used to measure whole body wet and dry weight (used to calculate % body water content) and whole body [Na+] (N=10), and gill NKA α1a and b mRNA isoform expression and NKA enzyme activity N=10 (W0-W4) or N=20 (W6-W20). Fish were targeted before, at and after yolk absorption (as an estimate of the time of natural SW migration) to measure gill NKA α1 mRNA isoform expression (W4, W12, W18 and W20; 709, 989, 1199 and 1269 ATU, respectively), and gill NKA activity post-SW transfer (W12, W18 and W20: 989, 1199 and 1269 ATU, respectively).

Fish Sampling Protocol

Fish were individually euthanized in a receptacle containing a lethal dose of buffered tricaine methanesulfonate (1.0 g L-MS-222; Syndel Laboratories, Vancouver BC, Canada). Once completely immobile, individual fish were rinsed with de-ionized water, blotted dry, transferred to a pre-weighed 15 mL polystyrene tube, weighed and then either dried at 65°C to constant weight and used for whole body Na+ measurement, or
frozen immediately in liquid nitrogen inside a 1.5 mL Eppendorf tube and stored at -80°C for future measurement of gill mRNA isoform expression and NKA activity.

**Analytical Techniques**

**Determination of % water content and WB [Na+]**

% Body water content was calculated as follows:

\[
[(\text{wet weight-dry weight})/(\text{wet weight})]\times 100.
\]

A method similar to that used by both Grant et al. (2009) and Sackville et al. (2011) was adopted here. Dried fish were digested at 65°C in 1M nitric acid (10X the wet weight of the fish). Fish were dissociated with a metal spatula to aid the complete digestion, and following at least 48 hours after the addition of nitric acid, the tubes were vortexed and contents were allowed to settle at room temperature overnight. The supernatant was then analyzed for Na+ content on a flame atomic absorption spectometer (Spectra AA-220FS; Varian, Mulgrave, VC, Australia) and then standardized to dry body mass for total body [Na+].

**Gill NKA activity**

A procedure modified from McCormick (1993) was used to measure gill NKA activity. This involved homogenizing gill samples and measuring the difference in the amount of phosphate released from the gill homogenates with and without the NKA specific inhibitor, ouabain (final concentration 1 mmol·l⁻¹). Gills were dissected from the frozen fish on ice and homogenized on ice in SEID buffer (pH=7.5, 150 mmol·l⁻¹ sucrose, 10 mmol·l⁻¹ EDTA, 50 mmol·l⁻¹ imidazole, 0.1% sodium deoxycholate) using a glass homogenizer. The homogenate was centrifuged for 1 min (4°C) at 5000 x g to remove
insoluble material and the supernatant was used in the assay of gill NKA. Protein concentration of the gill homogenate was measured using the Bradford method (Bradford 1976). All samples were run in triplicate and the average value for each fish was used in the statistical analyses.

**Gill mRNA Expression**

The protocol followed is described in Bystriansky et al. (2006) and Bystriansky and Schulte (2011). Briefly, a guanidine thiocyanate method (Chomczynski and Sacchi, 1987) was used to extract total RNA from gill samples using TriZol Isolation reagent (Invitrogen, Carlsbad, CA, USA). The isolated total RNA concentration was determined spectrophotometrically. RNA purity was confirmed by running 2 µg on an agarose gel (1%). First strand cDNA was synthesized from 2 µg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). Quantitative RT-PCR (qRT-PCR) was conducted using an ABI Prism 7000 sequence analysis system (Applied Biosystems Inc., Foster City, CA, USA). PCR reactions had 1 µl of cDNA, 150 pmoles of each primer and Universal SYBR green master mix (Applied Biosystems Inc., Foster City, CA, USA) (Bystriansky et al., 2006, Bystriansky and Schulte 2011). Forward and reverse primers used for each gene were isoform specific and tested to ensure that they amplified only a single target gene. Primer sequences used were: EF1-α forward 5’ GAG ACC CAT TGA AAA GTT CGA GAA G 3’, EF1-α reverse 5’ GCA CCC AGG CAT ACT TGA AAG 3’; NKA α-1a forward 5’ GGC CGG CGA GTC CAA T 3’ NKA a1-a reverse 5’ GAG CAG CTG TCC AGG ATC CT 3’, NKA α-1b forward 5’ CTG CTA CAT CTC AAC CAA CAA CAT T 3’ NKA a-1b reverse 5’ CAC CAT CAC AGT GTT CAT TGG AT 3’. For each gene sampled, the
relative quantity of mRNA was normalized to an endogenous gill reference (EF1a) and expressed relative to the mean value for pink salmon aged W4 (709 ATU), which was the earliest FW, control measured (Bystriansky et al., 2006, Bystriansky 2011).

**Statistical Analysis**

All data are presented as means ± SEM for N fish (8-10 unless otherwise specified on the figure). Statistical comparisons were made both between fish developing in FW, and between SW fish and their closest matched sample time in FW. For example, fish sampled after 24 h or 5 d were compared to the initial FW control, while those sampled after 2 wks, 4 wks and 8 wks in SW were compared to the same sampling dates for the FW control. A one-way analysis of variance (ANOVA) followed by a Holm-Sidak post-hoc test was used to compare groups when significance was found with ANOVA. Statistical analyses were performed on Sigmastat (version 10) and a level of p<0.05 was considered significant.

**Results:**

**Morbidity Post-SW Transfer**

Most alevins transferred from FW to SW at 0 or 2 wks post-hatch (W0; 568 ATU; W2; 639 ATU) died within 5 days and 2 weeks, respectively (Figure 2.2). At 4 weeks post-hatch (W4; 709 ATU), SW transfer resulted in 27% morbidity after 5 d and 41% after 2 weeks, (Figure 2.2). Of fish transferred at 4 wks post-hatch (W4; 709 ATU), 59 % survived in SW for 4 weeks at which time they were terminally sampled. Therefore, the majority of fish could survive an abrupt SW transfer starting 4 wks post-hatch.
In fish transferred to SW 6 to 14 wks post-hatch, survival approached 100%. In fish transferred to SW 6 weeks post-hatch (W6; 779 ATU), 89% of fish survived for 17 days, and in those transferred at 8 and 14 weeks post hatch (W8-W14; 849-1059 ATU) (Figure 2.2) 98% survived for 8 weeks. Beyond this period of nearly 100% survival, a modest rate of morbidity returned for SW transfers at 16, 18 and 20 weeks post-hatch (W16-W20; 1129-1269 ATU, Figure 2.2), but this morbidity never exceeded 25% (Figure 2.2).

**% Body Water Content**

In FW, % whole body water content increased significantly during development from 62% at hatch to about 82% by 14 weeks post-hatch (W14; 1059 ATU). % Whole body water content stabilized at between 81.2% and 82.8% from 1000 to 1500 ATU (W12-W26) (Figure 2.3a).

Due to the large observed effect of development on % body water content in FW, statistical comparisons of values obtained following SW transfer were restricted to time matched controls when possible. Furthermore, due to the large number of transfers to SW and subsequent sampling times in SW, data for all transfers are combined and grouped relative to time in SW (24 h, 5d, 2, 4 and 8 weeks) (Figure 2.3b).

In general, % whole body water content was remarkably similar between SW- and FW-held fish with the exception of transfer to SW shortly following hatch. Following 5 d in SW in fish transferred at 568 and 639 ATU (W0, W2), there was a large significant reduction in % whole body water content relative to time matched FW held fish, (Figure 2.3b). Additional significant differences are shown in Figure 2.3b, where in some
instances, % body water content following 5 days in SW was elevated relative to time matched FW-held fish (W8, W14, W16, W20; 849, 1059, 1129, 1260 ATU).

**Wet and Dry Mass**

In FW, wet mass almost tripled during the 26 wk experiment but this apparent growth was not always realized as dry mass (Figure 2.3c). In FW, dry mass decreased progressively from 0.055 g in recently hatched alevins (W0; 568 ATU), reaching a nadir at 0.028 g in fish aged 14 weeks post-hatch (W14; 1059 ATU; figure 2.3d), before regaining dry mass, which peaked at 0.08 g at W24 (1409 ATU). The initial loss of dry mass was compensated by a gain in water content, and the post-1100 ATU gain in dry mass reflected true anabolic growth.

Following SW transfer, wet body mass rarely differed from the FW values and similarly increased with ATU. However, SW transfers could produce small significant decreases in wet mass at 568 to 990 ATU (W0-W12) compared with the FW control, but these decreases did not necessarily persist in SW (Figure 2.3c). SW transfer after 1100 ATU could slightly delay but never prevent the anabolic growth reflected in increased dry mass (Figure 2.3c and 2.3d).

**Whole Body Sodium Levels**

Fish held in FW increased whole body Na⁺ by more than 5-fold between hatch (79.3 μmol Na⁺/g dry mass; W0; 568 ATU) and complete yolk sac absorption (and 427.7 μmol Na⁺/g dry mass; W12; 989 ATU) (Figure 2.4a). Whole body Na⁺ continued to increase but at a reduced rate through to 24 weeks post hatch (W24; 1409 ATU) when it
had reached 566.4 μmol Na⁺/g dry mass (Figure 2.4a) and declined slightly to 546.7 μmol Na⁺/g dry mass two weeks later (W26; 1479 ATU) (Figure 2.4a).

In general, few differences in whole body Na⁺ levels were observed in fish transferred to SW relative to those in FW until 8 weeks post SW transfer. After being in SW for 8 weeks all groups (W6-20; 779-1269 ATU) had between 489.8 and 590.2 μmol Na⁺/g dry mass, regardless of when (what age) they were transferred to SW (Figure 2.4b). Fish that had been in SW for 8 weeks displayed higher levels of whole body Na⁺ than time-matched FW counterparts, with the exception of those transferred at 18 and 20 weeks post-hatch (W18, W20; 1199, 1269 ATU), which had Na⁺ levels consistent with those of time-matched FW fish (Figure 2.4b). Significant increases in WB Na⁺ levels following 24 h or 5 d in SW were only observed in fish transferred to SW 16 weeks post-hatch (W16; 1129 ATU) and 18 weeks post hatch (W18, 1199 ATU) (Figure 2.4b).

The nadir in dry weight, the plateau in wet mass, the plateau in Na⁺ and stable level of water content that all occurred around 1000 ATU corresponded with the completion of yolk-sac absorption.

**Gill NKA Activity and Isoform Expression in FW**

In FW, gill NKA activity varied between 8.0 and 16.6 μmol ADP·mg protein⁻¹·hr⁻¹ over time, but the tendency of gill NKA activity to increase with ATU was not statistically significant (Figure 2.5).

In FW, the gill NKA α-1a isoform expression relative to EF1 α remained around 0.5 to 1.0 until it increased significantly at 1100 ATU. A secondary increase in gill NKA α-1a to approximately 2.0 became evident at 1409 ATU (W24) (Figure 2.6a).
In FW, gill NKA α-1b isoform expression tended to increase from the initial value of 1.0 at 709 ATU, but this did not reach statistical significance until 1100 ATU, by which time it had almost tripled. Thereafter, gill NKA α-1b isoform expression decreased and remained at about 1.0 up to 1479 ATU (W26) (Figure 2.6b).

The ratio of gill NKA α-1b/α-1a expression in FW changed over time (Figure 2.6c). Notably the ratio increased significantly from 2.6 at 709 ATU to a peak 4-5 times higher between 900 and 1100 ATU. Thereafter, the ratio declined significantly to the initial level of 2.0 or lower (Figure 2.6c).

**Gill NKA Activity and Isoform Expression After SW Transfer**

Gill NKA activity following transfer to SW almost doubled compared with the time-matched FW control at 1269 ATU, but not at 990 ATU (Figure 2.5).

Gill NKA α1-a isoform expression following SW transfer always decreased from between 0.4 and 1.4 to a very low level of 0.1-0.2 (Figure 2.6a) regardless of the life stage at transfer (W4, W12, W18, W20; 709, 989, 1199, 1269 ATU; figure 2.6a). In contrast gill NKA α-1b isoform expression following SW transfer always increased to between 2.7 and 4.1 regardless of life stage at transfer (W4, W12, W18, W20; 709, 989, 1199, 1269 ATU; figure 6b). As a result, the gill NKA α-1b/α-1a isoform expression ratio always increased following SW transfer. After 2 weeks in SW, the ratio always increased 8-30 fold: from 2.6 to 60.2 at W4 (709 ATU), from 12.1 to 95.0 at W12 (989 ATU), from 2.1 to 61.0 at W18 (1199 ATU) and from 2.3 to 47.4 at W20 (1269 ATU) (Figure 2.6c).
Discussion

This study successfully characterized the development of salinity tolerance in juvenile pink salmon from hatch through to 20 weeks post-hatch. Prior to this investigation it was unknown if pink salmon emerge from gravel redds ready for life in SW, or whether they adjust their hypo-osmoregulatory ability following SW entry. It was found here that developmental changes in individuals held in FW were associated with increased salinity tolerance (i.e. close to 0% morbidity post-SW transfer) around the completion of yolk-sac absorption (approximately 1000 ATU). Modifications included increased WB water content, increased gill NKA α-1b mRNA isoform expression and increased gill NKA enzyme activity. These changes appear to be endogenously controlled as fish were exposed to a constant photoperiod and temperature regime. The pattern of elevated α-1b/α-1a gill NKA expression at yolk sac absorption, followed by decreased expression of α-1b/α-1a and survival in SW thereafter, implies that a period of heightened salinity tolerance, similar to a smolt window, exists for this species. Furthermore, full strength SW acted as an environmental cue to further induce and improve hypo-osmoregulatory ability. Therefore, even though a FW preparatory period exists for this species, it differs from the classic smolt phase, as it does not fully prepare fish for SW.

At hatch, pink salmon alevins weighed an average of 0.146 g, which is typical for this species (Heard 1991, Honma 1982, Beacham 1991). By complete yolk sac absorption pink salmon from this study had an average wet mass of 0.192 g, which is within the range of 0.130 g-0.260 g, the size of migrant pink salmon fry throughout its
Pacific range as reported by Heard (1991) and others (Beacham 1991, Varnavsky et al., 1991, Varnavsky et al., 1993, Higgs et al., 1985, Honma 1982). Emergence of pink salmon fry from gravel redds generally occurs near complete yolk-sac absorption, typically between 900 and 1000 ATU (Heard 1991, Bailey et al., 1980, Bams, 1972). In the current study, the window of salinity tolerance likewise occurred at about 1000 ATU, concurrent with complete yolk sac absorption and the onset of exogenous feeding (W10; 924 ATU). Pink salmon from the same cohort used here, but held at the Quinsam Hatchery, started emerging at 971 ATU in 2009 (personal communication Pauline Scott) when yolk-sac absorption was mostly completed. Thus, we suggest that yolk-sac absorption is a useful life stage to indicate the time when coastal pink salmon are ready to out-migrate.

The test used here for SW readiness was an abrupt transfer to SW. In nature, SW exposure may be more gradual. Given the close to 100% morbidity observed in recently hatched alevins, it would be worth investigating the effect of more gradual SW exposure on alevin survival. It has previously been shown that pink salmon alevins exhibit high morbidity following direct SW transfers (Weisbart 1968, Honma 1982). The window of heightened salinity tolerance was identified using these abrupt SW transfers, indicating that fish transferred around the time of yolk-sac absorption that experienced close to 0% morbidity following SW transfer were in fact ready for life in SW.

Increased survival in SW appears to be associated with the ability to maintain water balance following SW entry and may be mediated by increased gill NKA enzyme activity. Gill NKA activity reached 14.4 µmol ADP·mg protein⁻¹·hr⁻¹ in FW in 8-week post hatch fish (W8; 849 ATU) and this corresponded with the time at which survival in SW was close to 100%. The values of gill NKA activity reported here for developing pink
salmon are similar to those previously reported for this species (Grant et al., 2009, Webster et al., 2009, Sackville et al., 2011) and chum salmon (Iwata et al., 2010), but lower than the 25-30 μmol ADP-mg protein⁻¹ h⁻¹ that has been measured in Atlantic salmon smolts (Nilsen et al., 2007, Stefansson et al., 2007).

Consistent with studies on FW-held smolting Atlantic salmon (Nilsen et al., 2007, Stefansson et al., 2007, Bystriansky et al., 2006), the expression of NKA α-1b increased while α-1a expression decreased, leading to maximum α-1b/α-1a mRNA expression at yolk-sac absorption (989 ATU). The subsequent decline in the ratio of α-1b/α-1a gill mRNA expression, and increase in morbidity following SW transfer suggests that pink salmon possess a window of heightened salinity tolerance around the time of natural SW-migration. The shift in this expression ratio is another good index of SW readiness in pink salmon.

Whole body Na⁺ levels were elevated shortly after SW transfer in fish transferred to SW at 16 and 18 weeks post-hatch (1129, 1199 ATU; figure 2.4b) and the morbidity observed in fry transferred to SW (W16-W20; 1129-1269 ATU; figure 2.2) could be related to ionic disturbance. Plasma osmolality or ion content post SW transfer is often used to measure a fish’s readiness for SW (ionoregulatory status) (24 h SW challenge, Blackburn and Clarke 1987), however plasma could not be readily obtained in 0.15-0.3g juvenile pink salmon. Instead, whole body Na⁺ was measured here, which has been previously used as a proxy for ionoregulatory status following direct transfer to SW in pink salmon (Grant et al., 2009, Sackville et al., 2011).

An interesting pattern of increasing WB Na⁺ was found in pink salmon developing in FW. It is not clear whether this represents a developmental trend that exists in all
salmonids or whether it is a trait unique to pink salmon. Developmental increases in whole body ion levels have been previously reported in FW-held Atlantic salmon (Rombough and Garside, 1984). Near to hatch, WB Na+ in Atlantic salmon was 64.4 ± 7.1 μmol/g dry weight, which is comparable to WB Na+ levels of 79.3 ± 3.6 μmol/g dry weight obtained here for pink salmon. WB Na+ levels in Atlantic salmon alevins then increased almost 3-fold to 220 μmol/g dry weight by near complete yolk-sac absorption (Rombough and Garside, 1984). In contrast, over a five-fold increase (79.3-427.7 μmol/g dry weight) in WB Na+ was found between recently hatched pink salmon and those at yolk-sac absorption in this study. Whether this dramatic increase in WB Na+ is somehow associated with preparation for SW entry is unknown but it is clearly worthy of further investigation. It is also worth noting that pink salmon sampled at complete yolk sac absorption exhibited a 1.5-fold increase in WB Na+ over those sampled just two weeks before, but the importance of this increase at yolk sac absorption is unclear. It is possible that this increase was related to the start of exogenous feeding, but a similar diet was previously found to have no effect on WB Cl− levels (Huang et al., 2007). In general few differences in WB Na+ existed between SW and FW held developing pink salmon, and both tended towards a plateau by the end of the sampling period.

Also associated with the completion of yolk-sac absorption was the attainment of a stable level of whole body water content, which plateaued at approximately 82% (Figure 2.3a and b), very close to the value of 82.5% previously reported for pink salmon from Quinsam hatchery (Grant et al., 2009). This plateau corresponded with the nadir in dry weight (Figure 2.3d) and the subsequent initiation of anabolic growth, which will be discussed below.
Developing pink salmon possessed a catabolic phase between 570 and 1000 ATU, characterized by the consumption of yolk (lipid), decreased dry mass, and increased wet weight related to the accumulation of water (Figure 2.3a). Wet mass should therefore not be used as an indicator of somatic growth in developing pink salmon until after yolk-sac absorption. An anabolic phase followed when both wet and dry mass increased. The growth rate of approximately 1.72% body mass-day\(^{-1}\) that occurred between 1100 and 1400 ATU was lower than that previously reported for juvenile pink salmon (Grant et al., 2009) reared at a higher temperature (10°C relative to 5°C in this study) in SW for a longer period.

The developmental changes including increased gill NKA \(\alpha\)-1b/\(\alpha\)-1a mRNA expression, and gill NKA enzyme activity, as well as those related to growth and increased WB water and ion levels, all occurred while pink salmon were held at constant photoperiod and temperature. This suggests that the typical short followed by lengthening photoperiod upon which many smolting species depend (Clarke and Hirano, 1995) may be unnecessary for pink salmon to prepare for SW, as is the case for other early ocean entry fish (chum and ocean-chinook, Clarke et al., 1989). The natural environment is of course far more complex than the laboratory environment employed here. Fish were reared in Heath trays and tanks as opposed to in gravel, and the experience of gravel emergence, yolk-sac absorption, the initiation of feeding, and exposure to the natural changes in light could only be partially replicated here. It remains possible that light exposure may be an important cue for pink salmon, and they are most receptive to such a cue during the observed window of heightened salinity tolerance. In fact, Seymour River Hatchery pink salmon without a visible external yolk-
sac increased gill NKA enzyme activity within 24 h after being transferred from complete darkness to a 12L:12D photoperiod in FW (Sackville 2010).

The preparatory increase in gill NKA activity associated with parr-smolt transformation is typically completed while smolts are in FW in order to allow them to move directly into full strength SW with little osmotic disturbance (Hoar 1988). The fact that changes in the transcriptional and activity levels of gill NKA occurred after transfer to full strength SW suggest that pink salmon are not fully prepared for SW while in FW. Within the window of heightened salinity tolerance (W10-W14; 919-1059 ATU) the magnitude of the response to SW demonstrated by gill NKA activity (Figure 2.5) and increased α-1b isoform expression (Figure 2.6b) tended to be less than that of fish transferred outside of this window. Therefore, the window for physiological compensatory changes that are stimulated by SW itself is plastic. Salmonids have previously been found to respond to SW with a heightened stimulation of gill NKA α-1b isoform expression as well as gill NKA activity when they are not as prepared for SW (Nilsen et al., 2007, Stefansson et al., 2007, Bystriansky et al., 2006). Nonetheless, the nearly 8-fold increase in α-1b/α-1a gill NKA isoform expression following 2 weeks in SW in fish transferred near 1000 ATU, demonstrates that even those ‘most ready’ for SW need to make adjustments once in SW and are likely undergoing de novo synthesis of α-1b and suppressing α-1a expression following SW entry.
Conclusions

The finding that pink salmon possess both an endogenously controlled preparatory phase during which they exhibit heightened salinity tolerance and an ability to enhance salinity tolerance directly in response to SW is in line with previous studies of SW tolerance in pink salmon, although some of the literature regarding the development of SW tolerance appeared to be contradictory. Sullivan et al. (1983) observed developmental increases, in FW held pink salmon, of gill NKA enzyme activity as well as plasma T4, both of which are normally associated with smolting in salmonids. I suggest these changes are likely associated with the window of endogenous heightened salinity tolerance identified here. Conversely Grant et al. (2009) suggested that the increases in gill NKA enzyme activity they observed in ocean-caught pink salmon signified that this species does not prepare for SW prior to entry. I suggest this change is likely associated with SW-stimulated heightened salinity tolerance. I can conclude that pink salmon that enter SW around the time of normal emergence and yolk absorption are more prepared for SW than those entering earlier or later, but they still need to further up-regulate their hypo-osmoregulatory machinery after out-migration into SW, as was reported by Grant et al. (2009).
Figure 2.1: A timeline of fish sampling and seawater (SW) transfers relative to accumulated temperature units (ATUs), weeks post-hatch (W) and the corresponding calendar date. Fish were sampled from freshwater (FW) every 2 weeks beginning at hatch on December 14, 2008, when fish were defined as 0 weeks post-hatch (W0), through to June 14, 2009, 28 weeks post-hatch (W28). No fish were sampled from FW on May 17, 2009 therefore one FW control group (W22) is missing. Fish were transferred to SW every 2 weeks from Dec 14, 2008 (W0) until May 3, 2009 (W20), and sampled at 24 h, 5 days, 2 weeks, 4 weeks, and 8 weeks following SW transfer. Fish were held at a constant temperature of 5°C, whereas temperature was more variable at the Quinsam hatchery explaining the disparity between the calendar dates at which fish reached approximately 1000 ATU (March vs. April).
Figure 2.2: Percent morbidity following transfer from freshwater to seawater in pink salmon at different stages of development defined as accumulated temperature units (ATUs). Groups of developing pink salmon were transferred to SW every two weeks, where W in the legend refers to weeks post-hatch. Thus, W2 refers to 2 weeks post-hatch. Yolk absorption in the fish represented above was complete by 989 ATU. Pink salmon from the same cohort but maintained at Quinsam River Hatchery emerged at 971 ATU. (See Figure 2.1 for further details).
Figure 2.3: (a) and (b) Changes in % whole body water content in pink salmon at different stages of development defined as accumulated temperature units (ATU’s) held (a) continuously in freshwater or (b) following different durations of seawater exposure (24 h, 5 days, or 2, 4 or 8 weeks) in fish that were transferred from FW to SW every 2 weeks following hatch (see Figures 2.1 and 2.2). In b) a regression line (R²=0.94) with 95% confidence intervals describing the FW controls presented in a) is included for comparison with SW transfer values due to the pronounced effect of development on % whole body water content. (c) and (d): Changes in wet and dry weight in pink salmon at different stages of development defined as accumulated temperature units (ATU’s) held continuously in FW or following different durations of SW exposure (24 h, 5 days, or 2, 4 or 8 weeks). (d) only fish that were transferred from FW to SW at 16, 18 and 20 weeks post hatch (W16, 1129 ATU; W18 1199 ATU; W20, 1269 ATU) are shown. Letters that differ indicate statistically significant differences (p<0.05). * depicts a significant difference from the initial FW control fish (24h and 5d) or depicts a significant difference from time matched FW control fish (2, 4 and 8 weeks) (p<0.05). No FW control exists at 1339 ATU (W22). Symbols indicate mean values ± SEM (n=10)
Figure 2.4: Changes in whole body [Na+] in pink salmon at different stages of development defined as accumulated temperature units (ATU’s) held (a) continuously in freshwater or (b) following different durations of seawater exposure (24 h, 5 days, or 2, 4 or 8 weeks) in fish that were transferred from FW to SW every 2 weeks following hatch. In (b) FW values from (a) are included (with letters indicating statistical differences omitted for clarity) for comparison with SW transfer values due to the pronounced effect of development on whole body Na+. No FW control exists at 1059 ATU (W14) or 1339 ATU (W22). (see Figure 2.3 for further details)
Figure 2.5: Gill Na⁺K⁺ATPase (NKA) activity (μmol ADP/mg protein*hr) of fish in FW and those transferred to SW at 12 and 20 weeks post hatch (W12, W20; 989, 1269 ATU). NKA activity represents mean ±SEM, n=3 except at 4 weeks post hatch (W4; 709 ATU)(n=3) and 8, 14, 16, 20 weeks post hatch (W8, W14, W16, W20; 8 * 1059, 1129,1260 ATU)(n=4). No statistically significant differences exist between NKA values from FW held fish. * depicts a significant difference between SW and time matched FW fish (p<0.05).
Figure 2.6: Changes in gill Na⁺K⁺-ATPase (a) α-1a, (b) α-1b and (c) ratio of α-1a/α-1b mRNA isoform expression relative to elongation factor (EF)-1α in pink salmon at different stages of development defined as accumulated temperature units (ATU’s). The first data presented in all panels is 4 weeks post-hatch (W4; 709 ATU; n=3) with subsequent values obtained every 2 wks in FW (closed circles in
a), b) and c). In a), b) and c) data are presented for fish transferred to SW (open symbols where fish were sampled at 24 h, 5 days, 2 and 4 weeks) at 4, 12, 18 and 20 weeks post hatch (W4; 709 ATU, W12; 989 ATU, W18; 1199 ATU and W20; 1269 ATU). Values in a) and b) are expressed relative to W4 fish (709 ATU). No FW control exists at 1339 ATU (W22). Symbols indicate means ± SEM (N=8, unless otherwise indicated on graph). (see Figure 2.3 for further details)


Chapter 3: General Discussion

Summary

My results provide strong evidence that pink salmon possess a window of heightened salinity tolerance at the time of yolk absorption, which corresponds to the time of natural emergence and out-migration. Furthermore, the pattern of gill NKA mRNA $\alpha$-1b/$\alpha$-1a isoform expression for pink salmon developing in FW confirms the existence of a FW preparatory phase prior to SW entry for this species, consistent with a smoltification phase. However, this physiological smoltification process continued in SW but not in FW, a result that confirms that pink salmon enter SW in a relatively precocial state. Fish held in FW beyond this time to increase body size at SW entry were no better at hypo-osmoregulating in SW, and thus a smoltification window exists and age rather than size at SW entry is more important for pink salmon.

Despite the existence of a FW preparatory phase, pink salmon are not fully prepared for SW upon entry, and SW exposure clearly stimulates further salinity tolerance. Following transfer to SW even the ‘most prepared’ pink salmon increased gill NKA activity and $\alpha$–1b/$\alpha$–1a mRNA expression. Pink salmon undergo the most extreme form of anadromy observed in salmonids; however, the findings from this investigation suggest that certain aspects of their transitional process from FW to SW may be similar to those of other smolting salmonids. The successful employment of early ocean entry has allowed pink salmon to maximize their exploitation of the nutrient rich ocean, and has possibly influenced their current abundance and successful proliferation throughout the Pacific Northwest.
The remainder of this chapter will provide further analysis and interpretation of these findings as well as address possible shortcomings of the results that were not discussed in Chapter 2, including those related to the artificial laboratory setting under which pink salmon were reared. Lastly, this chapter will include suggestions for future studies and a discussion regarding the significance future research in this area might provide.

Critique

The Use of Whole Body Ions

Juvenile pink salmon used in this study were too small to reliably obtain plasma Na\(^+\) measurements and consequently whole body Na\(^+\) was used as an indicator of hypo-osmoregulatory status following SW transfer. For a full justification of the utilization of this method refer to chapter 2 of this thesis, the limitations of this technique will be discussed here.

The use of WB Na\(^+\) data neither supported nor detracted from the finding that pink salmon are best able to tolerate SW at the time of yolk sac absorption and natural emergence. In fact, whole body Na\(^+\) levels post-SW transfer were rarely statistically different from those of FW-held fish. It is possible that the time of greatest ionic disturbance was missed because fish were not sampled for WB Na\(^+\) until after 24 h in seawater. It was previously found that a 3 h exposure to SW was best for detecting differences in muscle Na\(^+\) levels in pink salmon, compared with 12, 24, 48, 96 and 144 h (Varnavsky et al., 1993). Also, it has been reported for rainbow trout abruptly transferred to SW that whole body ion content does not change much, despite increases
in the plasma and tissues (Bath and Eddy, 1979). Furthermore, the ‘crisis period’ for these rainbow trout occurred between 0-10 h during which increases in [Cl{supershell}] were greater than increases in [Na{supershell}]. It is possible that measuring WB Na{superscript+} earlier than 24 h following SW transfer would have yielded different results. Also, measuring WB Cl{superscript−} may have provided a more complete picture of ionoregulation in juvenile pink salmon post-SW transfer.

**Potential for Growth**

A challenge in the present experiment was getting fish to accept artificial food, which is problematic since pink salmon normally undergo very rapid growth upon SW entry (Heard 1991, Grant et al., 2009). A connection between growth rate and salinity tolerance has yet to be characterized, but a linkage is possible through the actions of GH (Iwata et al., 2010). Obviously, a healthy growing fish is best for experiments. Feeding fish more natural prey items could expedite their taking to the feed, and allow for better growth. Temperature also has a direct effect on a fish’s capacity for growth, with warmer temperatures allowing for higher growth rates. Fish were reared at 5°C throughout the experiment and the lack of a seasonal temperature increase could have hindered optimal growth, and perhaps even osmoregulation in the later transfers. Future experiments of a similar nature should consider exposing fish to a more natural increasing temperature regime.
The Use of Quinsam River Fish

While a narrow window of heightened salinity tolerance was found for pink salmon around the time of complete yolk-sac absorption, this window likely varies between populations in both timing and flexibility depending on the distance of the natal spawning ground to the ocean. Increased FW residency would likely be beneficial to pink salmon that would naturally be in FW longer (due to having a further distance to travel before ocean-entry). It seems reasonable to hypothesize that pink and chum salmon would demonstrate superior salinity tolerance surrounding the time of normal ocean entry, and that the timing and length of this increased SW tolerance would vary among populations. Enhanced performance (growth, and survival) was observed in Quinsam River pink salmon reared at 10-12°C transferred to SW within 2 days post swim-up as opposed to 22 or 32 days post swim-up (Higgs et al., 1985). Beacham (1991) examined early juvenile survival and growth in fresh, brackish and marine water for coastal-spawning and interior-spawning populations of pink salmon. Interior-spawning populations had higher mean survival from emergence to 48 days when reared in FW. The coastal-spawning populations (including Quinsam River) didn’t have a substantial difference in mean survival in any of the three environments, but optimal growth and survival occurred in the brackish environment (Beacham 1991). The results obtained in this study may be more applicable to coastal populations of pink salmon.
**Future Directions**

**Hormones and Photoperiod**

Pink salmon partially prepare for SW while in FW, and possess a physiological smolt window centred around yolk-sac absorption, suggesting that this species has a smolt-like phase that is not entirely different than that of other anadromous salmonid species. It would therefore be interesting to assess whether pink salmon demonstrate other aspects of smoltification, and investigate whether the onset of smolt development is under the same environmentally mediated hormonal control. The fact that pink salmon were able to hypo-osmoregulate successfully in SW without being exposed to a simulated short, then increasing photoperiod suggests that they may be more similar to chum and ocean-type chinook salmon in this regard (Clarke et al., 1989). The extent to which this is true remains uncertain. Atlantic and coho salmon exposed to continuous light fail to make the necessary changes to undergo smoltification, likely due to an underdeveloped pre-optic area and light-brain-pituitary axis (Stefansson et al., 2007, Ebbesson et al., 2007). It would therefore be interesting to see whether continuous light exposure would disrupt a pink salmon’s ability to hypo-osmoregulate in SW.

Even if photoperiod is not a cue for pink salmon ‘smoltification’ it would be interesting to examine the role endogenous hormone cycles play in the initiation of the smolt process for this species. Interesting results would likely arise from examining developmental fluctuations in plasma cortisol, and GH both of which are known to be involved in increased SW osmoregulatory ability in smolting species (Björnsson et al., 1995, Björnsson 1997, McCormick 1994, McCormick 2001). Growth hormone-releasing
hormone (GHRH) has been implicated in triggering schooling and migratory behaviours in chum salmon (Ojima and Iwata, 2009), and it is possible that this would be the case for pink salmon as well.

It will be difficult to tease apart whether hormonal surges are related to development, increased salinity tolerance, or both. Sullivan et al. (1983) saw increases in plasma thyroxine in developing pink salmon but were unable to determine whether these were developmental or would aid in SW survival. Therefore, investigating both developmental changes, as well as artificially manipulating hormone levels could be useful. Prolactin has been associated with FW tolerance (McCormick 1994, Hoar 1988), so monitoring changes in GH and prolactin levels in developing pink salmon could elucidate mechanisms through which the endocrine system might influence both their window of heightened salinity tolerance, as well as their response to SW.

**Temperature**

Exposing pink salmon to a number of temperature regimes could illuminate the influence of temperature on the window of heightened salinity tolerance (identified here for pink salmon). In Atlantic salmon the duration of the smolt-window correlates with ATUs, (McCormick et al., 1999, Stefansson et al., 1998, Stefansson et al., 2008) and this is likely the case for pink salmon as well. It would also be useful to know at which temperature pink salmon perform optimally post-SW transfer.
Ion Balance and Euryhalinity in Developing Pink Salmon and Older Fry

Between hatch and complete yolk-sac absorption, a 5-fold increase in WB Na⁺ was found for pink salmon developing in FW. The importance of this increase is currently unclear. It would be useful to investigate the change in WB ions with development in other salmonid species in order to assess whether this trait is unique to pink salmon, and possibly related to early ocean entry.

It would also be interesting to conduct SW transfers on even older fry than were transferred here, in order to determine more conclusively whether increased size enhances salinity tolerance. The oldest fry in this study had the highest gill NKA α-1a isoform levels, thought to be associated with decreased salinity tolerance, but no SW transfers were conducted for this group. It is therefore unknown whether the decreased % survival post-SW transfer observed in the later transfers is related to a permanent reduction in hypo-osmoregulatory ability. While larger size may not immediately incur greater SW tolerance (as was found here), fish transferred in these experiments were relatively small, and therefore a larger size could eventually be beneficial to ionoregulation. Chum salmon have been found to remain relatively euryhaline after their normal migration to SW (Hasegawa et al., 1987) and it is possible the same is true for pink salmon, even though they appear to be ‘most ready’ at the time of yolk-sac absorption, emergence and out-migration.
**Comparative Studies**

Pink salmon are not the only species that employ early ocean-entry; chum, ocean-type chinook as well as some sockeye populations are all known to migrate to SW within 1 year after hatching. Comparing the mechanisms by which these other species go to the ocean ‘early’ could shed insight into the evolution of this trait.

It might also be worth comparing anadramous pink salmon populations to those that now live in the Great Lakes to determine whether they have retained any degree of euryhalinity, or perhaps now possess a better ability to grow and survive in FW.

**Early Ocean Entry: Ecological and Evolutionary Significance**

Pink salmon possess a window of heightened salinity tolerance around the time of natural SW-migration, and furthermore are able to respond directly to a SW cue, even outside of this window, enhancing hypo-osmoregulatory mechanisms. These findings could be significant from both an evolutionary and ecological context both of which warrant further discussion, and the possible benefits of employing early ocean entry will also be described.

Pink salmon represent the most-derived *Oncorhynchus* species and are also the most abundant and widely distributed, making it tempting to link their ecological success with their unique life history, in particular their extremely small size at ocean entry. While such speculation should be made with caution, there are obvious benefits to maximizing time spent in the ocean. Pink salmon likely employ early SW entry to
exploit the food abundant ocean and therefore to maximize their capacity for growth early on. This may be what allows pink salmon to mature after only 18 months of feeding, and also allows them to have a shorter life cycle than do species which depend on both FW and SW systems for food. Taking less time to reach maturity reduces generation time and could also lead to greater fitness, as an individual pink salmon would have a reduced probability of dying throughout the course of a shorter life-cycle (Quinn 2005). Furthermore, pink salmon are able to spawn closer to the ocean, which allows them to embark on shorter migrations to and from the ocean than those of other salmonid species (Quinn 2005).

Low productivity streams cannot support large numbers of feeding fry; therefore, producing offspring that will hatch and vacate their natal streams upon emergence allows for the production of more offspring (Quinn 2005). Because pink salmon do not feed in FW there is little risk of competition among pink fry and other resident salmon species (Quinn 2005).

Early ocean-entry could also influence homing, which is the predominant trait (compared to straying) in Pacific salmon and allows them to return to their natal stream to breed. It has been suggested that pink salmon may have higher stray rates than other Pacific salmon for three reasons: they often breed in unstable rivers such as small tributaries, they appear to be the least specialized in their FW habitats, and they have little variation in the age at maturity (other salmon may stray in time instead of space) (Quinn 2005). This hypothesis is also consistent with the fact that pink salmon generally have lower levels of genetic variation between populations; however, actual pink salmon stray rates are so far reported to occur at a level that is consistent with other species.
(Quinn 2005). The consequences of a higher stray rate could be beneficial, allowing for more genetic drift and therefore greater potential to exploit various habitats.

In order to successfully complete SW migrations and increase post-migratory growth and survival, it would be beneficial for pink salmon to align the time at which they are physiologically most ready for SW with ideal environmental conditions. We found pink salmon are able to increase their hypo-osmoregulatory ability in response to SW, even outside of their window of heightened salinity tolerance, and this might confer some flexibility in this regard. McCormick et al. (1998) suggested two aspects of migration timing will affect smolt survival (for Atlantic salmon), the first being the physiological smolt window, and the second an environmental smolt window, during which the seasonal changes of rivers, estuaries and the coastal environment allow for high smolt survival. To yield high adult returns the time of smolt migration should correspond with optimal environmental conditions (McCormick et al., 1998). Chittenden et al. (2010) examined coho salmon out-migrating from Quinsam River and found they will have better survival to maturity if they out-migrate in concert with plankton blooms, and this is likely the case for pink salmon as well. Hatcheries in Alaska have used plankton abundance in the ocean to aid in timing the release of pink and chum salmon (Eslinger et al., 2001). The initiation and duration of the window of heightened salinity tolerance is likely dictated by temperature, but the extent to which this is true is unknown. Pink salmon generally emerge after accumulating 1000 ATU (Heard 1991) but this will vary, ATUs at emergence will be higher following warmer winters, and might barely reach 1000 ATU in colder years (Personal communication with Dan Babchuck, Quinsam Hatchery). The ability to emerge and immediately out-migrate
allows Quinsam pink salmon to migrate during the spring freshet, and at times of high ocean productivity.

Despite the success of the pink salmon species as a whole, there can be high mortality rates among juvenile pink salmon (Heard 1991), and a changing climate will influence their predator/prey interactions (timing of plankton blooms) and probably also the length of their physiological smolt window. For this reason it will be essential to study the abiotic (photoperiod, temperature, flow) and biotic (prey availability, predator and parasite abundance) factors which will likely influence both salinity tolerance, and marine growth and survival. A better understanding of the smolt-like phase possessed by pink salmon may also lead to greater insight into the evolution of early ocean entry among salmonids, and perhaps a greater appreciation for this robust, but often overlooked salmonid species.
Bibliography


