Of Saline and Sea Lice: Hydromineral Challenges and Osmoregulatory Strategies Associated with Early Ocean Entry of Juvenile Pink Salmon, *Oncorhynchus gorbuscha*

by

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Abstract

Pink salmon (*Oncorynchus gorbuscha*) enter seawater (SW) following gravel emergence at a body mass of 0.2 g. Two hydromineral challenges associated with this remarkable early ocean entry were investigated: (1) initial exposure to a hyper-osmotic environment and (2) sea louse (*Lepeophtheirus salmonis*) parasitism.

To survive SW, pink salmon were hypothesized to develop hypo-osmoregulatory abilities as larval alevins prior to natural SW entry as post-larval fry. To test this, alevins and fry were transferred from freshwater (FW) in darkness to SW under a simulated natural photoperiod (SNP). Ionoregulatory status was assessed at 0, 1 and 5 days post-transfer. Alevins showed no evidence of hypo-osmoregulation, marked by a loss of water balance, a 35% increase in body $[Cl^-]$, and no change in gill Na^+/K^+-ATPase (NKA) activity. Conversely, fry maintained water balance and increased gill NKA activity by 50%. Fry gill NKA activity also increased by 50% following exposure to SNP in FW, providing the first evidence of photoperiod-triggered smoltification for pink salmon. A 15% increase in fry body $[Na^+]$ was observed as well, perhaps representing a novel mechanism for maintaining water balance during ocean entry.

Physical damage to the host epidermis is a primary proximal effect of louse infection. Such damage may exacerbate existing hydromineral flux in SW. To test this, ionoregulatory status was measured in pink salmon of varying size with and without attached-stage lice. In laboratory-infected fish (~1 wk SW; 0.2-0.4 g), body $[Na^+]$ increased by 12% when infected with 1 chalimus IV louse, and by 23% with 2-3 chalimus III lice. Mortality was 6%. In wild-infected fish (~4-12 wks SW; 0.5-1.5 g), body $[Na^+]$ did not differ from controls. Combining data sets revealed a "no effect" fish size threshold of 0.5 g for 1 chalimus IV louse. This threshold is partly due to increasing hypo-osmoregulatory ability.

Pink salmon thus appear to possess a novel hypo-osmoregulatory strategy where ion balance is sacrificed to maintain water balance prior to maximum ion excretion capacity. Out-migrating fish are particularly vulnerable to sea louse parasitism at this time, and as such, BC fish farms have relocated to minimize interactions during this critical period.

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Chapters 2 and 3 are co-authored as indicated by chapter footnotes. Research questions, experimental design, experimentation, data analyses and manuscript preparation were conducted by Michael Sackville under the co-supervision of Drs. Colin J. Brauner and Anthony P. Farrell. Laura Nendick and Stephen Tang assisted greatly with fish collection, care and experimentation for chapter 2.

Chapter 1: Introduction and Thesis Goals

The importance of Pacific salmon to the ecological, cultural and economic integrity of their surroundings has long been established. As keystone species of marine, freshwater and terrestrial ecosystems, they support multiple food webs across multiple habitats and serve as unique links in nutrient cycling between very distinct and often isolated systems (Quinn, 2005). Their highly productive populations have also significantly contributed to the economic development, success and well-being of coastal peoples for thousands of years, having sustained countless commercial, recreational and subsistence fisheries (Augerot and Foley, 2005; Quinn, 2005). As a result, these iconic fishes are intricately woven into the cultural fabric of virtually every coastal society within their distribution (Augerot and Foley, 2005).

The extent of these impacts stems largely from a truly remarkable life history strategy. As anadromous fishes, salmon spawn in freshwater, move to the ocean for a period of rapid growth, then return to natal streams to reproduce and die. Transition to more productive marine waters supports the rapid growth of large numbers of individuals by providing access to an abundance of space and food otherwise unavailable in most temperate freshwater systems (Gross et al., 1988; McDowall, 2008; McDowall, 1997). By facilitating this high productivity, successful transition to the marine environment is a major reason why these fishes have such dramatic effects on their environs, and without it, their impacts would be far less profound.

This thesis explores two hydromineral challenges associated with ocean entry that pink salmon (*Oncorhynchus gorbuscha*) must overcome to achieve their high-impact status: (1) the hyper-osmotic environment and (2) sea louse (*Lepeophtheirus salmonis*) parasitism. The strategies adopted to meet these challenges are investigated in pink salmon for reasons that are both basic and applied. The following is a brief thesis outline:

Chapter 2: The hyper-osmotic environment, basic research

Pink salmon represent the most extreme and perhaps derived form of salmonid anadromy, yet remain largely unstudied. Elucidating the mechanisms underlying their transition to marine waters may reveal a novel strategy and potentially provide insight into the evolution of salmonid anadromy. The physiological basis for ocean entry in pink salmon will serve as the focus of chapter 2.

Chapter 3: Sea louse parasitism, applied research

Recent pink salmon population declines have been attributed by some to sea lice of fish farm origin; however, the physiological effects of parasitism have yet to be determined. Chapter 3 aims to bring concrete measures of sub-lethal impact to this controversial issue, ideally promoting pink salmon conservation and sustainable industry practice.

The remainder of this introduction will further contextualize and explain thesis research drivers while listing specific research objectives.

1.1 OSMOREGULATORY DEMANDS OF ANADROMY

Anadromy has its obvious benefits with respect to species abundance and distribution, but it is not without obstacles. As osmoregulators, salmon homeostatically maintain very specific concentrations of water and ions within their extracellular fluid largely independent of the external environment (Evans, 2008; Krogh, 1939; Smith, 1932). Hydromineral balance within this internal milieu is essential to maintaining the electrochemical gradients required for nearly every cellular process imaginable; therefore, any disruption could have devastating effects at the cellular, tissue and even organismal level. Because the gill epithelium is permeable to both water and ions, fish must actively defend this balance against passive fluxes. In freshwater, where

osmolarity is 1/300th of plasma, teleosts passively gain water and lose ions to the external medium, thus requiring active ion uptake at the gills and excretion of excess water as dilute urine. In seawater, where osmolarity is roughly three times that of plasma, the scenario is reversed; fish combat passive ion gain and water loss with active ion excretion at the gills and water uptake at the gut. These two strategies, termed hyper- and hypo-osmoregulation respectively, demand very different and highly specialized machinery at the gills, gut and kidney (for reviews see Evans, 2008; Evans et al., 2005; Krogh, 1939; Smith, 1930). Anadromy and its many benefits require fish to switch between these opposing strategies, which is arguably the greatest physiological challenge associated with ocean entry. The failure to do so results in a rapid loss of hydromineral balance and, ultimately, death (Boeuf and Harache, 1982).

A successful switch to hypo-osmoregulation requires extensive physiological change at the osmoregulatory organs (for reviews see Boeuf, 1993; Folmar and Dickhoff, 1980; Hoar, 1988; McCormick and Saunders, 1987). In the intestine, specialized epithelial cells develop to create a favourable osmotic gradient that draws water into the extracellular fluid (ECF) paracellularly from the lumen (Grosell, 2006; Grosell et al., 2009; Grosell et al., 2007). The cells secrete bicarbonate (HCO_3^-) into the intestinal fluid which binds to calcium (Ca^{2+}) and precipitates out of solution, while sodium (Na^+) and chloride (Cl^-) are actively transported transcellularly into the ECF. This process is driven largely by Na^+/K^+-ATPase (NKA) on the basolateral membrane and by Na^+, K^+, Cl^--cotransporter (NKCC) and Cl^-/HCO_3^- anion exchanger on the apical side. Water does move into the ECF as a result, but an excess amount of Na^+ and Cl^- accumulates as well. To maintain ion balance, specialized mitochondria-rich cells (MRC's) situated on the interlamellar regions of the gill epithelium excrete Cl^- transcellularly while Na^+ leaves paracellularly down a local electrochemical gradient (Evans, 1993; Evans et al., 2005; Keys and Willmer, 1932; Potts, 1984). This process is driven largely by NKA and NKCC on the basolateral membrane and by an apical Cl^- channel. Divalent ions are primarily secreted

by the kidney, which also reduces urine production to conserve water in the hyper-osmotic environment (Smith, 1930). However, the associated changes to renal physiology are relatively understudied.

1.2 EVOLUTION OF ANADROMY AND SMOLTIFICATION

Depending on the nature and timing of the osmoregulatory switch, salmonids can generally be classified under one of two main types of anadromy; facultative or predictive. Facultative anadromy is common among more ancestral salmonids such as brook charr (*S. fontinalis*) of the genus *Salvelinus* (McCormick, 2009), and the switch to hypo-osmoregulation is triggered by direct exposure to seawater (McCormick and Naiman, 1984a; McCormick and Naiman, 1984b; McCormick et al., 1985). These fishes typically spend extended periods of time in brackish water while undergoing the necessary transformations for marine life (~3 weeks; McCormick and Naiman, 1984b), which results in considerable lag time between the onset of downstream migration and ocean entry. Conversely, predictive anadromy is common among more derived species such as members of the genera *Oncorhynchus* and *Salmo*, and the switch to hypo-osmoregulation is triggered by changes in photoperiod in anticipation of ocean entry (McCormick, 2009). This preparatory metamorphosis, which also includes a host of behavioural and morphological changes tailored to marine life, is termed smoltification (Boeuf, 1993; Folmar and Dickhoff, 1980; Hoar, 1976; Hoar, 1988; McCormick, 1994; McCormick and Saunders, 1987).

Smoltification facilitates the transition to marine waters by initiating the upregulation of hypo-osmoregulatory mechanisms almost two months prior to seaward migration (Dickhoff et al., 1985; McCormick et al., 1987; Nilsen et al., 2007; Stefansson et al., 2007). Controlled experiments demonstrate that preparatory increases in gill NKA activity and mRNA expression of the enzyme's α1 subunit seawater isoform greatly improve seawater adaptability, evidenced

by increased growth, survival and a reduction in both the duration and magnitude of ionic disturbances following seawater exposure (Boeuf and Harache, 1982; Boeuf and Harache, 1984; Brauer, 1982; Folmar et al., 1982; McCormick et al., 1987; Nielsen et al., 1999; Nilsen et al., 2007; Nilsen et al., 2003; Otto, 1971). Preparatory smolt changes run to completion within a matter of days to weeks of initial seawater entry, resulting in a significantly reduced acclimation period for this more derived form of anadromy.

Interestingly, transition to the marine environment within the predictively anadromous salmonids may occur earlier in development and at smaller fish sizes in those more derived species (Clarke, 1982; Hoar, 1976; Hoar, 1988; McCormick, 2009; McCormick and Naiman, 1984b; McKay et al., 1996; Oakley and Phillips, 1999; Quinn and Myers, 2004; Rounsefell, 1958). Consequently, there may be an evolutionary trend toward increasingly early ocean entry, from facultative to predictive anadromy, and from large body size to small. This earlier access to more productive marine waters may also be a successful strategy, as those more derived salmonids that enter the ocean earliest are generally the most abundant and widely distributed (Augerot and Foley, 2005; Quinn, 2005). Although predation is often considered the dominant selective pressure dictating size at ocean entry (Hargreaves and LeBrasseur, 1986; Holtby et al., 1990; Saloniemi et al., 2004), osmoregulatory challenges likely play a significant role as well (Clarke et al., 1989; McCormick and Naiman, 1984b). Investigating the strategies used to overcome the hydromineral challenges accompanying increasingly early ocean entry may thus provide insight into the evolution of salmonid anadromy. Similarly, physiological adaptations key to the productivity and associated high-impact status of these fishes may be revealed.

1.3 PINK SALMON OCEAN ENTRY

Pink salmon (*Oncorhynchus gorbuscha*) are an especially interesting salmonid in which to study ocean entry because they represent the most extreme, abundant, and perhaps derived

form of salmonid anadromy (Clarke, 1982; Hoar, 1988; McCormick, 1994; McCormick, 2009; Quinn and Myers, 2004; Rounsefell, 1958). Pink fry migrate seaward directly following gravel emergence at a size of 0.2 g (Grant et al., 2009; Heard, 1991), while most other anadromous salmonids do so after at least one year in freshwater at sizes between 2-30 g (Clarke, 1982; Rounsefell, 1958). Even chum and those runs of chinook and sockeye that migrate seaward in the same year of emergence are twice as large as pink fry and spend weeks to months in freshwater prior to ocean entry (Clarke, 1982; Hoar, 1988; McCormick and Naiman, 1984b; Quinn and Myers, 2004). Thus, pink salmon possess the smallest body size and shortest freshwater residency of any salmonid to regularly undergo this transition. As further described below, this exceptionally early ocean entry renders the hydromineral challenges explored in this thesis particularly daunting. Understanding how pink salmon overcome these challenges is therefore interesting from physiological, ecological and evolutionary perspectives alike.

1.4 HYDROMINERAL CHALLENGE 1: THE HYPER-OSMOTIC ENVIRONMENT

A major challenge associated with the early ocean entry of pink salmon is the demand for a sufficiently developed seawater tolerance upon yolk sac absorption and gravel emergence. This scenario is particularly daunting because of the high body surface area to volume ratio facing small pink fry and the relative lack of freshwater preparatory time commonly afforded to other salmonids. The near absence of post-emergent preparatory time has spurred speculation that pink salmon may employ a novel strategy in which they develop hypo-osmoregulatory abilities as larval alevins in the redd -perhaps without the usual photoperiod cue (Hoar, 1988; McCormick, 2009; Sullivan et al., 1983; Weisbart, 1968).

Despite this exceptional life history, smoltification and the development of seawater tolerance in pink salmon remains largely unstudied. Previous work has shown that although larval pink alevins fail to survive seawater exposure prior to yolk sac absorption, they do exhibit

signs of plasma ion regulation and possess a higher LT_{50} than other oncorhynchids, surviving almost three times as long as coho, chinook and sockeye alevins (Weisbart, 1968). While these results suggest larval hypo-osmoregulatory development, extra-branchial MRC's as seen on the yolk sac of chum alevins (Kaneko et al., 1995) could explain this effect. Further complicating matters is that fry experience a doubling of whole body Na^+ and Cl^- content that coincides with naturally timed seawater entry (Grant et al., 2009). This elevation in whole body ion levels persists until gill NKA activity peaks two months later, which is far longer than the mere hours to days reported for other salmonids (*O. keta, O. mykiss, O. kisutch, S. salar*; Black, 1951; Leray et al., 1981; Miles and Smith, 1968; Prunet and Boeuf, 1985) and may indicate an underdeveloped hypo-osmoregulatory capacity. Furthermore, the majority of the observed increase in gill NKA activity occurs after seawater entry (Grant et al., 2009; Honma, 1982), suggesting that compensatory rather than preparatory changes may occur. Evidently, when and how seawater tolerance and hypo-osmoregulatory ability of juvenile pink salmon develops remains unclear.

1.5 CHAPTER 2 OBJECTIVES

Chapter 2 thus has two objectives. **1)** To determine the extent to which pink salmon develop adult-like hypo-osmoregulatory abilities as larval alevins, and **2)** identify the physiological differences between alevins and post-larval fry critical to seawater survival. Studying the physiological mechanisms underlying early ocean entry in what might be the most derived oncorhynchid may provide valuable insight into the evolution of salmonid anadromy.

Chapter 2 Hypothesis

Pink salmon have adult-like hypo-osmoregulatory abilities as larval alevins.

Chapter 2 Predictions

1. Larval alevins increase gill NKA activity, α1b isoform expression and drinking rate following seawater exposure.

2. Post-larval fry exhibit increases in gill NKA activity, α1b isoform expression and drinking rate equal to or greater than those exhibited by larval alevins following seawater exposure.

3. Hydromineral balance is better maintained in post-larval fry than larval alevins.

1.6 HYDROMINERAL CHALLENGE 2: SEA LOUSE PARASITISM

Unfortunately for juvenile pink salmon, the hydromineral challenge that accompanies such an early transition to the marine environment can be exacerbated by other factors in the ocean. The ectoparasitic salmon louse, *Lepeophtheirus salmonis*, is one such factor that has become of particular concern for management and conservation groups as of late. The effects of louse infection on the ionoregulatory status of post-emergent pink salmon will serve as the focus for chapter 3 of this thesis.

1.7 SEA LOUSE BIOLOGY

Endemic to the Pacific salmon of British Columbia's coastal waters (Kabata, 1988), *L. salmonis* is a naturally occurring marine ectoparasite also commonly associated with Atlantic salmon in open net-pen fish farms (Orr, 2007). Lice first encounter their hosts as planktonic copepodids, drifting freely in the water column while surviving on limited internal energy reserves (Pike and Wadsworth, 1999; Tully, 1992; Wootten et al., 1982). Upon infection, copepodids anchor to the fish's surface with a chitinous filament and begin feeding on host mucus, epidermal tissue and blood (Brandal et al., 1976; Kabata, 1974). This on-host feeding fuels louse development through four attached stages (termed chalimus 1-4) and three unattached motile stages (termed pre-adult 1-2 & adult) to sexual maturity (Johnson and Albright, 1991b).

Growth from a 1mm copepodid to a 14 mm adult female is highly temperature dependent, ranging anywhere from four to twelve weeks (Johnson and Albright, 1991a; Tully, 1992).

Beyond the physical damage imparted by mouthparts while feeding, lice secrete proteases that suppress host immune function and aid tissue digestion (Fast et al., 2003; Fast et al., 2007a; Fast et al., 2007b; Fast et al., 2004; Fast et al., 2006; Firth et al., 2000; Wagner et al., 2008). As shown in 200 g Atlantic salmon and 90 g sea trout, this surface feeding can lead to significant hydromineral disruption (up to 60% increase in plasma electrolytes; Bjorn and Finstad, 1997; Bowers et al., 2000; Grimnes and Jakobsen, 1996; Nolan et al., 1999; Wootten et al., 1982). This disruption is thought to result from either epidermal lesions that damage the external osmotic barrier or from a stress-related increase in the osmo-respiratory compromise (Gonzalez and McDonald, 1994; Gonzalez and McDonald, 1992; Nolan et al., 1999; Randall et al., 1972; Wendelaar Bonga, 1997). The osmo-respiratory compromise is the trade-off between respiration and passive hydromineral flux across the gills. High permeability of the gill epithelium better facilitates the gas exchange required for respiration, but unfortunately also permits undesirable passive flux of water and ions. Furthermore, this trade-off increases with elevated respiration following a generalized stress response (Gonzalez and McDonald, 1992; Wendelaar Bonga, 1997). Regardless of origin, hydromineral imbalance is a major concern as it can lead to a myriad of associated complications; reduced swim performance (Brauner et al., 1994; Brauner et al., 1992), stunted growth (Brauer, 1982; Folmar et al., 1982; McCormick et al., 1987) and death (Boeuf and Harache, 1982) have all been linked to hydromineral disruption.

1.8 INCREASED LOUSE SUSCEPTIBILITY OF JUVENILE PINK SALMON

Adult pink salmon are believed to be well equipped to cope with the effects of sea louse infection in the wild (Beamish et al., 2005), but the case for out-migrating juveniles is far from clear. Foremost, pink salmon enter the ocean as small as 0.2 g and are thus three orders of

magnitude smaller than their adult counterparts upon first encountering lice (Grant et al., 2009; Heard, 1991; Jones and Hargreaves, 2007). Furthermore, ocean entry by these fish appears truly precocious, as hypo-osmoregulatory, immune and epidermal systems all still develop over the first few months at sea. Gill NKA activity peaks 2 months post-entry (Grant et al., 2009), immunocompetence is reached at approximately 2.5 months post-entry (Johnson et al., 1982), while scale formation remains incomplete until almost 3 months post-entry (Jones et al., 2008). Based on the aforementioned proximal effects of *L. salmonis* parasitism, the development of these physiological systems undoubtedly plays a critical role in successfully resisting and/or tolerating louse infection. Thus, juvenile pink salmon are likely particularly vulnerable to sea louse parasitism during this precocious out-migration.

With the exception of stickleback, *G. aculeatus* (Jones et al., 2006), most natural host populations are fortunately situated offshore during this critical period in pink salmon development (Gottesfeld et al., 2009; Quinn and Myers, 2004). This migratory allopatry may naturally restrict parasite exposure to more developed pink fry. However, open net-pen salmon farms often house adult *S. salar* in coastal waters year-round. These cultured fish can potentially serve as a nearshore parasite source that exposes out-migrating pink fry to unnaturally high and/or premature levels of *L. salmonis* infection (Orr, 2007). In fact, sea lice of fish farm origin have recently been implicated in pink salmon population declines and extinction predictions in British Columbia's Broughton Archipelago (Krkosek et al., 2007a; Krkosek et al., 2007b; Krkosek et al., 2006a; Krkosek et al., 2005; Krkosek et al., 2009; Morton and Routledge, 2005; Morton et al., 2004; Morton et al., 2005). Although the correlational data and mathematical models presented by these studies are compelling, major questions regarding parasite transmission and infection impacts on host physiology remain unknown. The resulting uncertainty has led to considerable debate in both scientific and public fora alike (Beamish et al., 2006; Beamish et al., 2007; Brooks, 2005; Brooks and Jones, 2008; Brooks and Stucchi, 2006;

Butterworth et al., 2008a; Butterworth et al., 2008b; Dill et al., 2009; Krkosek et al., 2008a; Krkosek et al., 2008b; Krkosek et al., 2006b; Morton and Routledge, 2008; Riddell et al., 2008). Two major unknowns are of particular interest: (**1**) the relative contribution of fish farms to local parasite populations and (**2**) the level of parasitism required to sub-lethally impair juvenile pink salmon. Chapter 3 of this thesis will address the latter.

1.9 LOUSE-INDUCED PERFORMANCE REDUCTIONS MAY BE LINKED TO IONIC HOMEOSTASIS

To date, only one study has examined the sublethal effects of louse infection on out-migrating juvenile pink salmon between 0.2 and 3 g in body mass (Nendick et al. 2010). Over this range, the effect of 1 motile louse per fish was size-related, significantly reducing swimming ability in fish below, but not beyond, a body mass of 0.7 g. Interestingly, this body mass threshold corresponds with the completion of pink salmon hypo-osmoregulatory development (Grant et al., 2009), indicating that performance impacts are perhaps linked to hypo-osmoregulatory ability. Further supporting this notion is the correlation between similar reductions in swimming ability and elevated plasma ions in 8-20 g *O. kisutch* following SW entry (Brauner et al., 1994; Brauner et al., 1992; Randall and Brauner, 1991) and 600 g *S. salar* infected with 80 lice/fish (Wagner and McKinley, 2004; Wagner et al., 2003; Wagner et al., 2004). Moreover, 200 g infected *S. salar* have also been shown to upregulate gill NKA activity by as much as 60%, likely as an energetically costly attempt to restore hydromineral balance (Nolan et al., 1999). Based on these studies, the proximal effects of louse infection and the precocious nature of juvenile pink salmon ocean entry, we hypothesize that louse-induced performance reductions are related to ionoregulatory status.

1.10 CHAPTER 3 OBJECTIVES

The ionic disruptions and ionoregulatory compensations associated with sea louse infection were measured in post-emergent pink salmon in field and laboratory settings. These data were subsequently used to determine a fine-scale threshold fish body mass for sub-lethal louse effects. These findings will hopefully promote more informed aquaculture management in British Columbia, ideally minimizing the interaction between open net-pen farms and wild pink salmon below the identified threshold.

Chapter 3 Hypothesis

Louse infection impairs ionic homeostasis.

Chapter 3 Predictions

1. Infected fish will increase gill NKA activity to compensate for the ionic disruption associated with increasing louse loads.

2. Infection levels beyond fish compensatory limits will result in a hydromineral imbalance.

3. Infection impacts will decrease as fish develop.

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Chapter 2: Water Trumps Ion Balance in Early Marine Survival of Juvenile Pink Salmon (*Oncorhynchus gorbuscha*)[1]

2.1 INTRODUCTION

Smoltification is a complex suite of behavioural, morphological and physiological changes that prepares anadromous salmonids for the transition from freshwater (FW) to the marine environment (for reviews see Boeuf, 1993; Folmar and Dickhoff, 1980; Hoar, 1976; Hoar, 1988; McCormick, 1994; McCormick and Saunders, 1987). Arguably the most dramatic change associated with this metamorphosis is the acquisition of seawater (SW) tolerance, in which fish switch from a hyper- to hypo-osmoregulatory strategy. To facilitate this remarkable shift, extensive remodelling of the major osmoregulatory organs is triggered by changes in photoperiod up to 2 months prior to seaward migration (Dickhoff et al., 1985; McCormick et al., 1987; Nilsen et al., 2007; Stefansson et al., 2007). The resulting preparation, which includes increases in gill Na^+/K^+-ATPase (NKA) activity and mRNA expression of the enzyme's α1 subunit SW isoform, has been shown to vastly improve SW adaptability (Boeuf and Harache, 1982; Boeuf and Harache, 1984; Brauer, 1982; Folmar et al., 1982; McCormick et al., 1987; Nielsen et al., 1999; Nilsen et al., 2007; Nilsen et al., 2003; Otto, 1971).

Pink salmon are an especially interesting animal system in which to study smoltification because they possess the smallest body size and shortest FW residency of any salmonid upon ocean entry. Pink fry migrate seaward directly following gravel emergence at a size of 0.2 g (Grant et al., 2009; Heard, 1991), while most other salmonids do so after at least one year in FW at sizes between 2-30 g (Clarke, 1982; Hoar, 1988; Quinn and Myers, 2004; Rounsefell, 1958). Even chum and those runs of chinook and sockeye that migrate seaward in the same year of

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emergence are at least twice as large and spend weeks to months in FW prior to ocean entry (Clarke, 1982; Hoar, 1988; Quinn and Myers, 2004; Rounsefell, 1958). Seaward migration is thus particularly challenging to pink fry because of the high body surface area to volume ratio and relative lack of post-larval FW preparatory time. The near absence of preparatory time has spurred speculation of a novel strategy where hypo-osmoregulatory development begins at larval stages in the redd -perhaps without the usual photoperiod cue (Hoar, 1988; McCormick, 2009; Sullivan et al., 1983; Weisbart, 1968).

Despite this exceptional life history, smoltification and the development of seawater tolerance in pink salmon remains largely unstudied. Previous work has shown that although larval pink alevins fail to survive seawater exposure prior to yolk sac absorption, they do exhibit signs of plasma ion regulation and possess a higher LT_{50} than other oncorhynchids (Weisbart, 1968). These results suggest larval hypo-osmoregulatory development, but extra-branchial MRC's as seen on the yolk sac of chum alevins (Kaneko et al., 1995) could explain this effect. Further confounding interpretation is that fry experience a doubling of whole body Na^+ and Cl^- content that coincides with naturally timed SW entry (Grant et al., 2009). This elevation in whole body ion levels persists until gill NKA activity peaks two months later, which is well beyond the hours to days reported for other salmonids (*O. keta, O. mykiss, O. kisutch, S. salar*; Black, 1951; Leray et al., 1981; Miles and Smith, 1968; Prunet and Boeuf, 1985) and may indicate an underdeveloped hypo-osmoregulatory capacity. Furthermore, the majority of the increase in gill NKA activity occurs after seawater entry (Grant et al., 2009; Honma, 1982), suggesting compensatory rather than preparatory changes. Evidently, when and how SW tolerance and hypo-osmoregulatory ability of juvenile pink salmon develops remains unclear.

The objective of this study was thus two-fold. 1) To determine if pink salmon possess adult-like hypo-osmoregulatory abilities as larval alevins, and 2) identify the physiological differences between alevins and post-larval fry critical to SW survival. To address these issues, I

measured ionoregulatory status of larval alevins and post-larval fry before and after 5 days of SW exposure.

2.2 MATERIALS AND METHODS

Experimental Animals

Pink salmon alevins aged 500 accumulated thermal units (ATU's) were transported from Seymour River Hatchery to the University of British Columbia on February 28th 2008. Fish were separated and held in two 90 L static charcoal-filtered glass aquaria in dechlorinated Vancouver city tap water ($[Na^+]$, 0.17 mM; $[Cl^-]$, 0.21 mM; hardness, 30mg L^{-1} as $CaCO_3$; pH 5.8-6.4) and maintained at a temperature of 3.5°C in total darkness to mimic gravel rearing conditions. Fish were treated according to University of British Columbia animal care protocol #A07-0055.

Transfer Protocol

At 540 and 600 ATU's, fish were randomly transferred to new 90 L static charcoal-filtered glass aquaria filled with either freshwater (FW; 3.5°C; composition listed above) or 100% seawater (SW; 3.5°C; 32.0 g L^{-1}, prepared in dechlorinated Vancouver city tap water with Instant Ocean sea salt mix); transfer tanks were maintained on a simulated natural photoperiod (SNP) of 12L:12D to mimic gravel emergence. A total of 38 fish (N=10 for wet/dry mass and total body $[Na^+]$ and $[Cl^-]$; N=10 for gill Na^+/K^+-ATPase activity; N=10 for drinking rates; N=8 for gill Na^+/K^+-ATPase α1 subunit mRNA expression) were sampled from the respective FW holding tanks pre-transfer (pre-transfer FW), and from FW and SW treatment tanks under SNP (FW+SNP, SW+SNP) at 1 and 5 days post-transfer.

Fish aged 540 ATU's, referred to as alevins, possess an externally visible yolk sac and do not naturally emerge from gravel at this stage in development to enter SW (M. Casselman; personal communication, Seymour River Hatchery). Fish aged 600 ATU's, referred to as fry, no

longer possess an externally visible yolk sac and do naturally emerge from gravel at this stage in development to enter SW. Due to a limited number of fish, the FW+SNP treatment was foregone during alevin transfers. Additionally, drinking rates were only measured in SW+SNP treatment groups, as they were expected to be very low or absent in FW.

Body Mass and Total Body [Na^+] and [Cl^-]

Fish were anaesthetized with a lethal dose of buffered tricaine methanesulfonate (0.8 g L^{-1} MS-222; Syndel Laboratories, Vancouver, BC, Canada), rinsed in de-ionized water, blotted dry and weighed. Fish were dried in pre-weighed 15 mL polystyrene tubes at 65°C until no further reduction in mass was observed. Dried fish were digested in 2 mL of 1 M nitric acid for 3 days at 65°C and the supernatant subsequently analyzed for Na^+ and Cl^- content. Supernatant [Na^+] was measured using flame atomic absorption spectroscopy (Spectra AA-220FS; Varian, Mulgrave, VC, Australia) and standardized to wet and dry body mass for total body [Na^+]. Supernatant [Cl^-] was measured using the colorimetric mercuric thiocyanate method (Zall et al., 1956) and standardized to wet mass for total body [Cl^-].

Gill Na^+/K^+-ATPase Activity

Whole gills were removed and stored at -80 °C following anaesthetization as described above. A modified version of the method outlined by McCormick (1993) was used to determine NKA activity. This method couples ouabain-sensitive ATP hydrolysis to the oxidation of NADH by way of pyruvate kinase and lactate dehydrogenase. Briefly, whole frozen gills were homogenized on ice in SEI buffer (250 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.3), centrifuged at 5000 *g* for 1 min and the supernatant removed. Supernatant ATPase activity was measured spectrophotometrically in the presence and absence of ouabain (1 mM), and activity was taken as the difference between these conditions. Protein concentration was measured using

the bicinchoninic acid method (Sigma-Aldrich) and bovine serum albumin standards. NKA activity is reported as μmol ADP mg^{-1} protein h^{-1}. All samples were run in triplicate within 2 h of homogenization.

Drinking Rates

Ten fish were placed in static polyethylene chambers containing 60 mL of aerated SW. To minimize disturbance, trials were completed in darkness and fish were permitted a 1 h chamber acclimation period. At trial onset, 90 μCi of [^{3}H]polyethylene glycol (PEG-4000; American Radiolabeled Chemicals Inc., St. Louis, MO, USA) was added to the system and 5 mL of water sampled. Following 4 h of exposure, fish were lethally anaesthetized as described above and an additional 5 mL water sample was taken. Fish were rinsed thrice in de-ionized water to remove residual PEG, blotted dry and weighed. Fish were digested with 2 mL of 10% perchloric acid for 48 h at 65^{o}C, then homogenized, vortexed and left to settle. 1 mL aliquots of clear supernatant and trial water samples were analyzed for radioactivity using a liquid scintillation counter (LSC-2000; Beckman-Coulter Inc., Fullerton, CA, USA). Water uptake was determined by dividing total counts per fish by the specific activity (μCi/ml H_2O) of reference water samples. Drinking rates were calculated by expressing total water uptake relative to fish mass and [^{3}H]polyethylene glycol exposure time. Drinking rates are reported as mL H_2O kg^{-1} h^{-1}.

Gill mRNA

Total RNA was extracted from whole gills using the Invitrogen TRIzol Reagent according to the manufacturer's instructions, and concentrations subsequently determined spectrophotometrically. First-strand cDNA was reverse transcribed according to Scott et al. (2004). Gene expression was assessed using quantitative real-time PCR (qRT-PCR) on an ABI Prism 7000 sequence analysis

system (Applied Biosystems). PCR reactions contained 1 μl of cDNA, 4 pmol of each primer and Universal SYBR green master mix (Applied Biosystems) in a total volume of 21 μl.

Statistical Analyses

Data are expressed as means ± s.e.m. A one-way analysis of variance (ANOVA) was used to analyze alevin data, and a two-way ANOVA was used for fry. The Holm-Sidak post-hoc test was subsequently applied for pairwise comparisons when effects were found to be significant. All data passed tests for normality and homogeneity of variance. All statistical analyses were conducted with Sigmastat (version 3.0, Systat Software Inc., San Jose, CA, USA), and a significance level of $P<0.05$ was used throughout.

2.3 RESULTS

Alevin SW+SNP Transfers

Alevin wet body mass was significantly reduced by 8% and 15% at 1 and 5 days post-SW+SNP exposure, respectively ($P<0.05$, Fig. 2.1A). No significant change in alevin dry body mass was observed post-transfer (Fig. 2.1B), indicating that reductions in wet mass were due to water loss.

Total body $[Na^+]$ of alevins expressed as a function of wet mass increased significantly by 15% relative to the pre-transfer FW control at 5 days post-SW+SNP exposure ($P<0.05$, Fig. 2.1C); however, total body $[Na^+]$ did not change when expressed as a function of dry mass (Fig. 2.1D). Total body $[Cl^-]$ expressed as a function of wet mass increased by 35% relative to the pre-transfer FW control at 1 day post-SW+SNP exposure and remained elevated at 50 μmol g wet $mass^{-1}$ for trial duration ($P<0.05$, Fig. 2.1E).

Gill NKA activity of alevins did not change significantly following SW+SNP exposure (Fig. 2.1F), but significant differences in α1 subunit mRNA expression were observed. The α1a/α1b ratio was almost completely inversed relative to the pre-transfer FW value by day 5,

decreasing significantly from 1.91 to 0.54 (Fig. 2.2A). When expressed relative to total RNA, this relationship appears to be driven by a significant 60% decrease in α1a (Fig. 2.2A), as α1b did not change significantly over trial duration (Fig. 2.2C). A similar pattern in α1a & b expression was observed when expressed relative to the control genes EF-1α and Ub (Table 2.1), but a significant increase in α1b also appears to be driving this relationship. It should be noted that trends expressed relative to EF-1α and Ub were affected by significant decreases in both of these control genes (Table 2.1).

Alevin drinking rates were measured as approximately 1.75 mL kg^{-1} h^{-1} at both 1 and 5 days post-SW+SNP transfer (Fig. 2.3).

Fry FW+SNP & SW+SNP Transfers

No significant changes in wet or dry mass were observed in fry relative to the pre-transfer FW values following FW+SNP and SW+SNP exposure (Fig. 2.4A,B), indicating that water balance was maintained.

Total body [Na^+] in fry expressed as a function of wet body mass increased significantly by 11% and 35% relative to the pre-transfer FW control at 1 and 5 days post-SW+SNP exposure, respectively ($P<0.05$, Fig. 2.4C). This increase exceeds that reported for alevins by more than two-fold. Additionally, values for fry 5 days post-SW+SNP exposure were significantly greater than time-matched FW+SNP values by roughly 20% ($P<0.05$, Fig. 2.4C). Total body [Na^+] in fry expressed as a function of dry body mass also increased significantly relative to the pre-transfer FW control at 5 days post-SW+SNP exposure, with SW+SNP values significantly exceeding FW+SNP by roughly 15% ($P<0.05$, Fig. 2.4D). This result contrasts the lack of change observed in alevins. However, total body [Cl^-] expressed as a function of wet mass in SW+SNP fry exhibited a pattern closely resembling that observed in alevins, increasing significantly above

pre-transfer FW and time-matched FW+SNP values to a plateau of approximately 50 μmol g wet mass^{-1} for trial duration ($P<0.05$, Fig. 2.4E).

While alevins failed to upregulate NKA activity following SW transfer, fry exhibited significant parallel increases relative to the pre-transfer value following both SW+SNP and FW+SNP exposure at 1 and 5 days post-transfer ($P<0.05$, Fig. 2.4F). The gill NKA α1a/α1b mRNA ratio expressed relative to total RNA also exhibited parallel changes following FW+SNP and SW+SNP exposure (Fig. 2.5A), matching the trend observed in SW+SNP alevins. The 1.49 pre-transfer α1a/α1b ratio decreased significantly to 0.54 by day 5 in FW+SNP and even further to 0.22 by day 5 in SW+SNP ($P<0.05$, Fig 2.5A). As in alevins, this relationship appears to be driven largely by significant decreases in α1a (Fig. 2.5B), as α1b did not change significantly over trial duration. When expressed relative to the control genes EF-1α and Ub (Table 2.2), similar patterns in the α1a/α1b ratio were observed, but as with the alevin trials, a significant increase in α1b also appears to be driving this relationship. However, as also seen in the alevin trials, the trends expressed relative to EF-1α and Ub are affected by significant decreases in both of these control genes (Table 2.2).

Fry drinking rates were measured as approximately 1.75 mL kg^{-1} h^{-1} at 1 and 5 days post-SW+SNP transfer and are not significantly different from those reported for alevins (Fig. 2.3).

2.4 DISCUSSION

The current study does not support the hypothesis that pink salmon possess adult-like hypo-osmoregulatory abilities as larvae. This point is clearly illustrated by a failure of larval alevins to maintain ion and water balance following SW exposure and a lack of upregulation in gill NKA activity. Furthermore, the key difference between larval alevins and post-larval fry critical to SW survival appears to be the maintenance of water balance, for which fry seemingly

sacrifice Na^+ balance in doing so. We also provide the first evidence to suggest that photoperiod may trigger smolt-like increases in gill NKA activity of pink fry.

Do Larval Alevins Develop Adult-Like Hypo-Osmoregulatory Ability?

Larval alevins show no evidence of adult-like hypo-osmoregulatory ability following SW exposure. Gill NKA activity does not increase as seen in post-larval stages (Honma, 1982, Grant et al. 2009), and levels remain low and comparable to those previously reported for FW pre-smolt salmonids (Madsen et al., 2009; Richards et al., 2003). A 35% increase in body $[Cl^-]$ persists 5 days post-SW entry, indicating a failure to maintain ion balance [the closely related chum regains $[Cl^-]$ balance 24 h post-transfer (Black 1951)], and the measures of wet and dry body mass clearly indicate a failure to maintain water balance.

The measured drinking rate of 1.75 ml kg^{-1} h^{-1} for alevins transferred to SW+SNP equals that reported for fry and exceeds that reported for many salmonids in FW (0.1 for S. salar, 0.25 for O. mykiss; Fuentes and Eddy, 1997). However, alevins still dehydrate, suggesting that water uptake may not be occurring. Several points further support this interpretation. First, the method employed to determine drinking rate estimates water ingested into the gastrointestinal tract and not that absorbed across the gut wall, thus it is not a true measure of water uptake. Second, the primary source of Na^+ uptake in SW salmonids is by active transport at the gut to facilitate water absorption (Grosell, 2006; Kirschner et al., 1974; Smith, 1930), yet $[Na^+]$ does not increase relative to dry mass. Finally, the lack of increase in gill NKA activity suggests that other changes typical of SW acclimation (i.e. intestinal remodelling for water uptake) may not take place, as they often co-occur (Collie and Bern, 1982; Loretz et al., 1982). Thus, the reported drinking rates may represent an ineffective behavioural response to the SW+SNP transfer.

Keys to Post-Larval Fry SW Survival

In contrast to alevins, and as expected, fry appear to hypo-osmoregulate at the whole animal level following SW exposure. A significant increase in gill NKA activity accompanied by a significant decrease in the α1a/α1b mRNA expression ratio indicates the induction of compensatory measures, while a significant increase in body [Na^+] coupled with a lack of change in wet and dry mass suggests that water balance is maintained by drinking. Maintaining water balance thus appears to be the key difference between alevins and fry critical to SW survival; however, fry unexpectedly experience a greater whole body ionic disturbance than alevins following SW transfer.

Anadromous salmonids are typically assessed as being prepared for the marine environment by the degree of ionic disturbance sustained at the plasma level following SW entry (24 h seawater challenge; Blackburn and Clarke, 1987; Clarke, 1982; Clarke and Blackburn, 1977). On a whole body level, water content may be a more suitable evaluation for pink salmon than a traditional ionic assessment as "prepared" fry experience an ionic perturbation three fold greater than "unprepared" alevins. Water balance therefore seems more critical to early marine survival, as pink fry actively sacrifice Na^+ balance (nearly a two-fold increase; Grant et al. 2009) by drinking to maintain hydration.

The prioritization of water balance does not imply that ion balance is unimportant to fry SW survival. Whole body levels still remain well below those of ambient SW, and the upregulation of gill NKA activity indicates an increased capacity for ion excretion. Although ion balance is not restored until gill NKA activity peaks eight weeks later (Grant et al. 2009), the degree of ion excretion afforded may be just as important to performance and survival if ion levels are kept below a certain critical threshold. This may explain the observed similarity between whole body [Cl^-] of alevins and fry despite the presumed increase in intestinal uptake by the latter.

Photoperiod May Trigger Smoltification in Pink Salmon

Upon controlled emergence from darkness to a natural photoperiod, changes in gill NKA activity and α1 isoform mRNA expression in FW fry parallel those of fry transferred directly to SW. These results demonstrate for the first time that photoperiod may trigger preparatory changes in pink salmon typical of smoltification. This differs from the current belief that ontogeny largely dictates the acquisition of salinity tolerance in pink salmon, which is supported by previously reported increases in gill NKA activity of FW fry following a surge in plasma thyroxine and yolk absorption (Sullivan et al., 1983). Although the present study lacks a suitable control (FW dark) to conclusively dissociate developmentally timed change from that triggered by environmental cues, the likelihood of coincidence between controlled emergence and ontogenetic change is low enough to merit speculation.

The observed drinking rates and changes in α1 mRNA expression reported for alevins following SW+SNP exposure also support the notion of smolt-like environmental cue sensitivity, as they match those observed in FW/SW+SNP fry and occur well before the proposed ontogenetic switch at yolk absorption. Pink salmon are thus clearly capable of responding to environmental cues at transcriptional and behavioural levels prior to yolk absorption, but fail to mount an effective change in whole animal performance. Instead of an ontogenetic switch for hypo-osmoregulatory development, we propose that yolk absorption may mark a point in pink development where fry become capable of fully translating environmental cues into a preparatory smolt response. This proposed smolt competence could be linked to endocrine development and certainly warrants further investigation. If our hypothesis is confirmed, future work should also determine whether pinks respond to a specific photoperiod or if light exposure is sufficient to cue the appropriate changes.

Summary

The current study sheds new light on the acquisition of SW tolerance in pink salmon. Contrary to long-held pre-conceptions, it was shown that pinks do not possess adult-like hypo-osmoregulatory abilities as larvae and that post-larval preparatory change might be triggered by a smolt-like photoperiod cue rather than ontogenetic change. Furthermore, maintaining water balance appears to be the key difference between alevins and fry critical to SW survival. Fry actively incur large ion loads in SW to maintain hydration prior to the completion of hypo-osmoregulatory development, which appears to be a novel strategy among salmonids. The ability to tolerate large, sustained ionic loads at the whole body level may be a key adaptation underlying this most extreme and perhaps derived form of salmonid anadromy.

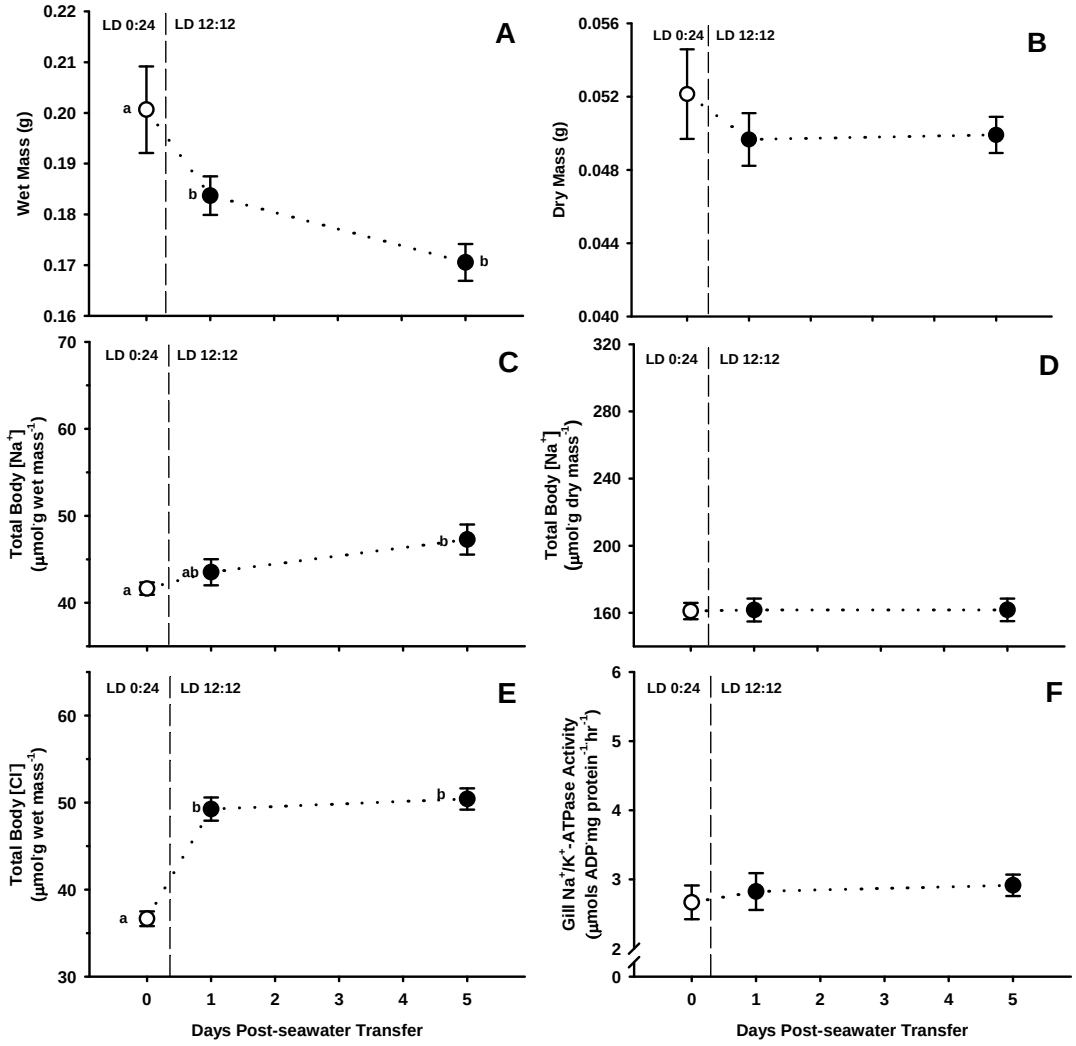

Figure 2.1. Wet mass (A), dry mass (B), body [Na^+] as function of wet and dry mass (C, D), body [Cl^-] as a function of wet mass (E) and gill Na^+/K^+-ATPase activity (F) of pink salmon alevins. Open circles represent alevins reared in freshwater under total darkness (pre-transfer FW), and closed circles represent alevins simultaneously exposed to seawater and a simulated natural photoperiod (SW+SNP). Values are means ± s.e.m. (n = 10); letters indicate significance ($P<0.05$).

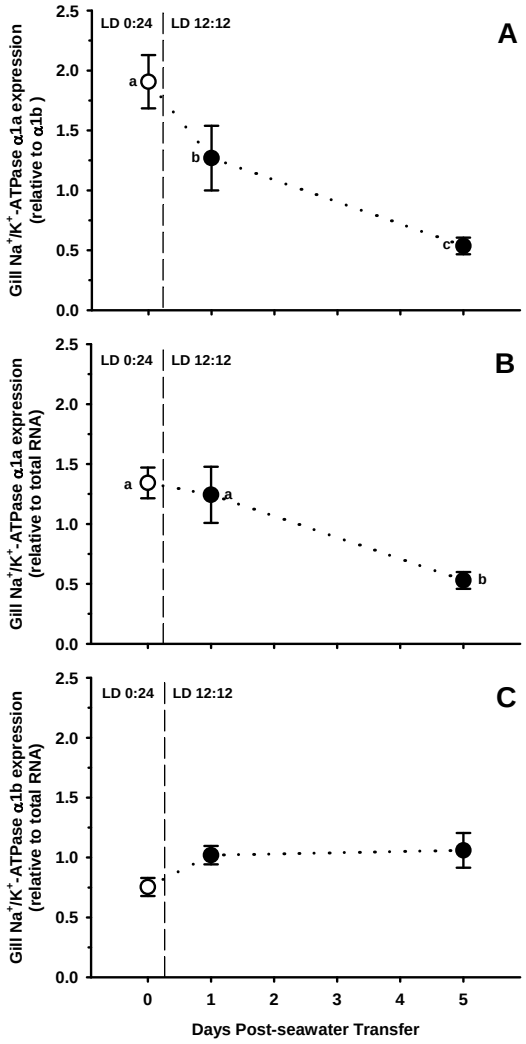

Figure 2.2. Gill Na^+/K^+-ATPase α1a mRNA expression relative to α1b (A) and total RNA (B), and α1b mRNA expression relative to total RNA (C) of pink salmon alevins. Open circles represent alevins reared in freshwater under total darkness (pre-transfer FW), and closed circles represent alevins simultaneously exposed to seawater and a simulated natural photoperiod (SW+SNP). Values are means ± s.e.m. (n = 6-10); letters indicate significance ($P<0.05$).

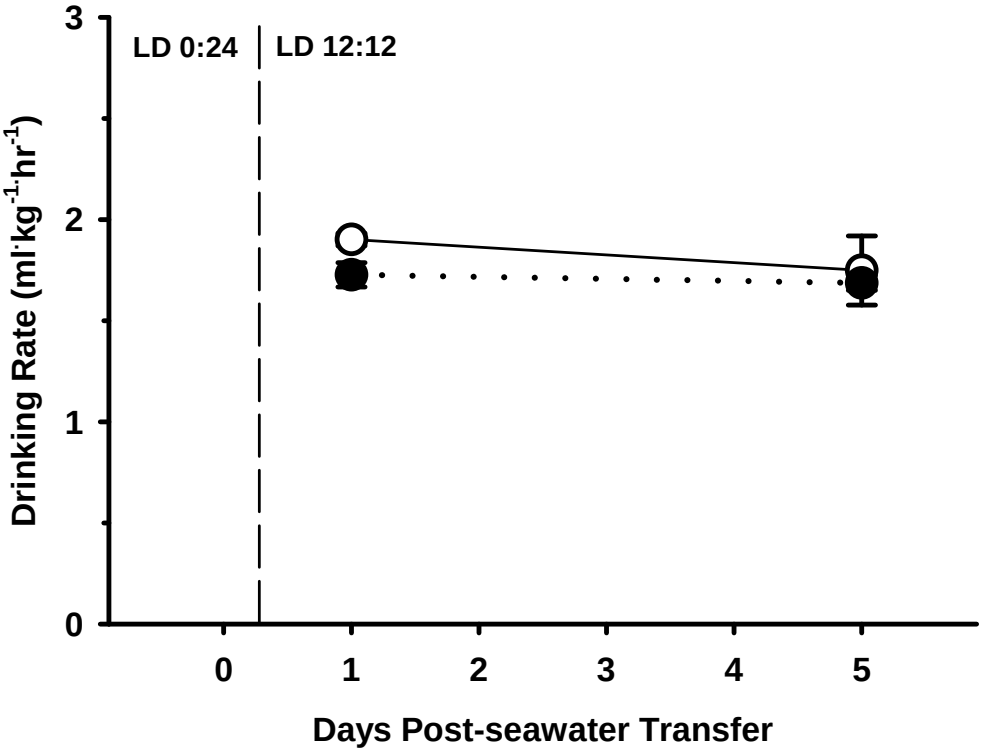

Figure 2.3. Drinking rates of pink salmon alevins (open circles) and fry (closed circles) following simultaneous exposure to seawater and a simulated natural photoperiod (SW+SNP). Values are means ± s.e.m. (n = 10). No significant differences were found between groups.

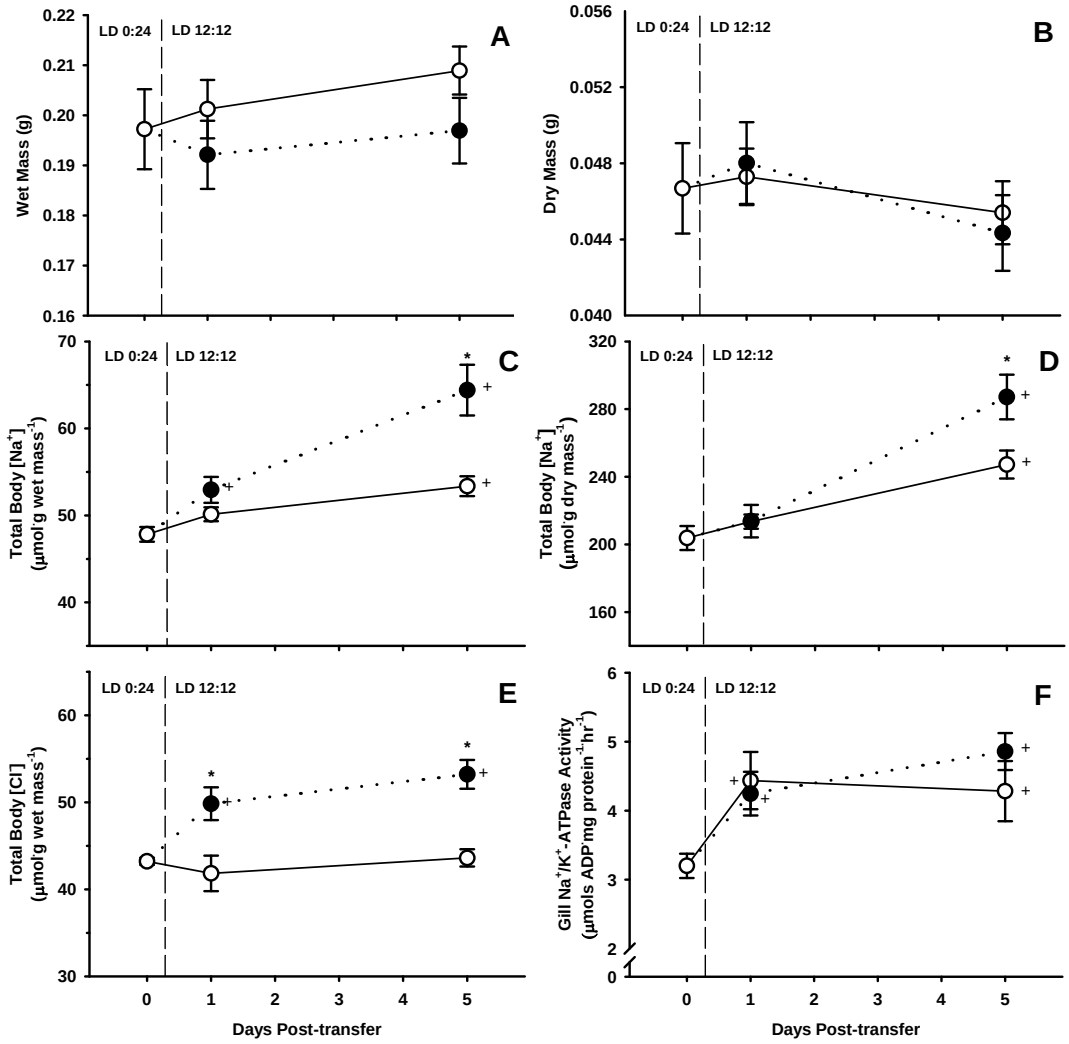

Figure 2.4. Wet mass (A), dry mass (B), body [Na^+] as function of wet and dry mass (C, D), body [Cl^-] as a function of wet mass (E) and gill Na^+/K^+-ATPase activity (F) of pink salmon fry. Open circles at day "0" represent fry reared in freshwater under total darkness (pre-transfer FW), open circles at day "1" & "5" represent fry exposed to a simulated natural photoperiod while still in freshwater (FW+SNP), and closed circles represent fry simultaneously exposed to both seawater and a simulated natural photoperiod (SW+SNP). Values are means ± s.e.m. (n = 10). "+" indicates a significant difference from the pre-transfer FW value; asterisks indicate significant differences between time-matched FW+SNP and SW+SNP values ($P<0.05$).

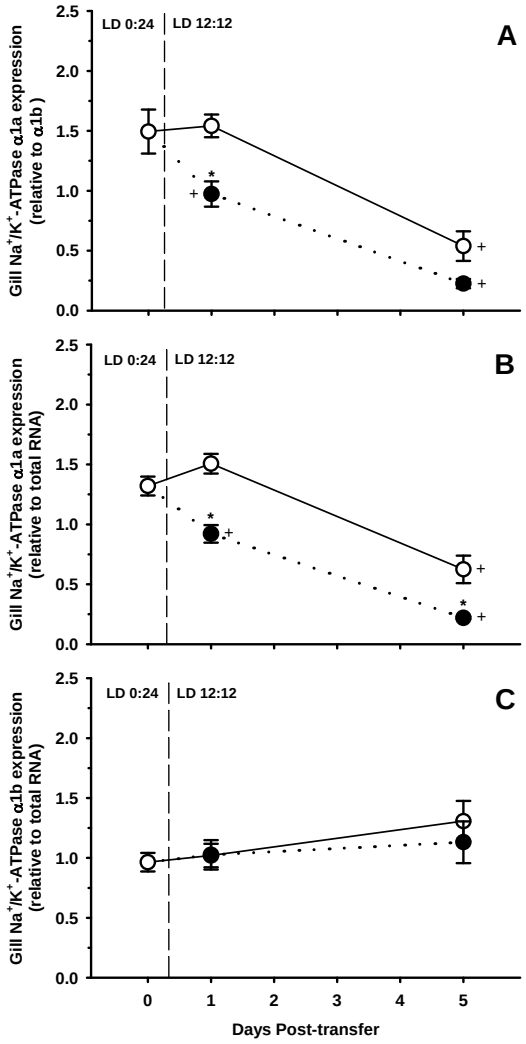

Figure 2.5. Gill Na^+/K^+-ATPase α1a mRNA expression relative to α1b (A) and total RNA (B), and α1b mRNA expression relative to total RNA (C) of pink salmon fry. Open circles at day "0" represent fry reared in freshwater under total darkness (pre-transfer FW), open circles at day "1" & "5" represent fry exposed to a simulated natural photoperiod while still in freshwater (FW+SNP), and closed circles represent fry simultaneously exposed to both seawater and a simulated natural photoperiod (SW+SNP). Values are means ± s.e.m. (n = 6-10). "+" indicates a significant difference from the pre-transfer FW value; asterisks indicate significant differences between time-matched FW+SNP and SW+SNP values ($P<0.05$).

Table 2.1. Expression of Na^+/K^+-ATPase α1a & α1b mRNA relative to EF-1α & Ub in pink salmon alevin gills pre- and post-SW+SNP exposure. Data are means ± s.e.m.; letters indicate significance.

Treatment	α1a/EF-1α	α1b/EF-1α	EF-1α/total RNA	α1a/Ub	α1b/Ub	Ub/total RNA
Pre-transfer FW	1.30±0.10 [a]	0.76±0.10 [a]	1.04±0.06 [a]	1.59±0.17 [a]	0.94±0.14 [a]	0.89±0.08 [a]
SW+SNP day 1	1.12±0.07 [a]	1.01±0.13 [a]	1.09±0.15 [a]	1.30±0.14 [a,b]	1.13±0.13 [a]	0.93±0.07 [a]
SW+SNP day 5	0.74±0.07 [b]	1.52±0.19 [b]	0.71±0.06 [b]	0.90±0.11 [b]	1.82±0.21 [b]	0.61±0.07 [b]

Table 2.2. Expression of Na^+/K^+-ATPase α1a & α1b mRNA relative to EF-1α & Ub in pink salmon fry gills pre- and post-FW+SNP & SW+SNP exposure. Data are means ± s.e.m.; "t" indicates significance from pre-transfer FW values; asterisks indicate significance between time-matched FW+SNP and SW+SNP values.

Treatment	α1a/EF-1α	α1b/EF-1α	EF-1α/total RNA	α1a/Ub	α1b/Ub	Ub/total RNA
Pre-transfer FW	1.36±0.06	1.04±0.13	0.97±0.04	1.59±0.14	1.13±0.08	0.86±0.04
FW+SNP day 1	1.35±0.08	0.91±0.07	1.17±0.14	1.56±0.12	1.07±0.14	1.00±0.08
SW+SNP day 1	1.02±0.05 ^{t}*	1.14±0.11	0.94±0.12	1.21±0.10	1.35±0.16	0.86±0.16
FW+SNP day 5	1.34±0.25	3.08±0.54 ^{t}	0.49±0.10 ^{t}	2.27±0.55 ^{t}	4.79±0.72 ^{t}	0.29±0.03 ^{t}
SW+SNP day 5	0.72±0.07 ^{t}*	3.53±0.33 ^{t}	0.31±0.02 ^{t}	0.83±0.13 ^{t}*	4.40±0.75 ^{t}	0.34±0.10 ^{t}

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Chapter 3: Pink Salmon (*Oncorhynchus gorbuscha*) Hypo-Osmoregulatory Development Plays a Key Role in Sea Louse (*Lepeophtheirus salmonis*) Tolerance[2]

3.1 INTRODUCTION

The ectoparasitic salmon louse *Lepeophtheirus salmonis* has been recently implicated in pink salmon (*Oncorhynchus gorbuscha*) population declines and extinction predictions in British Columbia's Broughton Archipelago (Krkosek et al., 2007a; Krkosek et al., 2007b; Krkosek et al., 2006; Krkosek et al., 2005; Krkosek et al., 2009; Morton and Routledge, 2005; Morton et al., 2004; Morton et al., 2005). Although these studies are compelling, major questions regarding parasite transmission and infection impacts on host physiology remain unknown. The uncertainty has led to considerable debate as to whether sea lice, specifically of farm origin, are negatively impacting local pink salmon populations (Beamish et al., 2006; Beamish et al., 2007; Brooks, 2005; Brooks and Jones, 2008; Brooks and Stucchi, 2006; Butterworth et al., 2008a; Butterworth et al., 2008b; Dill et al., 2009; Krkosek et al., 2008a; Krkosek et al., 2008b; Morton and Routledge, 2008; Riddell et al., 2008). A critical knowledge gap which the present study aims to address is the level of louse infection required to sub-lethally impair performance of out-migrating juvenile pink salmon weighing between 0.2 and 3 g.

Lice feed on host mucous, epidermal tissue and blood (Brandal et al., 1976; Kabata, 1974) as they develop through four attached stages (termed chalimus 1-4) and three motile stages (termed pre-adult 1-2 & adult) on the fish's exterior surface (Brooks, 2005; Johnson and Albright, 1991; Tully, 1992). As shown in 200 g Atlantic salmon and 90 g sea trout, this surface feeding can lead to significant increases in plasma electrolytes (up to 60%; Bjorn and Finstad,

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1997; Bowers et al., 2000; Grimnes and Jakobsen, 1996; Nolan et al., 1999; Wootten et al., 1982). This hydromineral disruption is thought to result either directly from epidermal lesions that damage the external osmotic barrier or indirectly from a stress-related increase in the osmorespiratory compromise (Gonzalez and McDonald, 1994; Gonzalez and McDonald, 1992; Wendelaar Bonga, 1997). Regardless of origin, hydromineral imbalance is a major concern as it can lead to stunted growth (Brauer, 1982; Folmar et al., 1982; McCormick et al., 1987), reduced swim performance (Brauner et al., 1994; Brauner et al., 1992), and even death (Boeuf and Harache, 1982).

While adult pink salmon are likely well equipped to cope with parasitism in the wild (Beamish et al., 2005), out-migrating juveniles may be less prepared. Foremost, fry enter seawater as small as 0.2 g (Heard, 1991) and are thus three orders of magnitude smaller than adult pinks when first exposed to lice (Jones and Hargreaves, 2007). Additionally, epidermal (Jones et al., 2008a), immune (Johnson et al., 1982) and hypo-osmoregulatory (Grant et al., 2009) systems all still develop during the first few months at sea. Since these physiological characteristics are presumably critical to *L. salmonis* infection resistance and tolerance, juvenile pink salmon are likely particularly vulnerable to parasitism during this precocious out-migration.

Currently, only one study has quantified the sublethal effects of infection on out-migrating, 0.2-3 g pink salmon. One motile louse was shown to significantly reduce swimming performance in fry below, but not beyond, a body mass of 0.7 g (Nendick et al. 2010). Interestingly, this body mass threshold corresponds with the completion of pink salmon hypo-osmoregulatory development (0.7-1.0 g; Grant et al., 2009), suggesting the observed effects might be linked to hypo-osmoregulatory ability. Similar reductions in swimming performance have also been correlated with elevated plasma ions in 8-20 g *O. kisutch* following SW entry (Brauner et al., 1994; Brauner et al., 1992; Randall and Brauner, 1991) and 600 g *Salmo salar* infected with 80 lice per fish (Wagner et al., 2003; Wagner et al., 2004). Furthermore, 200 g

infected *S. salar* have been shown to upregulate gill NKA activity by as much as 60% (Nolan et al., 1999), likely as an attempt to restore hydromineral balance. Based on these findings, we hypothesized that louse-induced performance reductions are ionoregulatory in origin. In a controlled laboratory setting and the wild, we investigated the ionic disruptions and ion regulatory compensations associated with sea louse-infection in post-emergent pink salmon for the first time. These data were used to determine a fine-scale threshold infection level for sub-lethal louse effects.

3.2 MATERIALS AND METHODS

Research Facility

All experiments were conducted at an autonomous field laboratory in the Broughton Archipelago, British Columbia, Canada between March and July of 2008. Constructed on a float-house adjacent to Doctor Islets in Knight's Inlet, the laboratory location provided easy access to juvenile pink salmon throughout the entirety of their near-shore migration (transport time from collection areas never exceeded 4 h). Laboratory holding tanks were equipped with flow-through aerated seawater (SW) drawn from depth at 30 m to ensure stable temperature (7.0-8.5 ^{o}C) and salinity (32-34 ppt), and the facility's translucent canopy permitted a natural photoperiod throughout study duration. All fish were treated in accordance with the University of British Columbia Animal Care Committee and the Canadian Council on Animal Care.

Series 1: River-caught (RC) Fish, Laboratory Infection

Experimental Animals

River-caught (RC) fish: Approximately 2,000 post-emergent pink fry were collected with rotary screw-traps from the Glendale River during their down-stream migration on March 27th 2008. Fish were immediately transported to the field laboratory where they were gradually

exposed to full strength SW (34 ppt) over a period of 24 h. Initially at an approximate size of 25 mm and 0.2 g, pink fry were divided into four 100 L fibreglass holding tanks in which they were fed commercial trout chow twice daily (Bio-Vita starter feed; Bio-Oregon, Longview, WA). Glendale River was selected as a fish source because it is highly representative of the region's fish, accounting for more than 35 and 85% of total Broughton pink salmon in even and odd years, respectively (Brooks and Jones, 2008). Out-migrating RC fish were used for laboratory-based infections for two reasons: (1) they had no prior exposure to sea lice; and (2) they represented the earliest and presumably most sensitive life-stage that could become infected in the wild given their precocious seawater entry.

Lepeophtheirus salmonis: Approximately 300 gravid female lice were collected from adult Atlantic salmon on March 24th 2008 during a commercial harvest at Marine Harvest Canada's Wicklow Point farm. Upon transport to the Doctor Islets laboratory, louse egg strings were incubated at 7 ^{o}C in four 4 L closed containers filled with aerated SW (34 ppt). Active nauplii were filtered out every two days with 96 μm plankton mesh and placed in separate 2 L rearing chambers. Incubation water was changed every other day and nauplii monitored daily for copepodid development. Active copepodid densities were estimated by averaging counts from five 10 mL aliquots sampled from each rearing chamber. Infection was initiated when a copepodid:nauplii ratio of 3:1 was reached.

Infection Protocol and Results

L. salmonis infection was initiated on April 3rd 2008 after RC fish had spent 1 week in SW. A total of 585 fish was divided into subgroups of ~75 and placed into 8 separate 11 L infection chambers (modified POS plastic totes; Rubbermaid). Each chamber was filled with 3 L of static aerated SW and approximately 1,700 active copepodids, yielding a bath density of ~560 copepodids $\cdot$ L^{-1} and a copepodid:fish ratio of 23:1. Chamber dimensions were such that infection bath depth was only 5 cm, minimizing fish/louse stratification and maximizing host/parasite

interaction. Louse exposure was carried out in darkness for a total of 4 h, followed by a switch to flow-through SW to flush the infection bath. An additional 150 fish were subjected to an identical sham infection without lice.

Approximately 48 h post-infection, all fish were lightly anaesthetized (0.05 $g \cdot L^{-1}$ MS-222) and sorted according to louse load. 511 of 581 fish were successfully infected (a prevalence of ~88 %): 129 individuals (22%) were infected with 1 louse; 101 individuals (17%) with 2 lice; 99 individuals (17%) with 3 lice; 182 individuals (31%) with 4^+ lice (where up to 20 lice were observed on some fish).

Sampling Protocol and Constraints

Following sorting, fish were placed in 10 separate 11 L holding tanks according to five categories of louse load (0, 1, 2, 3 and 4+ lice/fish; 2 replicate tanks/treatment). Each tank was equipped with flow-through SW (34 ppt, 7.0-8.5^oC), held no more than 75 fish and was supplied with commercial trout chow twice daily. Fish were sampled over the ensuing 24 days as sea lice developed towards adults. For the experiments described in this study, a total of 20 fish (10 for whole body [Na^+] and 10 for gill NKA activity) were sampled for four louse loads (0, 1, 2 and 3 lice/fish) at each of the four attached louse stages [chalimus 1-4; 3, 7, 15 and 24 days post-infection (DPI)]. All fish were examined by microscope at the time of sampling to determine louse number, developmental stage and surface area of epidermal tissue penetration.

As reported in previous laboratory studies (Jones et al., 2008a; Jones et al., 2008b), infected pink salmon fry experienced high rates of louse loss throughout trial duration. Consequently, the number of infected fish (especially those with more than one louse) declined with time. To increase infected fish numbers, fish that were initially placed in the 4^+ lice/fish group were re-sorted at 14 DPI (~chalimus 3 stage) and redistributed appropriately among the 1-3 lice/fish treatment tanks. A total of 99 fish were added to the sampling population (48 fish with 1 louse, 31 fish with 2 lice and 20 fish with 3 lice). Even with these additional animals, infected

fish numbers remained limited; hence measurements of gill NKA activity were necessarily restricted to fish with 1-2 lice of chalimus 1-3 stages, while measurements of whole body [Na^+] beyond the chalimus 3 stage were limited to fish with 1 louse. All reported louse loads correspond to infection levels at the time of sampling and, as a result, louse loads for some individuals would have been higher prior to sampling. Thus, if anything, the impacts of particular louse loads are overestimated.

Series 2: Ocean-caught (OC) Fish, Wild Infection

Over 10 000 wild pink salmon fry were captured by beach or purse seine during their near-shore migration through the Broughton Archipelago between April and June of 2008. These ocean-caught (OC) fish were graded directly following collection so that excess, uninfected fry could be released immediately on site and infected fry transported promptly back to the field laboratory. Fish were permitted a 24-h recovery period in the laboratory, then lightly anaesthetized (0.05 mg L^{-1} MS-222; Syndel) for sorting according to louse number and developmental stage. After an additional 24-h recovery period post-sort, fish were sampled for whole body [Na^+] and/or gill NKA activity, and the area of epidermal tissue penetration measured as in *Series 1*. Fish were only sampled if a minimum of 8 infected, size-matched fish of similar louse number and developmental stage were collected at the same location on the same date. A group of 10 uninfected, size-matched fish were also sampled from the same collection as a control. This sampling regime was designed to minimize any variation attributable to fish development and environmental experience. Total louse prevalence among this large sample of fish was roughly 10%, with an approximate intensity of 1. This low infection intensity restricted analysis to fish infected with 1 attached stage louse. As with *Series 1*, assay priority was given to whole body [Na^+] analysis.

Series 3: River-caught (RC) Fish, Mechanical Epidermal Abrasion

To mimic mechanical damage inflicted by motile stage lice, the epidermis of uninfected RC fish was subjected to various degrees of artificial abrasion following completion of *Series 1*. Using even pressure with a #2 scalpel blade, lightly anaesthetized fish approximately 0.7 g and 50 mm in size were subjected to: 1) 1 mm^2 surface scale removal, 2) 1 mm^2 epidermal penetration, or 3) 2 mm^2 epidermal penetration. The surface areas for abrasion were selected to match motile louse mouth sizes, and fish size corresponded to that of wild fish first encountering motile lice. All lesions were located posterior to the dorsal fin on the lateral line, and abrasion time never exceeded 30 s. A sham group was abraded in identical fashion with the scalpel handle to serve as a control.

Following abrasion, fish were held in duplicate tanks for each of the four treatment groups. Holding tanks, water conditions and fish care were identical to those of *Series 1*. For whole body [Na^+] and gill NKA activity, a total of 20 fish from each treatment were sampled pre-abrasion, and at 1 and 5 days post-abrasion as described below. Gill NKA activity was not measured in the 2 mm^2 abrasion group due to limited fish availability.

Assays

Wet & Dry Mass, Total Body [Na^+]

Fish were anaesthetized with a lethal dose of buffered tricaine methanesulfonate (0.8 g L^{-1} MS-222; Syndel Laboratories, Vancouver, BC, Canada), rinsed in de-ionized water, blotted dry and weighed for wet body mass. Individual fish were subsequently dried at 65^oC to a constant dry mass and digested in 1 M nitric acid (1 mL of acid for every 0.1 g of wet tissue mass). Digest supernatant was subsequently analyzed for Na^+ concentration ([Na^+]) using flame atomic absorption spectroscopy (Spectra AA-220FS; Varian, Mulgrave, VC, Australia). Total body [Na^+] is reported as μmol Na^+ g^{-1} wet mass.

Gill Na^+/K^+-ATPase Activity

Whole gills were removed from lethally anaesthetized fish (see above) and stored at -80°C. To determine gill NKA activity, a modified version of the method outlined by McCormick (McCormick, 1993) was used. Briefly, individual whole frozen gills were homogenized on ice in SEI buffer (250 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.3). Homogenates were centrifuged at 5,000 *g* for 1 min and the supernatant placed on ice. Supernatant ATPase activity was measured spectrophotometrically in the presence and absence of ouabain (1 mM), and taken as the difference between conditions. Protein concentration was measured using the bicinchoninic acid method (Sigma-Aldrich) with bovine serum albumin standards. NKA activity is reported as μmol ADP mg^{-1} protein h^{-1}.

Statistical Analyses

Data are expressed as means ± s.e.m. A two-way analysis of variance (ANOVA) was used throughout to determine significant differences, and the Holm-Sidak post-hoc test was subsequently applied for pairwise comparisons. All data passed tests for normality and homogeneity of variance. All statistical analyses were conducted with Sigmastat (version 3.0, Systat Software Inc., San Jose, CA, USA), and a significance level of $P<0.05$ was used throughout.

3.3 RESULTS

Series 1: River-caught (RC) Fish, Laboratory Infection

Fish Mortality, Growth and Louse-induced Damage

Of the initial 438 infected fish (1-3 lice/fish), only 25 animals died during the 24-day trial. There was no mortality among control fish, and so overall louse-induced mortality was approximately 6%. Of these 25 dead fish, 17 originated from the 4^+ lice/fish group and were

added to the 1-3 lice/fish groups following a re-sort at 14 DPI. These fish could have been initially infected with as many as 20 lice/fish. Mortality among fish originally infected with 1-3 lice/fish was therefore 2.4 %.

Control fish grew significantly from 0.224 $\pm$ 0.014 g to 0.358 $\pm$ 0.019 g over a 24-day period, yielding a specific growth rate (SGR) of approximately 2.26 $\pm$ 0.66 % body mass day^{-1} (Table 3.1). Growth of infected fish was similar, with SGR ranging from 1.23 to 1.86 % body mass day^{-1}. Fish mass only differed significantly from the control group at 7 DPI in the 2 lice/fish treatment ($P < 0.05$; Table 3.1).

Superficial, louse-inflicted epidermal damage was visible on host fish as early as 3 DPI (chalimus 1 stage), and epidermal penetration was present in all three treatments by 15 DPI (chalimus 3 stage; Table 3.1). The area of exposed subepidermal tissue increased significantly by 3-fold between 15 and 24 DPI in the 1 louse/fish treatment ($P < 0.05$; Table 3.1), during which time lice developed from the chalimus 3 to 4 stage. No significant differences among louse loads were found, but statistical power was low (0.187).

Total body [Na^+] and Gill Na^+/K^+-ATPase

As expected for a normal developmental trajectory in SW (see Grant et al., 2009), total body [Na^+] of control fish decreased steadily and significantly from approximately 81 $\mu mol \cdot g^{-1}$ at day 0 to 73 $\mu mol \cdot g^{-1}$ by 24 DPI ($P < 0.05$; Figure 3.1A). Total body [Na^+] of fish infected with 1 louse did not differ significantly from control values until 24 DPI (chalimus 4 stage, Figure 3.1 A; $P \leq 0.05$), when a 12 % increase was observed. A similar but larger and earlier significant increase in total body [Na^+] occurred in fish infected with 2 and 3 lice, where a 23 % increase was observed by 15 DPI (chalimus 3 stage, Figure 3.1A; $P < 0.05$). Thus, all three infection loads had a threshold louse developmental stage that triggered an ionic disturbance. This threshold was reached earlier in louse development when fish were host to more than one louse.

Although two lice triggered a greater ionic disturbance than 1 louse, no significant differences in total body [Na^+] were observed between 2 and 3 lice/fish.

As also expected for a normal developmental trajectory in SW, gill NKA activity of control fish increased steadily and significantly from approximately 7 μmol $ADP \cdot mg^{-1} \cdot h^{-1}$ at day 0 to 11 μmol $ADP \cdot mg^{-1} \cdot hr^{-1}$ by 15 DPI (Figure 3.1B; $P < 0.05$). In contrast, gill NKA activity of infected fish was significantly depressed as early as 3 DPI with just 1 louse (chalimus 1 stage, Figure 3.1B; $P < 0.05$). This approximate 40 % depression in enzyme activity was maintained independent of louse load until at least 7 DPI (chalimus 2 stage). By 15 DPI (chalimus 3 stage), gill NKA activity had recovered to control levels in all treatments. Therefore, rather than showing a compensatory increase in association with elevated ionic loads, infected fish exhibited an initial reduction in gill NKA activity.

Series 2: Ocean-caught (OC) Fish, Wild Infection

As with *Series 1*, epidermal penetration was not visible in infected OC fish prior to the chalimus 3 stage. Epidermal damage was also only visible in the smallest size class of OC fish (0.474 $\pm$ 0.043 g; Table 3.2.)

Total body [Na^+] and gill NKA activity of uninfected OC fish followed the same patterns observed for control fish of *Series 1*. As fish mass varied from ~0.5 g to ~1.5 g, total body [Na^+] decreased significantly from ~ 70 $\mu mol \cdot g^{-1}$ to ~ 50 $\mu mol \cdot g^{-1}$ (Figure 3.2A; $P < 0.05$). At the same time, gill NKA activity increased significantly from ~10 μmol $ADP \cdot mg^{-1} \cdot h^{-1}$ to ~14 μmol $ADP \cdot mg^{-1} \cdot h^{-1}$ (Figure 3.2B; $P < 0.05$). In contrast to *Series 1*, total body [Na^+] and gill NKA activity of infected OC fish did not differ significantly from size-matched uninfected OC fish (Figure 3.2; $P > 0.05$).

Series 3: River-caught (RC) Fish, Mechanical Epidermal Abrasion

The area of epidermal abrasion applied to RC fish exceeded the maximum area of louse-induced abrasion by nearly 10-fold (*Series 1 & 2)*. Despite this excessive damage, neither total body [Na^+] nor gill NKA activity differed significantly from respective time-matched controls (Figure 3.3; $P > 0.05$). Furthermore, total body [Na^+] and gill NKA activity were similar to size-matched control fish in other experiments.

Estimation of Threshold for Lice Effects

The data sets for total body [Na^+] from *Series 1 & 2* were pooled for analysis across a greater size range to determine the body mass threshold for louse-induced ionoregulatory disturbance. Second order polynomial regression lines were fitted to data of total uninfected and total infected fish for statistical comparison. Total body [Na^+] of fish infected with one chalimus 4 louse was significantly higher than that of uninfected fish when fish mass was below 0.5 g (Figure 3.4A), as clearly indicated by non-overlapping 95% confidence intervals. The observed difference in body [Na^+] between infected and uninfected fish below this threshold body mass of 0.5 g decreased with increasing fish mass and gill NKA activity (Figure 3.4A&B).

3.4 DISCUSSION

Using ecologically relevant infection levels of 1-3 attached stage lice/fish, we document some of the first sublethal physiological impacts of sea lice on post-emergent pink salmon. We conclude that beyond a threshold fish size of 0.5 g, one attached-stage louse (chalimus 1-4) no longer significantly disrupts hydromineral balance. Furthermore, we propose that this threshold is linked to pink salmon hypo-osmoregulatory development, which likely plays a key role in coping with the effects of any louse-induced damage or stress incurred. This notion is further strengthened by the maintenance of hydromineral balance in hypo-osmoregulatory advanced fish

(~0.7 g body mass) despite facing levels of experimenter-induced skin abrasion ten-fold that inflicted by lice in *Series 1* and *2*.

Results from the *Series 1* laboratory infection study clearly show that 1 louse at the chalimus 4 stage and 2 to 3 lice at the chalimus 3 stage are sufficient to significantly disturb total body Na^+ balance in pink salmon weighing less than 0.5 g. Because louse loads decreased over time in many fish, reported infection loads for a given sample point may have been less than actual infection histories. Similarly, louse numbers for our *Series 2* wild-infected OC fish also could have been greater prior to examination. Consequently, our measures of impact and the identified threshold, if anything, err on the side of caution.

We propose that this size-related louse tolerance is linked to pink salmon hypo-osmoregulatory development, adding to a previously suggested multi-factorial mechanism based on epidermal and immune system development (Jones et al., 2008a). A primary proximal effect of louse infection is physical damage to the host epidermis (Brandal et al., 1976; Kabata, 1974), which can potentially lead to hydromineral imbalance by disrupting the osmotic barrier and/or inducing a stress response (Wendelaar Bonga, 1997). While the immune system and epidermis combat proximal effects of parasitism (Jones et al., 2008a; Jones, 2001; Jones et al., 2008b), hypo-osmoregulatory ability enables fish to cope with the hydromineral consequences of any damage or attachment stress that might occur. The inverse relationship between total body Na^+ load and gill NKA activity of infected fish supports this notion, as does the absence of any ionic loading beyond attainment of maximum gill NKA activity. The more hypo-osmoregulatory advanced fish from *Series 3* even maintain Na^+ balance despite levels of skin abrasion ten-fold that inflicted by lice in *Series 1* and *2*. Although the nature of this damage differs considerably, this result suggests the potential to tolerate louse loads beyond those explored here upon reaching this developmental threshold. It should also be noted that our findings are consistent with the proposed role of epidermal and immune system development in louse resistance (Jones

et al., 2008a), as the epidermal damage for a given louse load decreases with increasing fish mass.

Louse-induced mortality of small, river-caught (RC) fish from the laboratory infection study was only 6% over 24 days. This does not exclude the possibility of increased lethality with further louse development, nor does it preclude the possibility that the observed sub-lethal ionic disturbances negatively impact fish performance and, ultimately, survival in the wild. An associated study found reductions in pink salmon swimming performance to occur at infection thresholds similar to those identified here for hydromineral balance (Nendick et al. 2010), indicating that the ion load may indeed impart a performance cost. Growth could also be negatively impacted, and although differences between infected and control fish were not observed here, low statistical power could be masking an effect (power = 0.112).

The mechanisms through which small juvenile pink salmon cope with this louse-induced ion load remain unclear. As in hydrominerally challenged rainbow trout, other areas of the body may buffer plasma and critical tissue osmolarity (Bath and Eddy, 1979; Wood and Randall, 1973a; Wood and Randall, 1973b; Wood and Randall, 1973c), potentially coming at a cost to aerobic scope, energy stores and/or recipient buffer tissue function. Any of these impacts could explain the previously reported reductions in swimming ability (Nendick et al. 2010). Even the larger, more developed fish from *Series 2 & 3* could be incurring substantial energetic costs despite maintaining ion balance following infection and abrasion. Measuring resting metabolic rate and metabolic scope would be a simple and effective way to quantify the energetic costs associated with louse infection and any resulting ionoregulatory challenge on a finer scale, thus making an excellent complement to future studies examining the effects of parasitism on fish development and survival.

Originally, gill NKA activity of infected fish was predicted to increase as a compensatory response to any resulting hydromineral disturbance. This was surprisingly not observed in any

louse-infected or abraded fish. In fact, gill NKA activity was temporarily lower in laboratory-infected RC fish. This reduction might be the product of a generalized stress response (Wendelaar Bonga, 1997), as stress-related elevations in cortisol have previously been paired with similar reductions in gill NKA activity of uninfected, confined chinook salmon (Strange et al., 1978). Alternatively, the observed reduction could reflect an infection bias toward weaker and less developed fish. A major limitation to studying louse impact is the inability to control which fish become infected and retain lice. As a result, infected fish may represent a subset of the population more susceptible to parasitism. Such fish may have less developed immune and epidermal systems, which may facilitate preferential louse attachment and retention (Jones et al., 2008a). If infected fish were relatively underdeveloped physiologically, hypo-osmoregulatory ability might also lag behind the population mean, thus potentially explaining the lower gill NKA activity. Sampling those fish that reject parasites or resist initial infection would help clarify this issue while providing further insight into the mechanisms underlying host susceptibility.

Summary

This study provides some of the first sublethal measures of physiological impact by sea lice on juvenile pink salmon in both a controlled laboratory setting and the wild. We clearly show that hypo-osmoregulatory development is important in tolerating the effects of sea louse parasitism and identify a threshold fish mass of 0.5 g below which one attached stage louse results in sub-lethal hydromineral disruption. This size threshold is also consistent with those previously based on louse-induced swimming performance reductions (0.7 g; Nendick et al., 2010) and the development of infection resistance mechanisms (immunocompetence and epidermis; 0.5-0.7 g; Jones et al., 2008a). From these data, we recommend that management decisions be made to minimize the anthropogenic-induced interactions between sea lice and

juvenile pink salmon prior to the completion of hypo-osmoregulatory development; specifically, fish weighing less than 0.7-1.0 g (Grant et al., 2009). These results should also be further integrated with surrogate measures of ecological fitness before more definitive conclusions regarding fish survival can be made. Despite low levels of fish mortality, high levels of louse rejection and the limitation of sublethal hydromineral disruption to the smallest of fish, the consequences of louse infection may prove to be quite different in the wild.

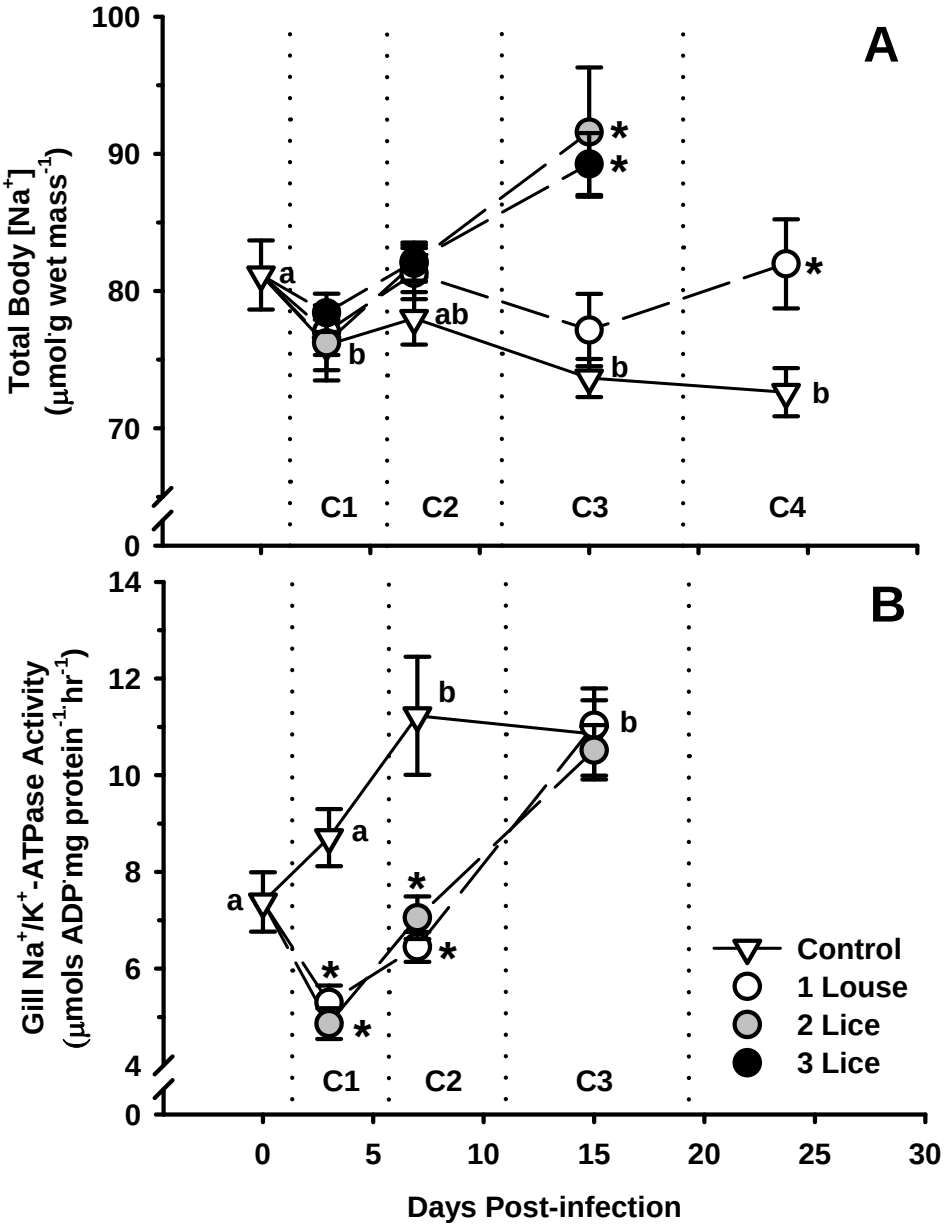

Figure 3.1. Total body [Na^+] (A) and gill Na^+/K^+-ATPase activity (B) of river-caught pink salmon artificially infected with *L. salmonis* copepodids (0-3 lice/fish) and held over time as indicated by days post-infection. Fish were sampled at each of the four chalimus developmental stages (C1-4). Data points represent mean ± SEM, n = 10. Asterisks indicate statistically significant differences from time-match controls; letters that differ indicate statistically significant differences within the control group ($P \leq 0.05$).

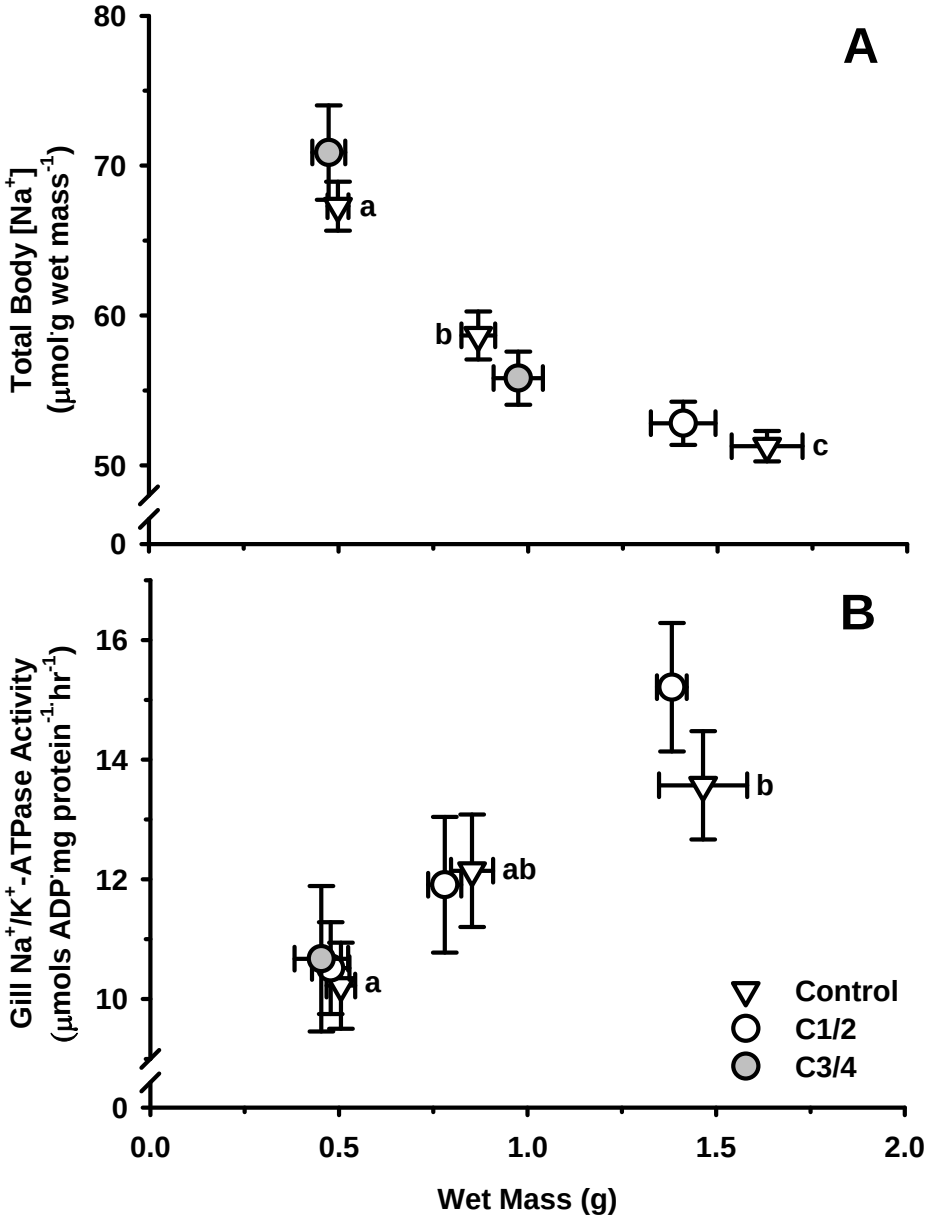

Figure 3.2. Total body [Na^+] (A) and gill Na^+/K^+-ATPase activity (B) of ocean-caught pink salmon naturally infected with one *L. salmonis* chalimus. Infected data are pooled according to louse stage [chalimus 1&2 (open circles) or chalimus 3&4 (shaded circles)] and expressed relative to fish wet mass. Data points represent mean ± SEM, n = 10. Infected fish do not differ significantly from size-matched controls; letters that differ indicate statistically significant differences within the control group ($P \leq 0.05$).

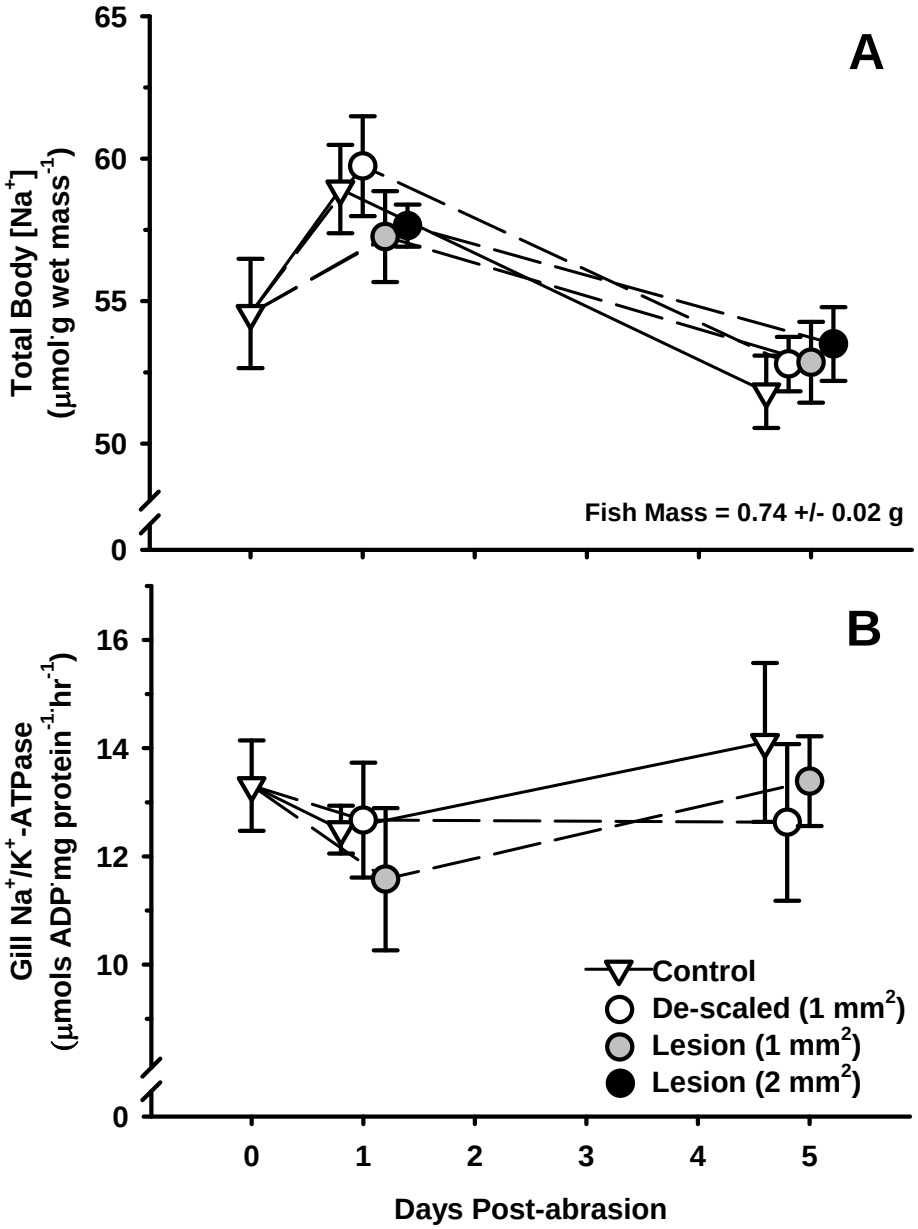

Figure 3.3. Total body [Na^+] (A) and gill Na^+/K^+-ATPase activity (B) of river-caught pink salmon following various degrees of mechanical epidermal abrasion. Mean fish mass is 0.738 g $\pm$ 0.019 SEM. Data are means ± SEM, n = 10; treatment groups did not differ significantly from control ($P \leq 0.05$).

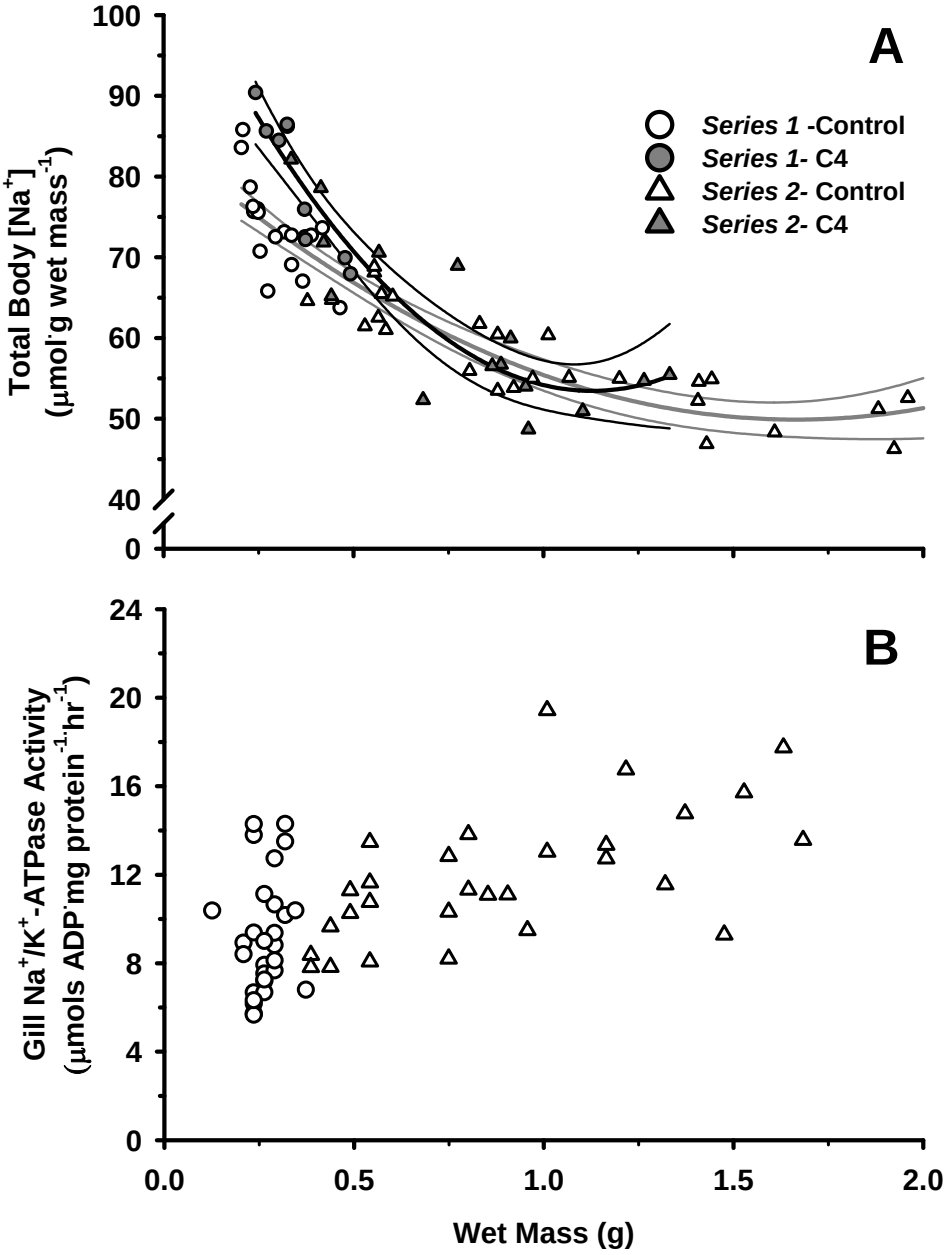

Figure 3.4. Total body [Na^+] (A) and gill Na^+/K^+-ATPase activity (B) of infected (shaded symbols) and uninfected (open symbols) pink salmon fry expressed as a function of wet mass. Circles represent individual river-caught fish from *Series 1* infected in a laboratory setting, and triangles represent individual ocean-caught fish from *Series 2* infected in the wild. All infected fish possessed a load of one stage 4 chalimus louse. Regression lines are second order polynomials based on total infected [white; $y = 84.6-(41.7x)+(12.5x^2)$; $R^2 = 0.87$; $P < 0.001$; $F = 140$] and uninfected [grey; $y =109.3–(99.0x)+(43.8x^2)$; $R^2 = 0.89$; $P < 0.001$; $F = 84.5$] data points. Thin lines represent 95 % confidence intervals.

Table 3.1. River-caught Fish, Laboratory Infection (*Series 1*): Fish mass and quantified area of exposed sub-epidermal tissue

DPI	Control	1 Louse	2 Lice	3 Lice
Day 0	0.224 ± 0.014 g ----	N/A	N/A	N/A
Day 3 (C1)	0.243 ± 0.009 g ----	0.224 ± 0.011 g ND	0.234 ± 0.009 g ND	0.235 ± 0.008 g ND
Day 7 (C2)	0.272 ± 0.011 g ----	0.254 ± 0.016 g ND	0.231 ± 0.015* g ND	0.249 ± 0.010 g ND
Day 14 (C3)	0.283 ± 0.010 g ----	0.313 ± 0.015 g 0.10 ± 0.05 ^{a} mm^2	0.279 ± 0.019 g 0.20 ± 0.06 ^{ab} mm^2	0.259 ± 0.014 g 0.15 ± 0.06 ^{ab} mm^2
Day 24 (C4)	0.358 ± 0.019 g ----	0.336 ± 0.031 g 0.30 ± 0.08 ^{b} mm^2	N/A	N/A
Specific Growth Rate	2.26 ± 0.66 % over 24 days	1.86 ± 1.00 % over 24 days	1.48 ± 0.86 % over 14 days	1.23 ± 0.34 % over 14 days

Mean ± SEM, n = 10. DPI refers to days post infection; C1-4 refers to chalimus developmental stages 1-4; ND = not detectable. Asterisks indicate significant differences from time-matched controls for fish mass; different letters indicate statistically significant differences from time-matched controls for tissue damage ($P \leq 0.05$). Specific growth rate is calculated as % body weight/day.

Table 3.2. Ocean-caught (OC) Fish, Wild Infections (*Series 2*): Fish mass and area of sub-epidermal tissue exposure

Fish Mass (g)	C1/2	C3/4
0.474 ± 0.043	ND	0.16 ± 0.11 mm^2
0.974 ± 0.065	ND	ND
1.409 ± 0.085	ND	N/A

Mean ± SEM, n = 10. C1-4 refers to chalimus developmental stages 1-4. ND = not detectable. All fish were infected with 1 louse. Reported damage is the total area of exposed sub-epidermal tissue.

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Chapter 4: General Discussion

This thesis is a blend of basic and applied research that investigates the hydromineral challenges associated with early ocean entry in pink salmon. Chapter two furthers our understanding of the most extreme and perhaps derived form of salmonid anadromy, while chapter three applies this knowledge to promote sustainable policy development within British Columbia's aquaculture industry.

4.1 CHAPTER 2 SUMMARY: THE HYPER-OSMOTIC ENVIRONMENT

The near absence of post-emergent freshwater preparatory time afforded to pink salmon makes their remarkable early ocean entry particularly daunting. To meet this challenge, pinks were hypothesized to undergo ontogenetically driven hypo-osmoregulatory development as larval alevins in the redd (Hoar, 1988; McCormick, 2009; Weisbart, 1968). Chapter two rejects this hypothesis, as alevins neither increase gill NKA activity nor maintain hydromineral balance following seawater exposure. Furthermore, evidence suggests pink fry may undergo a photoperiod-triggered smoltification similar to that shared by other members of the genus *Oncorhynchus*. Active ion loading to facilitate water balance is revealed as the true novel strategy underlying this unique life history. By successfully sustaining large ionic disturbances on a whole body level, pink fry maintain water balance and thus survive ocean entry two months prior to the completion of branchial ion excretion machinery development.

4.2 A NEW FORM OF ANADROMY?

Active ion loading to facilitate water balance is an essential component of what appears to be a distinct form of salmonid anadromy. Two major osmoregulatory hurdles accompany

increasingly early ocean entry: (1) an accelerated transition from FW to SW and (2) an increased body surface area to volume ratio (SA:V). Predictive anadromy meets the first challenge by significantly reducing the lag-time between downstream migration and ocean entry common to facultative anadromy (McCormick, 1994; McCormick, 2009). Likewise, increased hypo-osmoregulatory ability can overcome the greater osmoregulatory challenge associated with a higher SA:V in smaller fish (McCormick and Naiman, 1984). However, the internal ion loading of pink salmon may further combat both constraints by masking hypo-osmoregulatory deficiency and thus eliminating the need for post-emergent FW preparatory time. Entering seawater with underdeveloped ion excretion machinery distinguishes pink salmon anadromy from the more ancestral facultative and predictive forms. We therefore propose that this unique life history be termed "precocious anadromy."

4.3 EVOLUTIONARY IMPLICATIONS

Two relationships suggest that natural selection may favour early ocean entry among salmonids. First, those more derived species generally enter the ocean earlier and at smaller sizes (Clarke, 1982; Hoar, 1988; McCormick, 2009; McCormick and Saunders, 1987; McKay et al., 1996; Oakley and Phillips, 1999). Second, those species entering the ocean earliest are also the most abundant and widely distributed (Augerot and Foley, 2005; Quinn, 2005; Quinn and Myers, 2004). Although predation is accepted as the major selective pressure dictating size at ocean entry (Hargreaves and LeBrasseur, 1986; Holtby et al., 1990; Saloniemi et al., 2004), osmoregulatory challenges are also believed to play a significant role (Clarke et al., 1989; McCormick and Naiman, 1984). Internal ion loading could thus be argued as an adaptation for what is perhaps the most derived and successful form of salmonid anadromy, as it likely incurs selective advantages beyond those provided by predictive anadromy and increased hypo-osmoregulatory ability alone. For example, by facilitating earlier access to productive marine

waters, this trait could result in earlier rapid growth and maturation. Accelerated maturation could result in the short generation time possessed by pinks, which in turn benefits population growth and abundance. Shorter FW residency may also disrupt natal imprinting, perhaps leading to the observed high stray rate and increased distribution of pinks (Quinn, 2005). The implications are even more pronounced if this trait facilitates larval rearing in estuaries. Reduced spawning migration distance could increase fecundity by allowing adults to invest more into reproduction rather than migratory effort. Furthermore, reduced dependency on FW rearing conditions could improve the likelihood that strays encounter suitable habitat, thus increasing species dispersal. A seemingly limitless number of plausible scenarios can be generated to rationalize the benefits of internal ion tolerance to salmonid abundance and distribution.

4.4 FUTURE BASIC RESEARCH DIRECTIONS

Future research should investigate the suspected photoperiod response in pink fry and determine how they tolerate such high internal ion loads. The mechanism underlying this tolerance may resemble that observed in rainbow trout, where plasma osmolarity is buffered with other tissues following osmoregulatory stress (Bath and Eddy, 1979; Eddy and Bath, 1979; Wood and Randall, 1973a; Wood and Randall, 1973b; Wood and Randall, 1973c). If pink salmon are shown to possess a similar but greater internal buffer capacity, this trait could represent a constraint on size at ocean entry. Comparisons within and across salmonid species that vary in smolt size could perhaps reveal the adaptive role, if any, of this capacity in shaping salmonid evolution and anadromy in general. On a broader scale, this trait could be expressed to different degrees by many fishes, serving as a general strategy to overcome hydromineral challenges when whole body osmoregulatory ability is insufficient.

4.5 CHAPTER 3 SUMMARY: SEA LOUSE PARASITISM

Interestingly, the same life history facilitating early ocean entry in pink salmon appears to result in greater susceptibility to louse infection. Chapter three clearly shows an increased vulnerability to sea louse parasitism prior to the completion of hypo-osmoregulatory development, as evidenced by the inverse relationship between total body Na^+ load and gill NKA activity of infected fish. Hypo-osmoregulatory developed fish even maintain Na^+ balance despite levels of artificial abrasion ten-fold that inflicted by lice in our experimental trials. Although the immune system and epidermis likely combat proximal effects of infection, hypo-osmoregulatory ability clearly enables fish to cope with the hydromineral consequences of any damage or attachment stress that might occur.

4.6 APPLIED CONTRIBUTIONS

Generating a concrete threshold fish mass for sublethal louse impacts is perhaps this chapter's greatest contribution. Our identified window of sensitivity agrees with that proposed by Jones and colleagues (2008) and matches previous measurements of louse-induced performance reductions (Nendick et al., 2010). Together, these findings have directly influenced current aquaculture management in the Broughton Archipelago. Recommendations based heavily on these findings have led Marine Harvest Canada Ltd. to remove active fish farms along the pink out-migration route and initiate parasite control measures earlier in season. This chapter can therefore be deemed a success, as the ultimate goal of applied research is to positively shape industry practice.

4.7 FUTURE APPLIED RESEARCH DIRECTIONS

Sustainable aquaculture strives to conserve and enrich its surrounding natural and socioeconomic environments (Frankic and Hershner, 2003). Management decisions taken to

ensure the conservation of local ecological integrity thus require an understanding of the complex interactions between open-net pens and their environs. Although this chapter has already positively shaped aquaculture practice in British Columbia with respect to sea louse exposure, many important questions remain. Beyond the obvious repetition of our experiments with higher parasite loads and more developed lice, the additional measurements of resting metabolic rate and aerobic scope would provide excellent estimates of the total energetic cost associated with louse infection. These numbers, integrated with measures of performance and ecological fitness, would provide a more complete understanding of infection impacts for individual fish. However, exactly how fish farms contribute to local louse populations still remains unknown. This issue must be resolved if open-net pen aquaculture is to be managed confidently in the presence of wild fish populations.

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