SMOLTIFICATION AND GROWTH RETARDATION IN
NEW ZEALAND KING SALMON
Oncorhynchus tshawytscha (WALBAUM)

A thesis submitted in partial fulfilment
of the requirements for the Degree of
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By

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ABSTRACT

Growth retardation in King salmon *Oncorhynchus tshawytscha* (Walbaum) is a common and significant problem affecting marine farming operations in New Zealand. While the basic marine culture requirements for the King salmon species are well understood, the etiology of seawater adaptation and growth retardation remains understudied. Consequently, this study was established to investigate the physiological state and causative factors of growth retardation in collaboration with a leading New Zealand aquaculture company, New Zealand King Salmon Ltd (NZKS).

Hypoosmoregulatory indicators are not currently used by marine farmers in New Zealand due to the belief that King salmon are more adaptable to seawater than their more highly cultured counterparts, Coho and Atlantic, and can be transferred to seawater anytime after a critical weight is achieved. This study sought to investigate changes in hypoosmoregulatory ability and its relation to water temperatures commonly used in the hatchery environment. This was determined by changes in the activity of the predominating seawater-adapting gill enzyme Na⁺/K⁺-ATPase, as an indirect measure of its abundance during smoltification. Changes in plasma ion profiles and the ability to regulate ions after abrupt transfer were also measured and compared with enzymatic activity throughout the austral springtime smoltification period in commercial strains of under-yearling King salmon.

It was found that King salmon do undergo a distinct austral spring-time temporal increase in hypoosmoregulatory processes. This was characterised by a 2-fold increase Na⁺/K⁺-ATPase activity which was concomitant with reduced plasma Na⁺ in freshwater and following a seawater challenge in fish between fork lengths of 140-160 mm. Despite no consistent reduction in Na⁺/K⁺-ATPase activity during desmoltification, it was shown that the percent of ATP dependent activity specific to Na⁺/K⁺-ATPase diminished over time. Increased residual ATP dependent activity is hypothesised to be a result of apical H⁺-VATPase activity as a compensatory mechanism to rapidly normalise plasma Na⁺ during desmoltification concomitant with elevated basolateral Na⁺/K⁺-ATPase. Water temperature has been linked with the advancement and shortening of the smoltification period in several species. Gill Na⁺/K⁺-ATPase activity
and hypoosmoregulatory ability in King salmon were negatively affected by increasing water temperatures above 12°C in contrast to a constant 12°C. The level of growth retardation was dependent on the time of transfer to seawater and was found to increase during a period of reducing hypoosmoregulatory ability.

The transfer of growth retarded King salmon back to freshwater resulted in a complete reversal of the growth retarded state, comparable to that observed in Coho and Atlantic salmon. Growth retarded fish were able to readapt back to freshwater with higher survival and growth rates compared to the transfer of normal growing sub-adult King salmon, strongly demonstrating that growth retarded fish are more adapted to freshwater. Osmoregulatory physiology, and endocrinology during the transfer of growth retarded and normal growing fish were investigated. Overall, these results have fundamental implications for the aquaculture of King salmon that are able to be applied by industry to improve current husbandry practices.
PREFACE

This thesis is the final work of my M.Sc thesis study at the School of Biological Sciences, University of Canterbury, New Zealand. It serves as documentation of my work during the study, which has been made from September 2006 until April 2008. The study has been funded by the Foundation for Science Research and Technology New Zealand and The New Zealand King Salmon Company Ltd. The thesis consists of five chapters, which entails an introduction (Chapter 1), general methods (Chapter 2) and two main experimental chapters (Chapter 3 and Chapter 4). There is a final conclusion (Chapter 5) which considers both experimental sections in the context of growth retardation. It also serves to highlight the implications and future directions for the commercial salmon industry with regard to growth retardation and seawater transfer. The works presented here in this thesis are those of the authors unless otherwise stated.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine 5'-diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
</tr>
<tr>
<td>Kiwing</td>
<td>Alevin incubator containing artificial substratum</td>
</tr>
<tr>
<td>Biomass</td>
<td>Biological weight of population</td>
</tr>
<tr>
<td>Cm</td>
<td>Centimetre</td>
</tr>
<tr>
<td>K or CF</td>
<td>Condition Factor</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxy-ribonucleic acid</td>
</tr>
<tr>
<td>dH20</td>
<td>Distilled water</td>
</tr>
<tr>
<td>dd H20</td>
<td>Double distilled water</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>FCR</td>
<td>Food Conversion Ratio</td>
</tr>
<tr>
<td>FW</td>
<td>Freshwater</td>
</tr>
<tr>
<td>GDAS</td>
<td>Gastric dilation air sacculitis</td>
</tr>
<tr>
<td>Na⁺/K⁺-ATPase</td>
<td>Gill sodium, potassium-activated adenosine triphosphatase</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GR</td>
<td>Growth retardation</td>
</tr>
<tr>
<td>GRd</td>
<td>Growth retarded</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>hr</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Insulin-like growth factor-I</td>
</tr>
<tr>
<td>K</td>
<td>Fulton’s condition factor</td>
</tr>
<tr>
<td>Kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactate dehydrogenase</td>
</tr>
<tr>
<td>L</td>
<td>Litre</td>
</tr>
<tr>
<td>Vₘₕₙₓ</td>
<td>Maximal rate</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µL</td>
<td>Microliter</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mmolL⁻¹</td>
<td>Millimoles per litre</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>MRC</td>
<td>Mitochondria rich cell</td>
</tr>
<tr>
<td>mtDNA</td>
<td>Mitochondrial Deoxy-Ribonucleic Acid</td>
</tr>
<tr>
<td>M</td>
<td>Moles per litre</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometres</td>
</tr>
<tr>
<td>NEM</td>
<td>N-Ethylmaleimide</td>
</tr>
<tr>
<td>NAD⁺</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide (reduced form)</td>
</tr>
<tr>
<td>NZKS</td>
<td>The New Zealand King Salmon Company Ltd</td>
</tr>
<tr>
<td>PIT-tag</td>
<td>Passive integrated transponder-tag</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>%</td>
<td>Percentage</td>
</tr>
<tr>
<td>PEP</td>
<td>Phosphoenolpyruvate</td>
</tr>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>s</td>
<td>Seconds</td>
</tr>
<tr>
<td>NKCC</td>
<td>Sodium, potassium, two-chloride-adenosine triphosphatase</td>
</tr>
<tr>
<td>v/v</td>
<td>Solution by volume</td>
</tr>
<tr>
<td>SRIF</td>
<td>Somatostatin</td>
</tr>
<tr>
<td>SGR</td>
<td>Specific growth rate</td>
</tr>
<tr>
<td>TGC</td>
<td>Thermal growth coefficient</td>
</tr>
<tr>
<td>TJ</td>
<td>Tight junction</td>
</tr>
<tr>
<td>T₃</td>
<td>Triiodothyronine</td>
</tr>
<tr>
<td>T₄</td>
<td>Thyroxine</td>
</tr>
<tr>
<td>Inventory</td>
<td>Total number of individuals in a population</td>
</tr>
<tr>
<td>UC</td>
<td>University of Canterbury</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight by volume</td>
</tr>
<tr>
<td>H⁺-VATPase</td>
<td>Vacuolar-type hydrogen adenosine triphosphatase</td>
</tr>
</tbody>
</table>
CHAPTER ONE

General Introduction

Pacific King salmon, *Oncorhynchus tshawytscha* (Walbaum) sometimes known as Chinook is widely recognised as being one of the finest salmon species by discerning markets for their superior flesh quality. Despite it being highly sought after in Asian markets with a premium price paid above that of Atlantic (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch*), they are one of the least cultured. New Zealand is currently one of the largest suppliers of King salmon with close to half the world’s production (NZSFA, 2008). While the basic marine culture requirements for King salmon are well understood, the etiology of a problem known as growth retardation is not and currently results in a direct financial loss and potential product for farmers as juveniles fail to feed and grow to a marketable size.

Growth retardation is characterised as virtual cessation of growth and failure to feed that can occur in fish following transfer from freshwater to seawater. The condition becomes visually evident in some fish between 4 to 6 weeks, with most dying within 6 months to a year of entry. Salmon farmers will often grade cages several months after transfer to reduce size variability and feeding hierarchies within cohorts. It is also at this stage when growth retarded fish are removed and destroyed before they die and foul the cages.

Growth retardation also occurs in Atlantic and coho salmon in the Northern Hemisphere and is often referred to as stunting, running, parr reversion or failed smolt syndrome. Atlantic salmon farms in Tasmania (Australia) also suffer from a similar condition that is referred to as ‘pinheading’ (Purser, *et al.*, 2003). The cause of pinheading has also not been investigated fully but the condition appears surprisingly similar to growth retardation in King and coho salmon (Purser, personal communication). The term ‘running’ is frequently used by New Zealand salmon farmers to describe any fish that fails to grow at the same rate as other cohorts, despite some having a reasonable condition factor (NZKS, personal communication). There do not appear to be standard definitions for the conditions. Growth retardation (GR) and
growth retard (GRd) will be used in this thesis as collective terms to correspondingly describe the process and the individual fish which fail to grow after transfer to seawater.

1.1 LIFE HISTORY AND AQUACULTURE OF KING SALMON

Native to cold temperate waters, species within the family Salmonidae display unique behavioural and morphological homogeneity. It is generally agreed that Salmonidae are distributed within three monophyletic clades; *Salmo*, *Salvelinus* and *Oncorhynchus* (Figure 1.1). The genus *Oncorhynchus* consists of eight major species, which include King salmon, coho salmon, chum salmon (*O. keta*), pink salmon (*O. gorbuscha*), sockeye salmon (*O. nerka*), masu salmon (*O. masou*), rainbow trout (*O. mykiss*) and Cutthroat trout (*O. clarkii*) (Figure 1.1). Originally the term "salmon" was used for salmonid fish that migrate to sea, and the term "trout" was used for salmonid fish that remained exclusively in fresh water. Not surprisingly the usage of these terms confused phylogenetic relationships and led Pacific trout (rainbow and cutthroat trout) being classified as members of the genus *Salmo*. The reasons for a classification misnomer was due not only to their typical freshwater residency, they, like Atlantic salmon, have the unique potential for repeated spawning, in contrast to Pacific salmon which die after spawning once.

All species of Salmonidae reproduce in freshwater with the majority being anadromous – migrating between freshwater and seawater at some stage of their life. Some species of Salmonidae display both anadromous and entirely freshwater forms, a typical characteristic of their broad physiological, behavioural, and genetic flexibility. The unique life-history strategy of anadromy confers benefits to both reproduction and juvenile development in sheltered freshwater environments and rapid adult growth in the highly productive marine environment.
Salmonids undergo a series of morphologically distinct life stages as generally shown in for King salmon in Figure 1.2. After adults spawn in freshwater, eggs hatch into alevins, gradually developing to fry, parr then smolt. Smolt are morphologically distinct from earlier life stages and it is at this stage that they may move from freshwater to the sea (Healy, 1991). King salmon smolt are generally characterised as ocean-type or stream-type, representing the time of freshwater residency before their oceanic migrations (Healy, 1991). Ocean-type smolt migrate within the first year of hatching, typically within 3 months of emergence and remain in coastal waters much of their life before returning in the fall to spawn. In contrast, stream-type tend to remain in freshwater for one or more years before extensive oceanic migrations, returning to freshwater in the spring or summer months to spawn (Healy, 1983; Healy, 1991). Typically returning to their river of birth to spawn, adults are characterised by the season of freshwater entry (autumn or spring run).
Figure 1.2 A generalised life history strategy of King Salmon, *Oncorhynchus tshawytscha* (Walbaum) in freshwater and seawater phases. Smolts remaining in freshwater are termed here as parr-revertants.

King salmon are one of the largest, yet least abundant of all Pacific salmon (Healy 1991). Native populations are found on both the eastern and western seaboards of the Northern Pacific Ocean (Healy, 1991). North American King salmon are historically far more abundant than Russian populations, ranging from the Ventura River in Southern California to Kotzebue Sound in the north of Alaska. Populations have also been reported to exist as far north as the Coppermine River that feeds into the Coronation Gulf of the Canadian Arctic (Healy, 1991). On the western seaboard, King salmon are typically found between the Amur River to the Anadyr River, with the majority located on the Kamchatka Peninsula and Kuril Islands. Small populations also exist as far south as Hokkaido, Japan. Salmonids are not native to the Southern
Hemisphere, yet for more than a hundred years attempts have been made to establish self-perpetuating populations (land-locked and sea-run) outside of their natural ranges. Rainbow trout and three species of salmon (King, Atlantic and sockeye salmon) have been successfully introduced into New Zealand. Established anadromous and landlocked populations of King salmon can be found in New Zealand exclusively in the South Island. The current distribution includes rivers throughout Canterbury, Otago, Marlborough, Southland and the West Coast. Land-locked populations also exist in some Canterbury high country and West Coast lakes. Although the source of New Zealand original stocks cannot be explicitly determined, the introductions of ocean-type salmon ova most likely came from an undetermined tributary of California's Sacramento River basin during the early 1900s (McDowall, 1994). This has been substantiated by genetic studies using mtDNA of South Island populations, indicating a greater similarity to ocean-type Sacramento stock (Quinn, et al., 1996).

Historical introductions of King salmon between 1860 and 1910 were aimed at establishing recreational fisheries and sea ranching operations; however they never reached a commercially viable stage with low returns and high inconsistency. It was not until 1976 before New Zealand established its first commercial salmon farm at Waikoropupu Springs in Golden Bay. In 1983, British Petroleum New Zealand Ltd helped set up New Zealand’s first marine farm in Big Glory Bay on the shores of Stewart Island, which was soon be followed by the establishment of marine farms throughout the Marlborough Sounds. Although New Zealand’s aquaculture industry is still in its infancy, overall fisheries exports are steadily increasing and rank highly among the national export profile behind dairy, meat, horticulture and forestry (NZTE 2006). Currently, King salmon are the only farmed species of salmonid in New Zealand, with 26 freshwater/seawater sites in 2004, occupying 60 hectares of water space and producing approximately 7400 tonnes of fish (NZAC, 2005). Total exports of King salmon in year ended December 2007 were worth $ 36 million NZD. Japan and Australia accounted for the majority, with more than 70% of the total exports. The balance of the remaining exported product going to South East Asia and the USA (SeaFIC, 2008). New Zealand has steadily grown into one of the largest and niche market producers of King salmon with close to half the world’s production (NZSFA, 2008).
1.2 OSMOREGULATION

Salmonids are euryhaline, and occupy a wide range of environmental salinities, during which they must osmoregulate to maintain a blood composition within a narrow ionic and osmotic range. This homeostasis is crucial for proper cellular and whole body functioning.

1.2.1 Osmoregulation in Freshwater

Typical teleostean plasma is hyperosmotic (290-320 mosmol.kg$^{-1}$) to freshwater (20-40 mosmol.kg$^{-1}$) and consequently a constant outward diffusion of ions and passive influx of water via osmosis occurs. Osmotic balance is achieved in freshwater by several mechanisms, one of which is the ability to form copious hypotonic urine flows through high glomerular filtration rates in the kidney. Urine formed is not osmotically free and dominant electrolytes Na$^+$ and Cl$^-$ are lost in low concentrations (Potts, et al., 1970). Although active reabsorption in the proximal and distal tubules is thought to occur ionic gain is largely achieved through diet and the active uptake from surrounding water via specific transport enzymes in the gills (Johnson, 1973).

The fish gill epithelium is dominated by three main cell types including pavement cells (PVCs), mitochondria rich cells (MRCs) and accessory cells (ACs) all have been implicated as cellular sites for secretion and absorption of monovalent ions (Evans, et al., 1999; Evans, et al., 2005). August Krogh first proposed that the mechanisms of ionic uptake in freshwater fishes included the ions NH$_4^+$, H$^+$, and HCO$_3^-$ that may function as counterions to maintain electric neutrality across the epithelium (Krogh, 1937). It is now generally believed that Na$^+$ uptake by the freshwater fish gill occurs via an apical epithelial Na$^+$ conductive channel, electrochemically coupled to an apical vacuolar-type H$^+$-ATPase (hereafter H$^+$-VATPase refers to this enzyme) (Figure 1.3) (Fenwick, et al., 1999; Lin, et al., 1995; Marshall, 2002). The ATP dependent process of H$^+$ extrusion through H$^+$-VATPase creates the required internal negative potential (-mV) for inward diffusion of Na$^+$ though the epithelial Na$^+$ conductive channel. Protons (H$^+$) are provided by the rapid hydration of CO$_2$ by an intracellular carbonic anhydrase (CA). The energy stoichiometry of H$^+$-VATPase is still unclear and
may move 2 H\(^+\) per ATP (Steinmetz, et al., 1981) or 3 H\(^+\) per ATP consumed (Dixon, et al., 1980). The basolateral exit pathway for Na\(^+\) is believed to be carried out by a Na\(^+\)/K\(^+\)-ATPase, however it is yet to be confirmed (Evans, et al., 2005).

Both H\(^+\)-VATPase and epithelial Na\(^+\) conductive channels have been co-localised to mixed populations of pavement cells (Sullivan, et al., 1995) and mitochondria rich cells (MRCs) in the trout (Sullivan, et al., 1995; Wilson, et al., 2000) on the apical and basolateral membranes. The model for Na\(^+\) uptake has been supported in-vitro by the use of the selective inhibitor for H\(^+\)-VATPase, bafilomycin A1 (Lin, et al., 1991; Lin, et al., 1993). This has also been supported in-vivo by treatment with external bafilomycin A1 reducing whole body Na\(^+\) influx in young tilapia and young carp by up to 90 % and 70 % respectively (Fenwick, et al., 1999).

The hydration of CO\(_2\) by CA also produces bicarbonate ions (HCO\(_3^-\)). It is generally considered that that the apical excretion of HCO\(_3^-\) is coupled to Cl\(^-\) uptake (Cl\(^-\)/HCO\(_3^-\) anion-exchanger) although very few studies have shown this directly (Wright, 1991). Interestingly, heterologous antibodies to human pendrin (a non-anion exchanger) have been used and have been localised the apical region of H\(^+\)-VATPase rich, but not Na\(^+\)/K\(^+\)-ATPase rich cells in the branchial epithelium of the Atlantic stingray Dasayatis sabina (Piermarini, et al., 2002). Basolaterally located H\(^+\)-VATPases are thought to counteract the increased blood alkalosis by the reabsorption of H\(^+\) into the blood (Tresguerres, et al., 2006). Movement across the basolateral membrane for Cl\(^-\) into the blood is still unclear (Evans, et al., 2005).
Figure 1.3 Schematic model of Na\(^+\) and Cl\(^-\) uptake in freshwater teleostean gill epithelium. H\(^+\) and HCO\(_3^-\) are produced from carbonic anhydrase catalyzing CO\(_2\) and H\(_2\)O. The extrusion of H\(^+\) ions via H\(^+\)-VATPase creates and internal negative potential (-mV) for inward diffusion of Na\(^+\) though the epithelial sodium channel (ENaC) and Cl\(^-\) via apical Cl\(^-\)/HCO\(_3^-\) anion-exchanger down their electrochemical gradient. Exit pathways on the basolateral membrane are not known yet it is believed that Na\(^+\)/K\(^+\)-ATPase may be responsible for the basolateral transfer of Na\(^+\) (Modified from Evans, et al., 2005).
1.2.2 Osmoregulation in Seawater

Adaptation to the marine environment requires a reversal of many osmoregulatory processes. Plasma osmolality of seawater adapted salmonids becomes hypoosmotic to that of the external environment (>1000 mosmol kg\(^{-1}\)). Osmotic homeostasis must be maintained against a passive influx of ions and continual loss of water by way of osmosis. The loss of water is counteracted by a dramatic reduction in urine formation and active drinking of seawater. Water is absorbed via enterocytes across the epithelium of the intestine and pyloric caeca (Rawdon, et al., 1973; Veillette, et al., 2005) – the exact route of which is still unclear. The current hypothesis suggests that the major driving force of intestinal fluid transport is the net flux of Na\(^+\) and Cl\(^-\) uptake from the apical membrane and efflux of both through the basolateral membrane by a Na\(^+\)/K\(^+\)-ATPase (Movileanu, et al., 1998). Apically located NKCC proteins are thought to transport Na\(^+\), K\(^+\) and two Cl\(^-\) ions down the electrochemical gradient generated by the basally located Na\(^+\)/K\(^+\)-ATPase proteins. The active transport of Na\(^+\) out of the cell by Na\(^+\)/K\(^+\)-ATPase generates high local osmolality within the lateral spaces between adjacent enterocytes of the epithelium (Figure 1.4). High local osmolality draws water from the intestine, both transcellularly, presumably though aquaporins (AQP) and paracellularly though cell tight junctions between enterocytes (Evans, et al., 2005). Although AQP have been shown to be responsible for epithelial water uptake in the posterior intestine of the eel, (Aoki, et al., 2003) a transcellular transport role of AQP of salmonids has yet to be described. The primary role of Na\(^+\)/K\(^+\)-ATPase in water absorption been confirmed by the application of 10\(^{-4}\) M ouabain (specific Na\(^+\)/K\(^+\)-ATPase inhibitor), inhibiting 92% of fluid absorption in King salmon (Veillette, et al., 2005).
Figure 1.4. Schematic model of ion and water uptake in seawater adapted teleostean intestinal enterocyte. Dashed lines show passive transport, and solid lines active transport. Water uptake is achieved by high local osmolality of Na\(^+\) and Cl\(^-\) generated by basally located Na\(^+\)/K\(^+\)-ATPase and cAMP activated chloride channels (CFRT) respectively. Water may be drawn transcellularly through aquaporins (AQPs) and paracellularly though cell tight junctions (TJ) between enterocytes. K\(^+\) ions entering the cell are absorbed passively through K\(^+\) channels (K\(_{\text{w}}\)). (Modified from Evans, et al., 2005).

In seawater, external Na\(^+\) levels favors an inward-directed Na\(^+\) movement without the need an energetically expensive apical H\(^+\)-VATPase. Other primary ions including Cl\(^-\), SO\(_4\)\(^{2-}\), and Mg\(^{2+}\) also accumulate from intestinal absorption and diffusion across the gills and body surface. To obtain osmotically free water, excess monovalent ions (Na\(^+\), Cl\(^-\)) must be actively excreted by the gill and divalent ions (Mg\(^{2+}\), SO\(_4\)\(^{2-}\)) via the kidney. The Na\(^+\)/K\(^+\)-ATPase enzyme has been localised to the basolateral membrane of gill MRCs in the branchial epithelium of a variety of teleosts including salmonids (Karnaky, et al., 1976; Wilson, et al., 2000a).

The current model suggests that a basally located Na\(^+\)/K\(^+\)-ATPase strengthens the negative internal gradient to drive the NKCC co-transporter of Na\(^+\), K\(^+\) and two Cl\(^-\) ions down their respective electrochemical gradients (Figure 1.5). Within the cell Cl\(^-\) is secreted by a cyclic adenosine monophosphate (cAMP) activated chloride channel on the apical membrane. In turn the Na\(^+\)/K\(^+\)-ATPase generates high local concentrations of Na\(^+\) at the basal lamina forcing Na\(^+\) paracellularly down an electrochemical gradient to
the external medium through permeable tight junctions between MRCs and accessory cells (AC). Excess K⁺ ions enter the cell through NKCC and Na⁺/K⁺-ATPase, and exit passively by K⁺ ion channels on the basolateral membrane (Evans, et al., 1999).

**Figure 1.5** Schematic model of Na⁺ and Cl⁻ extrusion in seawater teleost transmembrane bound mitochondria rich cell (MRC) in the gill. Dashed lines show passive transport, and solid lines active transport. Entry of K⁺ via a Na⁺/K⁺-ATPase maintains the negative internal ionic potential (~mV) to drive the entry of two Cl⁻, Na⁺ and K⁺ ions into cell. Cl⁻ ions are apically expelled from the cell via a cAMP activated chloride channel (CFRT). Excess K⁺ entering the cell are recycled via K⁺ channels (Kᵢ). The secondary effect of Na⁺/K⁺-ATPase is the accumulation of high local concentrations of Na⁺ at the basal lamina which forces Na⁺ to leak paracellularly through permeable tight junctions between MRC and adjacent AC. (Modified from Evans, et al., 2005).
1.3 SMOLTIFICATION

The parr to smolt transformation referred to as ‘smoltification’ and the successful transition between the stream and ocean habitats are the most critical stages for many anadromous species (Healy, 1991). Smoltification is a collective term used to describe behavioural, morphological and physiological changes that occur in anticipation of the transition from the hypoosmotic environment of freshwater to the hyperosmotic marine environment.

The timing of smoltification and seaward migration is known to vary between and within salmonid species, and is generally determined by size, season and environment (Healy, 1991). The smoltification process is considered to be under endogenous control, as circannual rhythms have been observed under constant photoperiod regimes (Eriksson, et al., 1982). The prevailing hypothesis for most salmonids suggests that smoltification is controlled in synchrony with environmental and seasonal cues, such as photoperiod and water temperatures (Eriksson, et al., 1982; Solbakken, et al., 1994). Although not widely used, some commercial aquaculture operations do use contrasting photoperiod and water temperature regimes to produce off season smolts and control spawning to produce off season eggs (Duncan, et al., 1998; Duston, et al., 1995; Sigholt, et al., 1998).

The endocrine system controls and influences many of the processes involved in smoltification and osmoregulation (McCormick, 2001). Cortisol is the main corticosteroid in teleosts and functions far beyond the traditionally defined stress hormone, to more complex regulator of intermediary metabolism (hepatic gluconeogenesis & glycogenesis), smoltification and osmoregulation in fish (Mommsen, et al., 1999). Cortisol is secreted from adrenocortical cells located in the anterior portion of the tissue referred to as the anterior kidney or head kidney, specifically populating the dorsal posterior cardinal veins and their branches. Cortisol is a major regulator of osmoregulatory changes in both the gut as well as gill (Sundell, et al., 2003; Veillette, et al., 2004; Veillette, et al., 1995). Chronic cortisol stimulation has also been shown to increase H⁺-VATPase activity and subsequent Na⁺ uptake in freshwater trout but it has been shown to have little effect on seawater-acclimated rainbow trout (O. mykiss) (Lin, et al., 1993). The ability of cortisol to stimulate H⁺-
VATPase independent of other hormones, such as prolactin, is not known. However, synergistic effects of cortisol have been found with other hypoosmoregulatory hormones such as growth hormone (GH) and insulin-like growth factor-I (IGF-I) illustrated by increases in branchial MRC proliferation and Na\(^+\)/K\(^+\)-ATPase activities (Bisbal, et al., 1991; Külerich, et al., 2007; Madsen, et al., 1995; Mommsen, et al., 1999).

Considerable evidence suggests that thyroid hormones play an important function in the regulation of development, reproduction and growth of vertebrates (Clarke, et al., 1995; Power, et al., 2001). One of the most pronounced endocrine changes during smoltification is an increase in plasma levels of thyroid hormones (THs) (Ebbesson, et al.). The primary product secreted from the thyroid gland is thyroxine (T\(_4\)). T\(_4\) has relatively no direct action in comparison to its biologically active converted form triiodothyronine (T\(_3\)). Conversion of T\(_4\) to T\(_3\) is achieved by enzymatic removal of one of the iodide units of the outer ring of T\(_4\) in the liver and other tissues (Eales, et al., 1993). Functions of T\(_4\) and T\(_3\) are similar in most vertebrates; however, specific roles during smoltification are still unclear (Boeuf, 1993; Kulczykowska, et al., 2004).

Prolactin (PRL) and GH belong to the same family of adenohypophyseal hormones, both of which are elevated during smoltification (Agustsson, et al., 2003). PRL levels during smoltification appears varies between coho (Young, et al., 1989a) and Atlantic salmon (Prunet, et al., 1989). Nevertheless, PRL is primarily regarded as an important hyperosmoregulatory hormone in freshwater adapting teleosts as it is thought to decrease branchial extrusion of monovalent ions by reducing expression of gill Na\(^+\)/K\(^+\)-ATPase (McCormick, 1995). GH has been shown to increase in salmonids during spring smoltification (Björnsson, et al., 1989; Prunet, et al., 1989). However, debate exists as to whether or not changes in GH levels are seasonal, or primarily smoltification based, as GH has been shown to be elevated in fish in following years (Duan, et al., 1995; Perez-Sanchez, et al., 1999).

Although a basic design exists for salmonid smoltification, there is no one criterion to define a smolt. Superficially smolts are distinct from the earlier life stages of the parr, fry and alevin (Healy, 1991). During the transition to the smolt stage, Oncorhynchus species lose vertical parr marks, becoming silvery from the deposition of guanine and hypoxanthine (purine) crystals in the integument. The silversing of smolts in
the wild is an important morphological adaptation to pelagic marine life (Hoar, 1988). Silvering is under endogenous control but the rate of silvering is highly influenced by temperature (Hoar, 1988). Fin margins may also darken during smoltification (Mizuno, et al., 2004). Smolt generally show a more streamlined body form, indicated by a reduction in condition factor (weight per unit length). This reduced condition is due to the depletion of lipid stores, quantitative changes in lipid composition (Hoar, 1988) and continued growth of the caudal peduncle (Winans, et al., 1987). In a commercial hatchery, a reduction in condition may not be as evident due to the constant satiation with high energy feeds (Sundell, et al., 1998).

During smoltification, increased gill Na\(^+\)/K\(^+\)-ATPase activity acts to pre-adapt juveniles for entry to seawater. High Na\(^+\)/K\(^+\)-ATPase activity reduces freshwater hyperosmoregulatory processes and is suggested to result in considerable ionic imbalances during freshwater residency. The reduction in plasma primary ions (Na\(^+\) and Cl\(^-\)) during smoltification has been recorded in Atlantic (Primmett, et al., 1988; Reis-Henriques, et al., 1996) and coho salmon smolts (Folmar, et al., 1981). If smolts are prevented from reaching seawater, desmoltification or parr reversion take place, a process which appears to occur to maintain hydromineral balance and hyperosmoregulatory processes (Folmar, et al., 1982; Stefansson, et al., 1998). Desmoltification involves the loss of many of the characteristics described for smoltification. One of the more prominent and well documented changes is a reduction in gill Na\(^+\)/K\(^+\)-ATPase activity and euryhalinity (Handeland, et al., 2004; McCormick, et al., 1999; Zaugg, et al., 1973; Zaugg, et al., 1976). Although considered more as a physiological reversion to freshwater parr status, morphological characteristics attained during the smolting may also be partially reversible, the extent to which this is achieved is dependent on species and environment (hatchery or wild stock), and per se, is not be a reliable indicator of desmoltification or parr reversion.

Outside of the commercial environment, wild salmonids are facing dramatic disturbances, including climate change, habitat degradation, over-fishing and pollution. Over the past years there has been a large increase in scientific studies which have focused on various xenobiotic compounds that are suggested to interrupt the normal physiological and biochemical aspects of smoltification (Bangsgaard, et al., 2006; Brown, et al., 2003; Keen, et al., 2005; Madsen, et al., 2004). However there is little
information on the degree to which physiological and biochemical processes during smoltsification are affected by more natural environmental changes such as increased temperature, which in the face of global climate change should be a pressing issue for the study and conservation of marine life.

For teleosts, the rates of metabolic processes are constrained by the thermal environment. Accordingly in the wild their survival, health and natural distribution are inextricably linked to the thermal environment. Aside from the effects of temperature on wild stocks, elevated temperatures are commonly employed in commercial hatcheries to promote growth to produce of off-season and early entry smolts. Studies investigating gill Na⁺/K⁺-ATPase activity and water temperature during smoltsification in salmonids have shown that levels can be inhibited or decline at elevated water temperatures (Adams, et al., 1975; Hoar, 1988; Wedemeyer, et al., 1980). After 20 days at just 13°C Zaugg (1981) found inhibition of gill ATPase activity and impairment of continued migratory behavior in steelhead salmon. Clarke et al (1981) also reported reduced osmoregulatory performance in coho (O. kisutch) and King salmon held at 15°C and Atlantic salmon at 16°C (Duston, et al., 1991) in response to seawater challenge.

Another common feature of the hatchery environment is the use of constant thermal regime. It is suggested that a constant temperature may remove one of the major priming factors for smoltsification, distorting the normal development and inhibiting increases and detectable peaks of Na⁺/K⁺-ATPase activity (Wedemeyer, 1996; Wedemeyer, et al., 1980; Zaugg, et al., 1976) The effect of elevated (>12°C) water temperatures on smolt physiology has not been properly described for King salmon and warrants further investigation.

1.3.1 Seawater transfer

For both farmers and programmes involved in sea ranching, stocking, or cage rearing, a fundamental question must be raised regarding the development of smolt status with the timing of seawater entry. New Zealand sea-cage salmon farming involves the abrupt transfer of juveniles (NZKS, personal communication), without intermediary salinities that might be experienced in the wild (Healy, 1991).
Furthermore, the measure of their adaptability to seawater is solely based on morphological measures.

For successful adaptation of other salmonids (coho and Atlantic) farmers must ensure that the timing of seawater entry is synchronised with the development of elevated hypoosmoregulatory ability. The timing of smolt development and seawater regulation has traditionally been based on morphological characters, which include critical weight, integument silvering, darkening of fin margins, and reduced condition factor. Whilst these criteria remain valid characteristics of smolt-like juveniles, they can be strongly influenced by hatchery settings and do not reflect elevated seawater adaptability (Wedemeyer, 1996). Hatchery juveniles reared on a satiated diet may not exhibit a noticeable decline in condition in contrast to wild stock. Although silvering is not an accurate criterion of smolt status, its absence gives a clear indication that the process of smoltification has not occurred.

Various tests for seawater performance and smolt quality have been employed, including seawater challenges, gill Na\(^+\)/K\(^+\)-ATPase activity and changes in circulating plasma hormones – T\(_4\), cortisol and IGF-I. Many studies have shown that the time course increase of the gill Na\(^+\)/K\(^+\)-ATPase is a useful indicator of a peak in hypoosmoregulatory ability in Pacific and Atlantic salmon species (Ewing, et al., 1982; Franklin, et al., 1992; Hoar, 1988; Stefansson, et al., 1998) However, biochemical assays such as that for Na\(^+\)/K\(^+\)-ATPase, T\(_4\) and cortisol are often complex and expensive laboratory-based techniques and are not commonly applied by industry.

Seawater challenges including hypoosmoregulatory tests and salinity tolerance tests are inexpensive and relatively quick to perform by aquaculturists. A salinity tolerance test measures the ability of potential smolts to survive in hyper-saline water (35-40‰) typically for periods up to 96 hr. The disadvantage with salinity tolerance tests is that some fish may survive for short periods but fail to fully adapt in the long term, thus it fails to distinguish between seawater tolerance and seawater regulation. Hypoosmoregulatory tests, such as that developed by Blackburn and Clarke (1987), measure the ability of salmonids to regulate plasma ion concentrations after an abrupt transfer to seawater (35‰) for 24 hr. After 24 hr osmolality, Na\(^+\) or Cl\(^-\) ions can be measured in comparison to control readings. The average difference in ion levels from controls can be used over time to indicate an increasing or decreasing trend in
hypoosmoregulatory capacity. Salinities higher than typical seawater (>35‰) can give further accuracy to pronounced hypoosmoregulatory ability. It is essential that several predictors are used to accurately predict the optimal timing for transfer. Although these are common techniques in Europe and the Americas, none are currently used by New Zealand salmon farmers.

1.4 GROWTH RETARDATION

GR became recognised during the development of net pen culture in the early 1970s, but has been observed in wild stocks of coho (Varavsky, et al., 1992). GRd fish can be seen in the weeks to months following transfer to seawater by a continued arrest of growth in a proportion of the population. Frequently found separated from normal growing cohorts, they are easily distinguished by their poor appearance, emaciated muscles and low condition factor. The level of GR in marine farms has been previously recoded as affecting between 3 - 30% of fish transferred as under-yearling or yearling smolt (NZKS, personal communication). Consequently, in a commercial culture, GR results in direct loss of investment, resources and potential profit as fish die before reaching a marketable size.

Folmar et al (1982) characterised two forms of GR in coho salmon, both of which can develop from the inappropriate timing of transfer from freshwater to seawater. The premature transfer of coho parr to seawater before the typical period of entry can lead to ‘stunting’ (Bern, 1978; Folmar, et al., 1982). Secondly, ‘parr revertants’ can result from transfer during a period of desmoltification (Folmar, et al., 1982). GRd fish survive in a moribund state for several months yet will often die within six months to a year of entry.

The GR phenomenon is not limited to coho salmon (Clarke, et al., 1977; Collie, 1985; Folmar, et al., 1982; Varavsky, et al., 1992; Young, et al., 1989b). GR and abnormal growth patterns have been described in Atlantic salmon (Bjornsson, et al., 1988; Duston, 1994; Specker, et al., 1989; Timothy, et al., 2004) and only briefly mentioned in King salmon (Sheridan, 1988; Woo, et al., 1978). For unknown reasons GR in King salmon is often viewed differently, typically by industry, to that of other
closely related species such as coho (Figure 1.1). A variety of factors have been suggested by industry to explain GR, including intraspecific competition from larger cohorts and protozoan (Amoebic Gill Disease - AGD) or parasitic infestations – neither of which has been substantiated.

The physiological status of GRd fish is generally described as pan-hypoendocrine indicated by morphological changes in glandular tissues (pituitary, thyroid, interrenal, pancreas, corpuscles of Stannius and caudal neurosecretory system) and in some peripheral tissues (kidney and liver). Interrenal sensitivity to ACTH assessed in-vitro is depressed in GRd coho salmon (Young, et al., 1989a). Thyroid hormones (T₄ & T₃) and cortisol are significantly lower in GRd compared to normal growers (Folmar, et al., 1982; Nishioka, et al., 1982; Young, et al., 1989b). Although plasma ion profiles of GRd coho remain within normal ranges (Clarke, et al., 1977; Varnavsky, et al., 1992) elevations of K⁺ levels of hepatic tissue has been observed (Marini, et al., 1982). It was suggested that disruptions may result from the steady flux of ion rich blood that the hepatic portal system receives from the intestine. Kerstetter et al. (1989), as cited by Young et al (1989) also suggested that K⁺ loading may result from increased hepatic Na⁺/K⁺-ATPase activity and intestinal absorption dysfunction, neither of which has been substantiated.

The growth hormone and insulin-like growth factor-1 (GH/IGF-I) axis is an important regulator of growth and metabolism in vertebrates. The somatogenic action of GH is generally considered to be mediated by the hepatic synthesis of endocrine IGF-I (Mommsen, 2001). Interestingly, GRd coho and Atlantic salmon show a partial form of GH-resistance which is characteristic of mammalian Laron syndrome (also known as Laron-type dwarfism). Several studies have shown that hepatic IGF-I mRNA, plasma IGF-I and plasma insulin levels are significantly lower despite GH secretory activity and circulating levels being significantly elevated (Bjornsson, et al., 1988; Bolton, 1987; Duan, et al., 1995; Varnavsky, et al., 1995; Young, et al., 1989b). Starvation and dietary protein restriction has been shown to induce changes in the hormonal status of GH and hepatic GH receptors (Perez-Sanchez, et al., 1999) and is suggested to represent an adaptive response to shift metabolic actions for energy supply during starvation, but also prevent adiposity in actively feeding fish.
Inhibition of GH release in fish has been shown to be regulated by peptide hormones known as somatostatins (SRIFs) specifically SRIF-14 (Very, et al., 2007a). Found in the hypothalamus SRIF-14 is a 14-amino-acid long peptide that has been shown to be a potent inhibitor of GH secretion, subsequent IGF-I production and inducer of GR in rainbow trout (Very, et al., 2001). Using $[^{125}\text{I}]$-labelled tilapia (*Sarotherodon mossambicus*) growth hormone, Fryer and Bern (1987) revealed that GRd coho salmon have a decreased ability to bind GH, illustrated by a deficit of $[^{125}\text{I}]$-labelled GH-binding sites in liver and gill tissue. The decreased ability to bind GH was suggested by Young, et al., (1989a) to be a result of $\text{K}^+$ loading in hepatocytes, however, it has recently been shown that SRIF-14 can also reduce target organ sensitivity to GH by a down regulation of GH receptors and GH receptor mRNA biosynthesis (Very, et al., 2007a). In addition SRIF-14 treatment reduces target organ expression of IGF-I receptor mRNA, including $[^{125}\text{I}]$-IGF-I binding *in-vitro* (Very, et al., 2007b). Not surprisingly, significantly higher levels of naturally occurring SRIF-14 have been found in GRd coho salmon concomitant with elevated GH levels (Sheridan, et al., 1998). The lack of inhibitory effect of SRIF-14 on pituitary GH release suggests a reduced pituitary GH sensitivity (Sheridan, et al., 1998). SRIF-14 appears to work in an extra-pituitary manner on target organs reducing sensitivity to IGF-I by reducing IGF-I receptors and receptor biosynthesis ultimately retarding growth.

Several studies have shown that GRd coho and Atlantic salmon transferred to freshwater have resumed feeding and growth (Bjornsson, et al., 1988; Clarke, et al., 1977; Young, et al., 1989b) which clearly shows that the hyperosmotic marine environment is responsible for the inhibition of adaptation and growth of these fish. It is unclear if this also occurs in King salmon. More detailed studies of the time course of endocrine and osmoregulatory changes during freshwater transfers may help unravel the initiation of GR. Further etiological studies on the mechanisms involved in the initiation, maintenance and reversal of the retarded state are needed. Its elucidation has practical importance for not only commercial sea cage operators, but also for programmes involved in sea ranching or wild stocking of salmonids.
1.5 THESIS AIMS

Smoltification processes have been studied in considerable detail in Atlantic and coho salmon, primarily due to their commercial significance in aquaculture. The physiology of smoltification commercial strains of King salmon is extremely understudied. GR of coho and Atlantic salmon is well known to arise in a proportion of the population when transferred outside of a normal period of smoltification (Bjornsson, *et al.*, 1988; Folmar, *et al.*, 1982). GR has become a significant problem in New Zealand commercial operations yet no detailed studies have revealed its cause. It is hypothesised that GR occurs in King salmon due to inappropriate timing of seawater transfer and/or poor development in the hatchery environment.

The primary aim of this thesis is to address a gap in our understanding of smoltification and how this relates to seawater adaptability and GR in King salmon. Therefore, the initial objective was to characterise the normal spring/summer smoltification period to a post-smolt stage in a fast growing strain of under-yearling King salmon stock in a typical freshwater production environment. Concomitantly, the effect of a common hatchery water temperature on smoltification processes was compared in groups of fish reared using a constant 12°C water temperature and a steadily increasing water temperature above 12°C (Chapter 3). Physiological predictors of smoltification which included plasma ion analysis, hypoosmoregulatory tests, growth indices and gill Na⁺/K⁺-ATPase assays were used on a weekly basis to assess smolt status. A reference to long term performance of production stock in seawater was also made (Chapter 3).

GRd fish die within the first year of seawater residence, yet the transfer of GRd coho salmon back to freshwater has resulted in a restoration of several endocrine components (Young, *et al.*, 1989b) growth and condition (Bjornsson, *et al.*, 1988; Clarke, *et al.*, 1977). The second objective of the thesis was to therefore determine if the phenomenon of GR in King salmon is reversible and comparable to that reported in coho and Atlantic salmon. Changes in osmoregulatory physiology, growth and overall survival of GRd and normal growing cohorts were compared during transfer back to freshwater (Chapter 4). The overall conclusions of both sections are discussed in Chapter (5).
This project was initiated by industry to investigate GR with an aim to reduce levels and improve commercial standards. Implications and answers for the New Zealand salmon industry are also discussed (Chapter 5).
CHAPTER TWO

General Methods

Research was conducted at the New Zealand King Salmon Co. Ltd (NZKS) Tentburn Hatchery, Southbridge, Canterbury and the NZKS Te-Punga Bay and Ruakaka cages located in the Marlborough Sounds. Analysis was carried out at the University of Canterbury and Canterbury Health Laboratories, Christchurch, New Zealand. This chapter details various methods referred to in experimental chapters 3 & 4.

2.1 Water Temperature

Freshwater temperatures were monitored using two Onset HOBO data loggers (Onset Computer Corporation. Bourne, MA) at five minute intervals over the study period. Data loggers were positioned in the centre of the raceways in mid-water to allow an average raceway temperature to be logged. Water temperatures at sea cages were logged at a depth of 5 m.

2.2 Morphological Analysis

Prior to weighing, fish were exposed to (6 ppm) AQUI-S® (Aquí-S New Zealand Ltd, Wellington, New Zealand) for a duration sufficient to reach anaesthesia. All fish collected were individually counted, measured for fork length ± 0.1 cm, weight ± 0.1 g and external colourations were determined before blood was obtained by the severance of the caudal peduncle. Body parameters were then used to calculate the Fulton’s condition factor $K$ using the following equation:

$$K=100 \times W \times \left(\frac{1}{L^3}\right)^{-1}$$

where $W$ is individual weight (g) and $L$ is individual fork length (cm).
Specific growth rate (SGR) was calculated using the following equation by Kreeger (1995):

$$SGR = (\ln W_2 - \ln W_1)(t_2 - t_1)^{-1} \times 100$$

where \(\ln\) is the natural logarithm of the initial weight \(W_1\) (g) and last recorded weight \(W_2\) at the period being calculated, and \(t\) is the time in days at \(W_1\) and \(W_2\). Fish growth was measured in relation to temperature using thermal-unit growth coefficients (TGC) (Cho, et al., 1998). TGC was calculated using the following equation.

$$TGC = (W_f^{1/3} - W_i^{1/3})/\sum (T \times (t_f - t_i)) \times 100$$

\(W_f\) and \(W_i\) are final and initial body weights in kg, respectively. \(T\) is the sum of the average daily water temperatures (°C) calculated between \(t_f\) (final) and \(t_i\) (initial) sampling dates. Average feedout (see Table 3.1, Chapter 3) was used as a general measure of intake calculated as percent of body weight per day.

Each fish was categorised by body colouration (banded, intermediate or silver) similar to that described by Negus (2003). The ‘banded’ designation means that all parr marks are visible. The ‘intermediate’ designation means that parr marks closest to the head are gone, but marks are still visible near the caudal fin. The ‘silver’ designation means that all parr marks are gone, or those remaining near the caudal fin are extremely pale.

2.3 Tissue Water Content

A block of white muscle tissue was taken immediately below the dorsal fin. A scalpel was used to excise the muscle from skin, blood and any red muscle. Tissues were carefully blotted and weighed on an analytical balance (Adventurer, Ohaus Corp, NJ) to the nearest 0.001g. The tissues were then dried in foil for 24 hr in an oven at
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80°C and the dry weight of the residual tissue was determined. Water content is expressed as the percentage difference between dry and wet weight.

2.4 Haematocrit

Haematocrit was determined from freshly drawn heparinised blood collected in micro-haematocrit tubes (Vitrex Medical A/S Denmark). After sealing, tubes were centrifuged for 5 min at 12,000RPM in a microhaematocrit haemofuge (Heraeus, Baxter). Haematocrit (Hct) was expressed as a percentage of red blood cells to total blood volume.

2.5 Hypoosmoregulatory Tests

Hypoosmoregulatory ability of fish was assessed by measuring plasma Na\(^+\) levels (mmolL\(^{-1}\)) after 24 hr in seawater. Filtered natural seawater was adjusted to 35‰ by the addition of sea salts (Dominion Salt Ltd, Lake Grassmere), measured using a temperature compensating type refractometer (ATAGO, Model ATC-S/Mill-E) and rechecked using a digital salinity meter (Orion, Model 115). Temperature of the seawater tanks was adjusted to 12 ± 0.5°C. Tanks were placed in a cool darkened room to stabilise water temperatures and minimise any distractions or stressors. All food was withheld from fish 24 hr prior to challenges. Ten randomly selected fish from experimental groups were placed into the tanks for 24 hr, during which time all tanks were vigorously aerated to maintain high oxygenation >8mg/L\(^{-1}\) and prevent stratification. After 24 hr fish were removed and sampled according to the following protocol.

2.5.1 Plasma Ion Analysis

At each sampling time, 10 fish were overdosed using the aquatic anaesthetic AQUI-S\(^{®}\). Fish were washed in freshwater and dried using a clean paper towel before blood collection to reduce sample contamination. Weight, length and external appearance were recorded. Blood was collected by caudal severance or ammonium...
heparinised disposable syringes as stated in experimental chapters 3 and 4. Initial pooled blood from caudally severed fish was discarded to reduce contamination from muscle plasma and spinal fluid. Plasma was obtained by centrifugation (7000g, 5 min) and stored at -20°C until analysis at the University of Canterbury. Sodium levels Na⁺ (± 1 mmolL⁻¹) were measured by flame photometry (Sherwood, Model 410) using duplicate 25 μL samples diluted in 5 mL of ddH₂O. Pipetting large volumes (5 ml) was found to be highly variable (± 0.05ml). With large dilution factors the accuracy of the volumes are essential to prevent erroneous results. Therefore to ensure precise dilutions, sampling vials were tared on a scale (Adventurer, OHAUS) before ddH₂O was pipetted to the nearest 5 ± 0.001 g.

2.5.2 Tissue Ion Analysis

After euthanizing fish, an incision was made from the gills to the anal fin and viscera and swim bladder removed. Blocks (2 - 3 g) of anterior kidney and hepatic tissue were carefully excised, blotted dry and snap frozen in liquid nitrogen and stored at -20°C until analysis of ion content could be conducted. Kidney and hepatic tissue samples were dried at 80°C for 24 hr, removed weighed to ± 0.1 mg (Adventurer, OHAUS) and digested in 1ml of HNO₃ at room temperature for a further 24 hr with constant agitation. Sodium Na⁺ and potassium K⁺ levels (± 1 mmolL⁻¹) were measured by flame photometry. Total tissue concentrations of Na⁺ and K⁺ were expressed as µM/mg⁻¹ of dry weight tissue. Samples were run in duplicate and were not pooled.

2.6 Enzymatic Assays

Fish were euthanised using an overdose of AQUI-S®. Measurements including: weight ± 0.1 g, length ± 0.1 cm, external colourations and any abnormalities were taken. Plasma was obtained by the severance or puncture of the caudal peduncle in the same manner as described. Two gill arches on the left side were immediately excised, washed, stored in ice-cold SEI buffer (150 mmolL⁻¹ sucrose, 10 mmolL⁻¹ EDTA, 50 mmolL⁻¹, imidazole pH 7.3); snap frozen and transported in liquid nitrogen before being stored at -80 °C at the University of Canterbury.
2.6.1 Gill Na\(^+\)/K\(^+\)ATPase Assay

Gill Na\(^+\)/K\(^+\)-ATPase activities were measured by a method developed by McCormick (1993) with the following modifications. The sequence of the reaction is shown in Figure 2.1. The assay solution A which is used as a control, contained 2.8 mM PEP (2-(Phosphonoxy)-2-propenoic acid trisodium salt), 0.7 mM ATP, 0.22 NADH, 4U/mL lactic dehydrogenase, 5U/mL pyruvate kinase from rabbit muscle and 5 mM imidazole. Assay solution B was the same as above but also contained 0.5 mmolL\(^{-1}\) of the Na\(^+\)/K\(^+\)-ATPase inhibitor ouabain. Assay solutions A & B were separately diluted in a salt solution (50 mM imidazole, 189 mM NaCl, 10.5 mM MgCl\(_2\) and 42 mM KCl) at a ratio of 3:1 and warmed in a 25°C water bath prior to use.

![Figure 2.1](image)

**Figure 2.1 Sequence of the ATPase coupled enzyme reaction.**

Gill filaments were first rapidly thawed and the storage SEI buffer discarded. Gill tissue was then homogenised with 1-2 mL of ice-cold SEI buffer (150 mmolL\(^{-1}\) sucrose, 10 mmolL\(^{-1}\) EDTA, 50 mmolL\(^{-1}\) imidazole, 0.5% sodium deoxycholate pH 7.5) and immediately centrifuged (5,000 g for 30s) to remove insoluble material. 10 µL of the supernatant from the gill homogenates were added to each of four wells in a chilled 96-well plate. A sub-sample of the remaining homogenate was then removed for later analysis of protein content. To two wells of each sample, 200 µL of the assay A-plus-salt solution were added and to the remaining two wells 200 µL of the assay B-plus-salt solution were added. The plate was shaken immediately for 30 seconds before being read in a FLUOstar OPTIMA BMG Labtech microplate reader (Offenburg, Germany) at 25°C, the reduction of absorbance of NADH (coupled to the hydrolysis of ATP) was read at 340 nm for 10 minutes. To ensure optimal activity of the coupled
assay, 20 nmolL⁻¹ and 10 nmolL⁻¹ of adenosine 5'-diphosphate sodium salt in ddH₂O was used as a standard. As described in the protocol of McCormick (1993) this ensured that the activity or rate of the selected enzyme was not limited by any part of the assay solutions. The slope of the standard curve at 340 nm was between -0.019 to -0.020 OD unit:nmolL:ADP⁻¹:well⁻¹. All solutions were used within 24 hr of being prepared. The inter-assay coefficient of variation of specific activity of same gill homogenate was 2.1% (n=10). The average intra-assay coefficient of variation of duplicate gill samples was 3.9% (n=180).

As a control, all substrates and coupling enzymes (pyruvate kinase, lactic dehydrogenase), were measured as described above for 10 mins with the exclusion of protein samples (gill homogenate) to determine background rates of NADH oxidation. There was no significant reduction in the absorbance of NADH at 340nm (n=5). The resulting Na⁺/K⁺-ATPase activities were calculated from the averaged differences in rate of activity with and without ouabain from the linear rate of the stable slope before being normalised for protein concentration to yield the $V_{max}$ of ouabain-sensitive Na⁺, K⁺-ATPase activity. Measurements are expressed as micromoles of ADP per milligram of protein per hour ($\mu$mol ADP · mg protein⁻¹ · hr⁻¹). All chemicals and reagents used were purchased from Sigma-Aldrich. The protein concentrations of the gill homogenates were measured in duplicate using a Bicinchoninic Acid (BCA) kit (Pierce Rockford Il) standardised to bovine serum albumin (BSA).

### 2.6.2 Gill NEM-Sensitive H⁺-VATPase and Na⁺/K⁺-ATPase Assay

ATPase activities were measured by a method developed by McCormick (1993) with modification by Lin and Randall (1993) to concurrently determine both ouabain sensitive Na⁺/K⁺-ATPase and H⁺-VATPase activities.

N-Ethylmaleimide (NEM) is a sulphydryl alkylating agent that has been used to inhibit both vacuolar type ATPases (H⁺-VATPase) and plasma membrane type (P-type H⁺ATPases) in an ATP-protectable manner (Lin, et al., 1995). Although NEM is not a specific blocker for H⁺-VATPase like the macrolide antibiotic bafilomycin A1, NEM does block V-type H⁺ ATPase with a 10- to 100-fold selectivity (Forgac, 1989). The
sensitivity of this assay using NEM to block H⁺-VATPase has been validated in gill homogenates from rainbow trout by comparisons to bafilomycin A1, and the plasma membrane ATPase inhibitor, vanadate (Lin, et al., 1993). The contribution of additional unrelated ATPase activity was minimised through the use of sodium azide as an inhibitor of mitochondrial H⁺-ATPase and ouabain, a specific Na⁺/K⁺-ATPase inhibitor. Lin et al (1993) recommended the use of the strong Ca²⁺ chelator EGTA to eliminate unrelated Ca²⁺ATPase activity in the assay. BCA was preferred for protein determination due its accuracy of quantitative concentrations between 20 - 2,000 µg/mL⁻¹. However, due to the incompatibility of EGTA with the BCA reagents (Pierce. Rockford, Il) we compared its ability to a similar, yet compatible chelator EDTA (≥10 mmolL⁻¹) to reduce residual specific activity in gill homogenates.

Gills taken from two seawater adapted fish were washed in ddH₂O and homogenised in 500 µL of SI buffer (150 mmolL⁻¹ sucrose, 50 mmolL⁻¹ imidazole). Homogenates were centrifuged (5000g, 30s) and 100 µL of the supernatant of each fish were diluted into 1 mL of SI buffer containing either 10 mmolL⁻¹ EDTA or 12.5 mmolL⁻¹ EGTA. These were then frozen in liquid nitrogen and stored at -80°C. Respective homogenates were then compared in quadruplet using the ouabain sensitive Na⁺/K⁺-ATPase assay to determine residual specific activities. EGTA at 12.5 mmolL⁻¹ did not significantly reduce unrelated activity in comparison to 10 mmolL⁻¹ EDTA in both homogenates (t₆=0.02575, p=0.9803) (t₆=0.1556, p=0.8815) (Student's t-test). As a result the following assay was carried out with 10 mmolL⁻¹ EDTA in substitute for EGTA.

Three treatments were used for each sample (control + sodium azide, ouabain, ouabain + NEM). The control assay solution A contained 2.8 mmolL⁻¹ PEP 20.7 mmolL⁻¹ ATP, 0.22 mmolL⁻¹ NADH, 4U/mL⁻¹ LDH and 5U/mL⁻¹ PK from rabbit muscle, 5 mmolL⁻¹ imidazole, 5.0 mmolL⁻¹ sodium azide. The second assay solution B contained control assay, plus 0.5 mmolL⁻¹ of the inhibitor ouabain, and the third assay solution C contained control assay plus 0.5 mmolL⁻¹ ouabain, 1.0 mmolL⁻¹ NEM. Prior to the assay, the assay solutions (A, B and C) were separately diluted in a salt solution (50 mM imidazole, 189 mM NaCl, 10.5 mM MgCl₂, 42 mM KCl) at a ratio of 3:1 and warmed in a 25°C water bath prior to use.
Gill filaments were rapidly thawed prior to the assay and the storage SEI buffer discarded. Gill tissue was then homogenised with 1-2mls of ice-cold SEID buffer (150 mmolL⁻¹ sucrose, 10 mmolL⁻¹ EDTA, 50 mmolL⁻¹ imidazole, 0.5% sodium deoxycholate pH 7.5) and immediately centrifuged at 5,000g for 30 s to remove insoluble material. 10 µL of the supernatant from the gill homogenates were added to each of six wells in a chilled 96-well plate. A sub-sample of the remaining homogenate was removed for later analysis of protein content. To two wells of each sample 200 µL of the assay A- plus-salt solution, B-plus-salt solution and C-plus-salt solution were added. The plate was shaken immediately for 30 seconds before being read in a FLUOstar OPTIMA BMG Labtech microplate reader (Offenburg, Germany) at 25°C, the reduction of absorbance of NADH was read at 340 nm for 10 minutes. As described for the Na⁺/K⁺-ATPase assay, standards using 20 nmolL⁻¹ and 10 nmolL⁻¹ of adenosine 5'-diphosphate sodium salt in ddH₂O were used to ensure optimal activity. Intra-assay coefficient of variation of samples was 4.2% (n=30). All solutions were freshly prepared and used within 24 hr. As described for the previous Na⁺/K⁺-ATPase protocol, background oxidation of NADH in solution over a 10 min period was not significant (n=5).

The resulting Na⁺/K⁺-ATPase activities were calculated from the averaged differences in rate of activity with and without ouabain from the linear rate of the stable slope before being normalised for protein concentration to yield the $V_{\text{max}}$ of ouabain-sensitive Na⁺, K⁺-ATPase activity. NEM sensitive H⁺-VATPase activities were calculated by calculating the averaged differences in the linear rate of the stable slope between the A, B and C treated samples. Measurements are expressed as micromoles of ADP per milligram of protein per hour (µmol ADP · mg protein⁻¹ · hr⁻¹). Without the use of a specific inhibitor, activity sensitive to 1 mmolL⁻¹ NEM is expressed as NEM-sensitive H⁺-VATPase. The protein concentrations of the gill homogenates were measured in duplicate using a Bicinchinonic Acid (BCA) kit (Pierce. Rockford, II) standardised to bovine serum albumin (BSA).
2.6.3 Pyloric Caeca and Posterior Intestine Na\(^+\)/K\(^+\)-ATPase Assay

Na\(^+\)/K\(^+\)-ATPase activity of the pyloric caeca and posterior intestine of fish in sea cages were determined as a measure of intestinal osmoregulatory ability. To open the peritoneal cavity a longitudinal incision was made on the ventral surface from the gills to the anal fin. The viscus was removed and several pyloric caeca and the posterior section of the intestine were excised. The section of posterior intestine was located distal to the ileoceleal valve and marked by a pronounced bulge in the intestine. After excision, all samples were cut longitudinally to expose the serous membrane of the tissue, they were then rinsed thoroughly in ice-cold SEI buffer and placed in cryotubes containing fresh SEI buffer. These were then snap frozen in liquid N\(_2\) before being stored at -80°C. Immediately prior to the assay, samples were rapidly thawed and the storage SEI buffer discarded. Tissues was then homogenised with 1-2 mL of fresh ice-cold SEI buffer (150 mmolL\(^{-1}\) sucrose, 10 mmolL\(^{-1}\) EDTA, 50 mmolL\(^{-1}\) imidazole, 0.5% sodium deoxycholate pH 7.5) and immediately centrifuged (5,000 g for 30s) to remove insoluble material. The resulting supernatant was used for determination of Na\(^+\)/K\(^+\)-ATPase activity described above in section 2.6.1, with modification of pH, ion concentrations and detergent concentration as optimally described by Veillette, et al (2004).

2.7 Enzyme Linked Immunosorbent Assay (ELISA) of Plasma Cortisol using Monoclonal Antibodies and Dexamethasone suppression.

In teleosts cortisol is the main interrenal hormone and is secreted in response to stress through the Hypothalamic-Pituitary-Interrenal Axis (HPI-axis). Plasma cortisol levels were determined in this study during acclimation to freshwater (Chapter 4), measured using a modified Enzyme Linked Immunosorbent Assay (ELISA) similar to that described by Lewis, et al (1992). This assay employs the use of a monoclonal antibody (A2) in which cortisol samples and standards compete for immobilised antibody-binding sites. Cortisol bound to microtiter plates is detected using anti-mouse immunoglobulin conjugated to horseradish peroxidise. After washing, substrate is
added, and the colour produced is measured using a microplate reader and quantified against a generated standard curve using known concentrations of cortisol. Initial studies using a standard binding curve, based on known amounts of cortisol in stripped human plasma, revealed inaccurately high resting levels of cortisol. The inconsistency of the ELISA was resolved to be related to the differential binding properties of salmon plasma against standardised cortisol in human plasma. Consequently, the ELISA was standardised directly to King salmon plasma to produce a highly accurate measure of plasma cortisol levels. Dexamethasone suppression was employed to obtain standard salmon plasma free of cortisol (Pickering, et al., 1987).

Dexamethasone is often used as model analogue for cortisol as it preferentially activates pituitary glucocorticoid receptors with a higher binding potency than cortisol (Knoebl, et al., 1996). In response to a stressor, adrenocorticotropic hormone (ACTH) is released from the anterior pituitary gland to stimulate cortisol secretion. The release of ACTH is controlled in part by a negative feedback system through increases in plasma cortisol (Mommsen, et al., 1999). However, the preferential binding of dexamethasone at the pituitary level inhibits the release of cortisol’s principle secretagogue ACTH and therefore inhibits cortisol secretion.

Dexamethasone sodium phosphate was rapidly introduced via an intraperitoneal injection at a dose of 3µg.g⁻¹ body weight. Pickering, et al. (1987) showed that for brown trout Salmo trutta L, an oral dose of 3µg.g⁻¹ body weight had a strong suppressive effect for up to 72 hr on cortisol response even when exposed to acute confinement. Pickering, et al. (1989) chose oral administration instead of intraperitoneal injections to eliminate the probable result that handling stress would stimulate cortisol release. Intraperitoneal injection was chosen for this study because it would guarantee a sufficient dose. Any cortisol released during the handling and injection process would have been catabolised or taken up by tissues during the 24 hr (Mommsen, et al., 1999). This was later validated as dexamethasone suppressed plasma was undetectable for cortisol (< 0.5 nmoL⁻¹) by ELISA. Although ACTH is the primary secretagogue for cortisol, there are several other hormones that can stimulate cortisol secretion (Mommsen, et al., 1999). Therefore, to avoid any possible stimulation of cortisol release, fish were kept isolated from disturbances. 24 hr after dexamethasone administration fish were rapidly anesthetised with AQUI-S® and blood was collected.
via a caudal puncture – the whole procedure taking less than 1 min. Plasma was obtained from whole blood by centrifugation (13,000g, 10mins at 4°C), snap frozen in liquid N₂ and stored at -80°C.

Monoclonal antibodies (A2) produced via immunization of RBF/Dn mice with cortisol-21-acetate-3CMO-BSA (Steraloids Inc. Wilton,NH, USA) have shown low cross-reactivity to other C₂₁ steroids (19 % 11-deoxycortisol) and have previously been used for the direct determination of cortisol through ELISA (Lewis, et al., 1992). The following describes the modifications to the ELISA protocol of Lewis, et al. (1992).

ELISA plates (Falcon 3912 Microtest III, Becton Dickinson, Oxnard, CA, USA) were coated overnight at 4°C with 100 µL of cortisol-thyroglobulin in conjugate/well (1µg/mL in 6 M aqueous guanidine hydrochloride). The next day the plates were washed four times with a solution of phosphate buffered saline (PBS), 0.05 M NaH₂PO₄, 0.1M NaCl adjusted to pH 7.4 with 5 M NaOH, containing 0.1% Tween 20 (v/v). To avoid further absorption of the protein, the plates were blocked for 1 hr at 20°C with PBS assay buffer (150 µL/well), which contained 0.1% Tween 20 (v/v) and 0.1% gelatine (w/v). After draining the plates, 45 µL of the standard or 45 µL of assay buffer was added to appropriate wells, followed by either 5 µL of dexamethasone suppressed salmon plasma, to the standard wells, or 5 µL of salmon plasma sample to the assay buffer wells. Antibody solution was then added (50 µL /well) for a 60 min incubation at room temperature. Antibody solution was prepared by adding 200 µL of cortisol monoclonal antibody (Clone A2) and 14 µL of sheep anti-mouse IgG-peroxidase to 14 mL of assay buffer. The plates were then washed four times and substrate solution added. Substrate was prepared by dissolving tetramethylbenzidine at 1.4562 mmolL⁻¹ in methanol, this was then mixed in a 2 : 3 ratio of ddH₂O containing 166.61 mmolL⁻¹ sodium acetate, 31.23 mmolL⁻¹ citric acid (pH 5.5) and 833.33 µL/L H₂O₂. Colour development was terminated by the addition of 100 µL of 0.9 M HCL to each of the wells. The absorbance was then read at 450nm using a BMG Fluostar plate reader (Offenburg, Germany).

ELISA assay standards were prepared by appropriate dilution of cortisol primary standard (1 mg/mL in ethanol) in assay buffer. Human plasma was stripped using Amberlite® XAD®-2 polymeric adsorbent columns to obtain cortisol free human plasma. Steroids were obtained from Sigma Chemical (St. Louis, MO, USA) and all
other chemicals were of analytical grade. All samples and standards were run in triplicate and final cortisol levels expressed nmoL$^{-1}$. To illustrate the differences in binding properties of human stripped plasma and dexamethasone suppressed salmon plasma, corrected curves from 0 – 2800 nmoL$^{-1}$ are shown in Figure (2.2).

![Figure 2.2](image)

**Figure 2.2** Point to point standard binding curves for plasma cortisol ELISA using: stripped human plasma or cortisol free plasma obtained via dexamethasone suppression. Values interpolated from the curve are corrected by a dilution factor of 10.
CHAPTER THREE

Characterisation of Smoltification and Desmoltification in Commercial Stocks of Under-Yearling King Salmon (Oncorhynchus tshawytscha)

3.1 INTRODUCTION

The majority of global salmon producers mirror the natural life cycle of grow-out in seawater. However, for optimal growth and survival in the marine environment it is critical that fish being transferred have undergone and completed the smoltification process.

Smoltification and the development of hypoosmoregulatory ability whilst in freshwater pre-adapt the anadromous salmonids for life at sea. Physiologically, smoltification involves a temporal increase in hypoosmoregulatory ability, the basis for which is significant increases in gill Na\(^+\)/K\(^+\)-ATPase activity, which is widely accepted as the principal enzyme involved in osmotic and ionic regulation of marine teleosts (Hoar, 1988; McCormick, 1995). Increased Na\(^+\)/K\(^+\)-ATPase activity during smoltification is a preparative mechanism to prevent excess Na\(^+\) and Cl\(^-\) accumulation during initial seawater entry. Whilst residing in freshwater, increased Na\(^+\)/K\(^+\)-ATPase activity in the gill reduces hyperosmoregulatory capacity which can lead to a continuous and possibly critical efflux of primary ions. Low plasma ion levels during smoltification have been observed in Atlantic (Primmett, et al., 1988; Reis-Henriques, et al., 1996) coho salmon (Folmar, et al., 1981) and King Salmon smolts (Franklin, 1989).

If smolts are prevented from reaching seawater a process of desmoltification or parr reversion is known to occur and is theorised to be a necessary process to normalise hyperosmoregulatory processes (Duston, et al., 1991; Handeland, et al., 2004; McCormick, et al., 1999; Stefansson, et al., 1998). Desmoltification involves the partial reversal of physiological characteristics associated smoltification and marine adaptation, the most prominent of which is a reduction in gill Na\(^+\)/K\(^+\)-ATPase activity and hypoosmoregulatory capacity. A proportion of Atlantic and coho salmon which are transferred prematurely to seawater or become delayed and are transferred during a period of desmoltification will generally survive for many months but fail to feed, ultimately becoming growth retarded. It is hypothesised that this process is also true for
King salmon. GR is a significant problem for the culture of King salmon in New Zealand. The development of GR in stocks can result in not only direct financial losses of productivity but also hinder development in highly intensive systems.

For the above reasons, the development of species specific indicators to determine optimal timing of transfer is critical for salmonid aquaculturists and furthermore, wild enhancement programs. There are several recognised indicators that are used to measure smoltification of salmonids which include elevations in in-vitro activity of gill Na\(^+\)/K\(^+\)-ATPase, changes in thyroid hormones, cortisol, GH and IGF-1. The seawater hypoosmoregulatory test and to a lesser extent the salinity tolerance tests are commonly employed by salmonid aquaculturists in the Americas and Europe.

New Zealand’s marine salmon farmers currently use traditional morphological measurements of smoltification such as body silvering and size which, although are valid in some cases, are less effective at determining heightened hypoosmoregulatory ability. Previous studies commissioned by the NZKS Ltd (unpublished data) using seawater challenges failed to accurately predict a smolt episode resulting in unpredictable post-smolt seawater performance. The use of multiple and more accurate smolt indicators to predict optimal transfer times may result in improved initial growth, reduced GR percentages, inventory control and more importantly ethical husbandry.

Smoltification, especially in commercial stocks of King salmon, is less well studied in comparison to the highly cultured species of Atlantic and coho salmon. For all salmonids, environmental factors such as photoperiod and temperature are recognised as major modulators of smoltification. However, in juvenile ocean-type King salmon the completion of smoltification is suggested to be independent of photoperiod (Clarke, et al., 1981) and that size and temperature are the major factors driving smolt development (Clarke, et al., 1981; Franklin, et al., 1992). This is of particular interest to commercial operations, where elevated temperatures and high energy feeds are used to produce under-yearling and off season smolts (Duncan, et al., 1998; Duston, et al., 1995; Sigholt, et al., 1998). High temperatures may reduce the time and investment spent at this stage and for stock management reasons allows staggered production and harvest at sea. Although this appears a useful production strategy, there is increasing evidence to suggest that high water temperatures above 12°C can strongly influence smoltification and subsequent ability to adapt to seawater (Adams, et al., 1975; California Department of
Water Resources, 1988; Hoar, 1988; Wedemeyer, et al., 1980). After 20 days at 13°C in steelhead (Salmo gairdneri) Zaugg (1981) found that gill Na\(^+/\)K\(^-\)-ATPase activity became reduced and continued migratory behaviour was impaired. Clarke et al (1981) also reported reduced osmoregulatory performance in coho (O. kisutch) and King salmon held at 15°C in response to seawater challenge.

Another common feature of hatchery environments is constant water temperature which diverges from the natural environment where water temperatures follow a seasonal pattern. The concurrent seasonal increase in water temperature and photoperiod are reported to be a greater stimulus to smolt development than either independently. Dickhoff, et al (1989) showed that in Atlantic salmon gill Na\(^+/\)K\(^-\)-ATPase activity was significantly higher in naturally fluctuating water compared to groups reared at a constant 11°C. Björnsson et al (1989) also reported that smolts exposed to a temperature increase from 6 to 11 °C, showed a five-fold increase in growth hormone and improved seawater tolerance. The use of constant rearing temperatures in hatcheries is suggested to distort normal development and inhibit increases or detectable peaks of gill Na\(^+/\)K\(^-\)-ATPase activity (Wedemeyer, 1996; Wedemeyer, et al., 1980; Zaugg, et al., 1976). Higher temporal resolution studies of gill Na\(^+/\)K\(^-\)-ATPase activity has shown that relatively small changes in the timing and level of smolt development from environmental manipulation such as temperature can produce significant differences in the long term post-smolt seawater performance (Handeland, et al., 2004).

To date, there have been no studies in New Zealand specifically investigating the freshwater rearing regime on smoltification processes in King salmon. Both Franklin (1989) and Quinn (1999) investigated seawater temperature on adaptability of King salmon. Franklin (1989) revealed that King salmon and Sockeye were able to survive and successfully osmoregulate at temperatures up to 19 °C from a transfer of 13 °C. However, this study did not include a temperature history during smoltification prior to acclimation (13°C, 4 days) or long term performances (> 1 month).

The current study sought to investigate possible interactions between physiological processes during the development of hypoosmoregulatory ability and growth under elevated temperature regimes of under-yearling production stock. Juveniles were reared on two temperature regimes under a normal production situation. The temperatures used were a constant temperature of 12°C which is recognised as being the
upper limit for smoltification in King salmon (California Department of Water Resources, 1988; Wedemeyer, *et al.*, 1980). The second temperature used was a temperature regime that increased progressively above the optimal 12°C limit. Small groups of fish were retained in freshwater to monitor the post-smolt stage, whilst the remaining stock was transferred to seawater at normal production times. Gill Na⁺/K⁺-ATPase activity, plasma ion concentrations, hypoosmoregulatory challenges and morphological analysis were used as indices of smolt status between temperature groups to the post smolt stage. Plasma concentrations of Na⁺ in unchallenged fish were measured to determine if natural reductions could be measured during smoltification, and if so, do reductions accurately coincide with elevations of gill Na⁺/K⁺-ATPase activity and hypoosmoregulatory ability. Groups that were transferred to sea cages with normal production stock were also monitored for their long term performances, specifically levels of GR.
3.2 METHODS

3.2.1 Fish Maintenance

A commercial scale experiment was carried out at the NZKS freshwater production facility in Canterbury, New Zealand. All rearing and husbandry practices were carried out in accordance with current industry and company standards. King salmon (*Oncorhynchus tshawytscha*) from the NZKS S1 brood lines were used for this experiment. S1 fish are transferred to sea as under-yearlings during the austral spring/summer. Since 1994 the NZKS Company Ltd has been developing a family selective breeding programme in which S1 stocks have been bred using microsatellite markers for maximum growth and early two year maturation (NZKS, personal communication). This strategy takes advantage of the growth spurt known to occur prior to maturation to produce a larger fish earlier.

S1 Fish were spawned from the 17\(^{th}\) of April until the 19\(^{th}\) of April 2006 and incubated for approximately 28 days. Immediately prior to hatching, eggs were transferred to larger incubators (Kiwingers) which contained an artificial substratum of plastic mesh. Alevins were left for 36-38 days until the yolk sacs became all but depleted. At this time they were transferred to concrete raceways (30m×5m×1m length×width×depth) and slowly introduced to a crumble feed (Prima 1# Alitec, Puerto Montt, Chile). Oxygen concentrations were maintained at all times above 8 mg/L\(^2\) and flows adjusted accordingly to increasing densities. All eggs, alevins and fry were incubated and reared on water sourced from an aquifer with a constant temperature of 12.1 °C

3.2.2 Experimental Setup

All production stock was size graded and electronically counted (Vaki Aquaculture Systems Ltd. Kópavogur, Iceland) between the 5\(^{th}\) and 13\(^{th}\) of September 2006. Only the largest 50% of all fish were retained. The top 20% of this were classed as extra large fish with the remaining 30% separated into four groups of approximately 28000 fish each.
The two temperatures profiles used are shown in Figure 3.1. The experimental groups are herein referred to as increasing and constant temperature regimes. The average temperatures from the 19th September 2006 to the 16th January 2007 were 12.40 ± 0.95°C and 11.95 ± 0.29°C and a total of 1762.6 and 1696.1 degree days for the increasing and constant and regimes respectively. The maximum and minimum daily temperatures recorded during the study for the increasing regime were 17.2°C and 10.4°C and for the constant regime 13.4°C and 10.9°C, respectively.

Before normal production transfers to seawater cages were carried out, approximately 500 fish from each group were electronically counted (Vaki, Iceland) and retained in freshwater (Table 3.1) to investigate physiology during the post smolt stage. Screened dividers were placed in raceways to keep densities similar to a normal production environment. All groups were reared on natural photoperiod and fed using electronic spinners and hand supplementation. Fish were constantly monitored and fed accordingly, to ensure they were satiated during daylight hours. Fry were first fed on a crumble Prima 1# Alitec (Puerto Montt, Chile). Larger fry, parr and smolt were fed on Alitec Golden Activa (semi-float food). Between 2-3 weeks before saltwater entry all fish were fed a saltwater diet; Orient 50 Skretting (Nutreco Holding N.V). Groups of fish retained in freshwater were fed Alitec - Golden Activa (semi-float food) until the end of the study. All food was withheld 24 hr prior to sampling.
Figure 3.1 Average daily water maximum, mean and minimum temperatures over the study period 19th September 2006 – 17th January 2007. Constant temperature regime (top graph) and increasing temperature regime (bottom graph). Arrows indicate the start of sampling (19th September 2006). Mean temperatures are calculated from 288 daily sample points.
Chapter 3 Characterisation of Smoltification and Desmoltification in Commercial Stocks of Under-Yearling King Salmon (*Oncorhynchus tshawytscha*)

Table 3.1 Summary of duplicate temperature groups increasing temperature (I1, I2), constant temperature (C1 & C2) and extra large (XL). Spawning dates, grading date, total numbers initially stocked in each raceway, raceway density kg/m³, daily feed out percentage (% body weight. day⁻¹) seawater transfer date and number of fish held to post smolt stage in freshwater, destination seawater cages (NT denotes not transferred). Average seawater entry weight (n=200) and total number of fish stocked in each cage.

<table>
<thead>
<tr>
<th></th>
<th>I1</th>
<th>I2</th>
<th>C1</th>
<th>C2</th>
<th>XL</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Increasing</td>
<td>Constant</td>
<td>Constant</td>
<td>Increasing</td>
</tr>
<tr>
<td>Date spawned</td>
<td>17-Apr-06</td>
<td>17-Apr-06</td>
<td>19-Apr-06</td>
<td>19-Apr-06</td>
<td>21-Apr-06</td>
</tr>
<tr>
<td>Date size graded</td>
<td>05-Sep-06</td>
<td>05-Sep-06</td>
<td>12-Sep-06</td>
<td>12-Sep-06</td>
<td>13-Sep-06</td>
</tr>
<tr>
<td>Inventory at grade</td>
<td>26,009</td>
<td>28,518</td>
<td>28,429</td>
<td>28,690</td>
<td>28,787</td>
</tr>
<tr>
<td>Stocking Density (kg/m³)</td>
<td>1.79</td>
<td>1.8</td>
<td>2.35</td>
<td>2.03</td>
<td>2.7</td>
</tr>
<tr>
<td>Maximal Density (kg/m³)</td>
<td>12.14</td>
<td>13.31</td>
<td>13.27</td>
<td>13.2</td>
<td>8.26</td>
</tr>
<tr>
<td>Feedout Percentage</td>
<td>1.50%</td>
<td>1.40%</td>
<td>1.30%</td>
<td>1.10%</td>
<td>1.47%</td>
</tr>
<tr>
<td>Transfer Date</td>
<td>28-Nov-06</td>
<td>05-Dec-06</td>
<td>13-Dec-06</td>
<td>N/T</td>
<td>25-Oct-06</td>
</tr>
<tr>
<td>Inventory held in freshwater</td>
<td>523</td>
<td>543</td>
<td>576</td>
<td>577</td>
<td>-</td>
</tr>
<tr>
<td>Destination Cage</td>
<td>T61B</td>
<td>T61C</td>
<td>T61D</td>
<td>-</td>
<td>T61A</td>
</tr>
<tr>
<td>Transfer weight (g) (n=200)</td>
<td>74.0</td>
<td>75.7</td>
<td>77.8</td>
<td>-</td>
<td>45.2</td>
</tr>
<tr>
<td>Cage Inventory</td>
<td>199,052</td>
<td>198,773</td>
<td>198,808</td>
<td>-</td>
<td>180,378</td>
</tr>
</tbody>
</table>

3.2.3 Sampling

Approximately 50 - 70 fish were removed and sampled from each of the four groups (I1, I2 and C1, C2) on a weekly basis during the austral spring/summer from the 19th of September 2006 to the 17th of January 2007. Ten fish from each netted group were randomly selected and immediately transferred to 35‰ seawater to assess hypoosmoregulatory ability for 24 hr. After 24 hr fish were removed and euthanised using an overdose of Aquis®. Individual fish were then measured for fork length ($L_f$) ± 1 mm, weight ($W_i$) ± 0.1 g and external colourations before blood was obtained by the severance of the caudal peduncle. Plasma Na⁺ levels were measured using flame photometry and expressed in mmolL⁻¹.

For determination of gill Na⁺/K⁺-ATPase activities and plasma Na⁺ levels in unchallenged fish (control plasma) an additional 10 fish from the netted groups were randomly selected. Using a dip net, fish were quickly euthanised using an overdose of
Aquis®. All fish were measured for fork length ($L_f$) ±1 mm, weight ($W_i$) ±0.1 g and external colourations before blood was obtained by the severance of the caudal peduncle. Two gill arches on the left side were immediately excised, washed and stored in ice-cold SEI buffer (150 mmolL⁻¹ sucrose, 10 mmolL⁻¹ EDTA, 50 mmolL⁻¹, imidazole pH 7.3). Samples were immediately frozen in liquid N₂ and stored at -80°C at the University of Canterbury. Gill Na⁺/K⁺-ATPase activities were measured by a method developed by McCormick (1993) with modifications described in Chapter 2. Remaining fish (≥ 40) from each group were used for morphological analysis to determine weekly changes in group mean weight, length, condition factor, specific growth rate (SGR) and thermal growth coefficient (TGC) as described in Chapter 2.

Within a typical population, precocious maturation of a very small number of males can occur during the parr stage as under yearlings, yearlings (1+ years) and older (Bagliniere, et al., 1985; Saunders, et al., 1982). As these fish do not undergo proper smoltification they were visually characterised and excluded from any sampling.

Sampling from XL production grades were partially included in this project to be used as a post smolt performance comparison between early and late seawater entries. Due to logistical and production difficulties, extra large grades (XL) were only subjected to hypoosmoregulatory challenges and plasma ion analysis for two weeks prior to SW transfers. XL grades were only reared using an increasing temperature regime (Figure 3.1)
Chapter 3 Characterisation of Smoltification and Desmoltification in Commercial Stocks of Under-Yearling King Salmon (*Oncorhynchus tshawytscha*)

Table 3.2 Reference for week number and sampling dates during the 2006-2007 study period.  

<table>
<thead>
<tr>
<th>Week number</th>
<th>Sampling Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17th September</td>
</tr>
<tr>
<td>2</td>
<td>26th September</td>
</tr>
<tr>
<td>3</td>
<td>3rd October</td>
</tr>
<tr>
<td>4</td>
<td>10th October</td>
</tr>
<tr>
<td>5</td>
<td>17th October</td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24th October</td>
</tr>
<tr>
<td>7</td>
<td>31st October</td>
</tr>
<tr>
<td>8</td>
<td>7th November</td>
</tr>
<tr>
<td>9</td>
<td>14th November</td>
</tr>
<tr>
<td>10</td>
<td>21st November</td>
</tr>
<tr>
<td>11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28th November</td>
</tr>
<tr>
<td>12&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5th December</td>
</tr>
<tr>
<td>13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>12th December</td>
</tr>
<tr>
<td>14</td>
<td>19th December</td>
</tr>
<tr>
<td>15</td>
<td>26th December</td>
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<tr>
<td>16</td>
<td>2nd January</td>
</tr>
<tr>
<td>17</td>
<td>9th January</td>
</tr>
<tr>
<td>18</td>
<td>16th January</td>
</tr>
</tbody>
</table>

<sup>a</sup> transfer date of XL grade fish,  
<sup>b</sup> transfer date of increasing temperature group 1 (I1),  
<sup>c</sup> transfer date of increasing temperature group 2 (I2),  
<sup>d</sup> transfer date of constant group (C1)
3.2.4 Long term performance

Transfers were made to the NZKS Te-Punga sea cage, located in the Marlborough Sounds. Transferred groups were combined with NZKS production stock to create cages of 180,000–200,000 fish of a similar size range (Table 3.1) but not temperature regime. Approximately 11 months post transfer, grading, counting and weight assessments were carried out. GRe fish were counted and removed from normal growing stock. Due to the sea cages in this study being made up from groups reared on mixed freshwater rearing regimes, general conclusions on seawater performance is assessed by evaluating overall smolt status at the time of seawater transfer.

3.2.5 Statistical Analysis

Statistical analysis was carried out using R (version 2.5.1) and GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance level of 5% (α=0.05) was used. All values are expressed as means ± 1 SEM, unless stated otherwise. Data were tested for homogeneity of variance and data transformed where necessary.

Differences in Na\(^{+}/K^{+}\)-ATPase, natural plasma Na\(^{+}\) and plasma Na\(^{+}\) following hypoosmoregulatory challenges length, weight, and condition factor (CF; 100 \times W L^{-3},) were compared using ANOVA within groups over sample weeks and between groups on single dates. When factors were found to be statistically significant, post hoc comparison using Tukey HSD-tests and Dunnett's multiple comparison tests were carried out. For changes in gill Na\(^{+}/K^{+}\)-ATPase, natural plasma and plasma Na\(^{+}\) following hypoosmoregulatory challenges a three-way analysis of covariance (ANCOVA) was used to determine whether sampling week or temperature regime had a significant effect on these variables, length was used as a covariate. Models were simplified using Akaike's information criterion (AIC).

To investigate changes in natural plasma Na\(^{+}\) concentrations, and plasma Na\(^{+}\) concentrations following hypoosmoregulatory challenges mean values were plotted in relationship to not only time, but also plotted against mean fork length values. This was carried out to investigate a best fit model of changes in Na\(^{+}\) concentrations throughout
time or by changes in size (fork length) as occasionally some fish sampled were the same or a smaller size than a previous week. Third order polynomial curves were then fitted to each model.

### 3.2.6 Animal Ethics

All animal experimentation in this study was carried out with the prior approval of the University of Canterbury Animal Ethics Committee (2007/12R).

### 3.3 RESULTS

#### 3.3.1 Growth and Morphology

All groups attained characteristic external colourations of smolt. During the month of November (weeks 10, 11, 12) all fish were externally smoltified with little or no indication of parr marks (Figure 3.2). However, faint vertical markings were gradually regained after this time. Weight and length increased progressively from September 2006 to January 2007 in all groups (Figure 3.3). Using a two-way ANOVA there was also a significant difference between group weight ($F_{1,2844}= 281.9, p <0.0001$) and length ($F_{1,2844}= 336, p <0.0001$). There was also a significant difference between weeks on weight ($F_{17,2844}= 1234, p <0.0001$) and length ($F_{17,2844}= 1971, p <0.0001$). Using Bonferroni corrections no significant differences were found between groups weight and length at week 1 ($17^{th}$ September 2006). After 6 weeks ($24^{th}$ Nov) groups reared on increasing temperature regimes became significantly larger in both weight ($t=3.565, p<0.01$) and fork length ($t=4.997, p<0.001$) than constant temperature fish. With the exception of no difference of weight at week 7, groups reared on an increasing temperature regime were significantly larger in weight and length for the remainder of the study.

The change in condition factors ($K$) of both groups are shown in Figure 3.4. There was a significant effect of week on condition factor in both increasing ($F_{18,1422}=54.97, p$
<0.0001) and constant \( (F_{18,1422}=81.46 \ p <0.0001) \) groups using a one-way ANOVA. 
Groups reared on increasing temperatures did not show a significant drop in condition factor \((p> 0.05\), Dunnetts Multiple Comparison test\) remaining consistent for an initial 8 weeks. Constant temperature groups did show a significant drop in condition factor during initial weeks \((p< 0.05\), Dunnetts Multiple Comparison test\). Both constant and increasing temperature groups remained significantly higher after week 13. A two-way ANOVA showed a significant difference between groups \((F_{1, 284}= 43.27 \ p <0.0001)\). Bonferroni corrections revealed that initially (week 1) groups reared on constant temperatures had a significantly lower condition factor in comparison to increasing temperature groups \((t=3.304 \ p <0.05)\) and during week 6 (24th October) to week 11 (28th of November) \((t= 5.681 \ p <0.001, t=3.738 \ p <0.01, t=4.543 \ p <0.001, t=7.301 \ p <0.001, t=4.820 \ p <0.001 \text{ and } t=3.195 \ p <0.05 \text{ respectively})\).

Specific growth rate (SGR) and thermal growth co-efficient (TGC) are shown in Figure 3.5. Both constant and increasing groups showed positive SRG and TGC but were highly variable throughout the weeks. Over the 18 week period mean SGR ± S.E was 2.06 ± 0.89 % day\(^{-1}\) and 1.97 ± 0.57 % day\(^{-1}\) for increasing and constant temperatures respectively. Taking into account the effect of temperature mean TGC was 1.68 ± 0.66 % day\(^{-1}\) and 1.62 ± 0.48 % day\(^{-1}\) for increasing and constant temperatures respectively.
Figure 3.2 External colourations as percent of sampled populations in increasing groups and constant groups. Separated by three body colour characteristics, banded (heavy parr marks), intermediate (faint parr marks) and smolt (no visible parr marks) (n=40).
**Figure 3.3** Weight and length of increasing and constant temperature treatment groups. * denote significant differences (p<0.05) between temperature groups using Bonferroni correction (two-way ANOVA). Values are mean ± 1 SEM, n = 80.
Figure 3.4 Average condition factors (K) of increasing — and constant — temperature treatment groups. Condition factors (CF) were calculated as 100 *(body weight in g) × (length in cm) ^ -3. * denote significant difference (p<0.05) from initial condition factor (week 1) using Dunnett's Multiple Comparison test. Values are mean ± 1 SEM, (n=80).
Figure 3.5 Increasing and constant temperature treatment group average specific growth rate (SGR) and thermal growth coefficient (TGC) over sample weeks. SGR and TGC are not calculated for the initial (1) sampling week of each group, since their previous growth history was not available. Specific growth rates (SGR) were calculated as 100 * (ln Wf − ln W0) (feeding days)^−1, where Wf and W0 are initial and final weights of the fish, respectively. TGC was calculated as = 1000 * (Wf^1/3 − W0^1/3) (number of day degrees)^−1. All points are mean values, n=40.

3.3.2 Osmoregulation

The ability to regulate plasma Na⁺ during a 24 hr hypoosmoregulatory test increased in all groups from early October until November (Figure 3.6). Survival of both groups was extremely high with only two mortalities occurring during 24 hr hypoosmoregulatory tests. Both mortalities occurred in the first weeks (Week 1 group C2, Week 2 group I2). There was a significant difference in plasma Na⁺ levels between weeks in increasing (F_{17, 341}=12.29 p<0.0001) and constant (F_{17, 341} 31.57 p<0.0001) temperature treatment groups using a one-way ANOVA. An ANCOVA using individual fish length as a covariate indicated that there was a significant effect of sampling week (F_{17,681} F= 29.0466 p <0.001) temperature regime (F_{1,681} F= 11.9509 p<0.001) and length (F_{1,681} F= 5.7751 p <0.05) on Na⁺ levels of challenged fish. There was also a significant
interaction effect between sampling week and temperature ($F_{17, 681} = 3.5903, p<0.001$) which meant that the effect of temperature on Na$^+$ levels differed throughout the weeks.

All groups were able to regulate Na$^+$ levels $<170$ mmolL$^{-1}$ by early October. The lowest plasma Na$^+$ levels were recorded in the fish reared on a constant 12°C temperature regime (Figure 3.6). Due to differences in fish size, mean plasma Na$^+$ levels were plotted against mean fork size (Figure 3.7). Constant temperature groups had a slightly higher correlation between plasma Na$^+$ and fork length ($R^2 = 0.3861$) compared to increasing temperatures ($R^2 = 0.2251$). Based on third order fitted polynomial curves maximal hypoosmoregulatory ability (155-165 mmolL$^{-1}$) was achieved between fork lengths of 140-160mm of both groups. Elevated hypoosmoregulatory ability of both groups continued to around week 8 (7$^{th}$ November) before plasma Na$^+$ concentrations started rising to previous levels ($>170$ mmolL$^{-1}$) comparable to parr in weeks 1 and 2.

Hypoosmoregulatory tests were carried out on XL grade fish one week preceding and immediately prior to seawater transfer on October the 25$^{th}$. There were significant differences in Na$^+$ values following hypoosmoregulatory challenges between weeks ($t_{38} = 2.122, p=0.0404$) and natural values in unchallenged fish between weeks ($t_{38} = 3.460, p=0.0013$). Natural plasma Na$^+$ levels and Na$^+$ levels following challenges did not differ significantly to constant or increasing temperature groups.

**Table 3.3** Summary of an extra large grade fish weight, length condition factor and Na$^+$ levels of freshwater controls and fish challenged in 35‰ seawater for 24 hr. All Na$^+$ levels are mean values ± SE, $n=20$. Morphometrics are mean values ± 1 SEM $n=40$. Different letters (a - b) denote significant differences between weeks using unpaired two-tailed t-test ($p<0.05$).

<table>
<thead>
<tr>
<th>Week</th>
<th>Weight (g)</th>
<th>Length (mm)</th>
<th>K</th>
<th>Freshwater Na$^+$ mmolL$^{-1}$</th>
<th>Seawater Na$^+$ mmolL$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Freshwater</td>
<td>Seawater</td>
</tr>
<tr>
<td>5</td>
<td>35.29± 0.83 a</td>
<td>141.69± 0.97 a</td>
<td>1.23 ± 0.01 a</td>
<td>136.57 ± 0.61 a</td>
<td>161.88 ± 1.98 a</td>
</tr>
<tr>
<td>6</td>
<td>39.48± 0.98 b</td>
<td>147.56± 1.07 b</td>
<td>1.22 ± 0.01 a</td>
<td>132.93 ± 0.86 b</td>
<td>167.31 ± 1.62 b</td>
</tr>
</tbody>
</table>
Figure 3.6 Plasma $\text{Na}^+$ levels (mmolL$^{-1}$) following hypoosmoregulatory challenge for 24 hours in seawater (35 %) of Increasing and constant temperature groups. * denote significant difference ($p<0.05$) from initial values (week 1) using Dunnett’s Multiple Comparison test. Values are mean ± 1 SEM, $n=20$. 

*Increased brain size, suggests a larger brain capacity and cognitive ability.*
Figure 3.7 Plasma $\text{Na}^+$ levels (mmolL$^{-1}$) and fork length $L_F$ (mm) in groups challenged for 24 hours in seawater (35 ‰). Third order polynomial lines are fitted to the data. Increasing $R^2 = 0.2251$ and constant temperature $R^2 = 0.3861$. Values are mean ± x,y SEM, n=10.
Natural plasma Na\(^+\) levels were comparable to hypoosmoregulatory challenged fish exhibiting a notable decline and progressive rise over time (Figure 3.8, 3.9). There was a significant difference in natural plasma Na\(^+\) between weeks in increasing (F\(_{17, 342}\)=33.04 \(p<0.0001\)) and constant (F\(_{17, 342}\)=51.84 \(p<0.0001\)) temperature treatment groups using a one-way ANOVA. ANCOVA using individual fish length as a covariate indicated that natural Na\(^+\) levels were significantly affected by week (F\(_{17, 700}\)=46.488 \(p<0.001\)) and temperature regime (F\(_{1, 700}\)=30.628 \(p<0.001\)) which was partly mediated by the effects of length (F\(_{1, 700}\)=336.944 \(p<0.001\)). However, the significance of week was not affected when controlling for either length or temperature in the models.

Natural plasma Na\(^+\) levels progressively declined below 140 mmolL\(^{-1}\) in both constant and increasing water temperature groups before returning to 150~160 mmolL\(^{-1}\) by week 18 (Figure 3.8). Natural plasma Na\(^+\) levels demonstrated a significant polynomial relationship with fork length of both temperature groups (Figure 3.9). As with Na\(^-\) levels following hypoosmoregulatory challenges, constant temperature groups exhibited a slightly higher relationship between fork length and natural Na\(^+\) levels (R\(^2\)= 0.6026) in comparison to increasing temperatures (R\(^2\)= 0.5254). Based on third order fitted polynomial curves, lowest natural plasma Na\(^+\) levels were achieved in both groups between approximate fork lengths of 130-150mm.
**Figure 3.8** Natural plasma Na$^+$ levels (mmolL$^{-1}$) in freshwater of increasing and constant temperatures groups. * denote significant difference ($p<0.05$) from initial values (week 1) using Dunnett’s Multiple Comparison test. Values are mean ± 1 SEM, n=20.
Figure 3.9 Natural plasma Na\(^+\) levels (mmolL\(^{-1}\)) and fork length L\(_F\) (mm) of freshwater groups. Third order polynomial lines are fitted to the data. Increasing temperatures \(R^2 = 0.5254\) and constant \(R^2 = 0.6026\). Values are mean ± x, y SEM, n=10.


**3.3.3 Gill \(Na^+/K^+\)-ATPase Activity**

Gill \(Na^+/K^+\)-ATPase activity increased in all groups over the study period. A Bonferroni correction following a two-way ANOVA indicated that the constant temperature groups showed significant elevations in gill \(Na^+/K^+\)-ATPase activity from mid-October at weeks 5, 6, 9, 12, 14, 15, 16, 17 (\(t=3.218 \ p<0.05\), \(t=3.334 \ p<0.05\), \(t=3.168 \ p<0.05\), \(t=3.592 \ p<0.01\), \(t=4.664 \ p<0.001\), \(t=3.804 \ p<0.01\), \(t=5.917p<0.001\) and \(t=6.183 \ p<0.001\) respectively) (Figure 3.10). ANCOVA using length as a covariate indicated that there was a significant effect of sample week on \(Na^+/K^+\)-ATPase activity (\(F_{17,699}=5.3182 \ p<0.0001\)) which was partly mediated by changes in length over time (\(F_{1,699}=130.85 \ p<0.0001\)). Sample week had the same effect on \(Na^+/K^+\)-ATPase activity when controlling for either length or temperature. However, the significant effect of temperature (\(F_{1,699}=178.13 \ p<0.0001\)) explained more of the total variance when controlling for the effects of length, as differences in temperature on gill \(Na^+/K^+\)-ATPase activity was partly mediated by the differences in length on a given date.

\(Na^+/K^+\)-ATPase activity increased almost two-fold in both temperature groups by week 10 as seen in Figure 3.10, however the percentage of total activity attributable to \(Na^+/K^+\)-ATPase using the specific inhibitor ouabain declined significantly over weeks (increasing \(F_{17,342} =16.28 \ p<0.001\); constant \(F_{17,342} =10.41 \ p<0.0001\)). Significant reductions in the total activity attributable to \(Na^+/K^+\)-ATPase activity were observed in both temperature groups by week 18 as shown in Figure 3.11. During initial weeks (1 - 4) the proportion of \(Na^+/K^+\)-ATPase activity rose to a maximum of 62% and 56% in smolts reared in increasing and constant temperature regimes respectively. Despite a continued increase in gill \(Na^+/K^+\)-ATPase activities (Figure 3.10), consistent and significant reductions after the 24\(^{th}\) October (week 6) were observed (Figure 3.11). By the end of the study in mid January (week 18) gill \(Na^+/K^+\)-ATPase activity accounted less than half - approximately 40% of the overall activity.
Figure 3.10 Gill Na⁺/K⁺-ATPase activity of Increasing and constant temperature treatment groups. * indicates significant difference (p<0.05) between groups using two-way ANOVA and Bonferroni corrections. Values are mean ± 1 SEM, n=20.
**Figure 3.11.** Percent of total activity inhibited by ouabain in gill homogenates of groups reared on increasing and constant temperature regimes. * indicate significant difference from initial values (week 1) using Dunnett’s multiple comparison test. Values are mean ± 1 SEM, n=20.
3.3.4 Seawater Performance

Seawater stock was graded, counted and weight sampled 11 months after entry (October 2007). Performance was measured in terms of overall survival, level of GR and growth rate (TGC and SGR) shown in Table 3.4. Although performance of specific groups cannot be ascertained due to fish being combined with production stock to make up larger pens of approximately 200,000 fish, performance is discussed in reference to overall timing of entry.

Extra large grade fish (XL) transferred on October 25th 2006 had high survival (90.37%) and low levels of GR (3.93 %) in comparison to stocks transferred later in the year (Table 3.4). Extra large grade (XL) fish entered seawater at a daily average temperature of 12.2°C on the 25th of October (Figure 3.12). Increasing and constant and temperature treatment groups which were transferred between the 29th November and the 13th of December experienced progressively lower survival rates and higher levels of GR. The final transfer on the 13th of December (cage T61D) recorded the poorest result with 80.84 % survival and GR in 12.34 % of the population. These groups did experience slightly higher daily averages of water temperature between 14.1 – 14.2°C (Figure 3.12). Using TGC to account for these differences in water temperature between transfer dates it was observed that growth rates were also highest for fish transferred in October (1.68 % day⁻¹, Table 3.4). Late November and December transfers experienced appreciably lower growth rates (TGC) of between 1.25 and 1.53 % day⁻¹.

| Table 3.4 Summary of post-smolt seawater performance of group transferred. Performance measured 11 months after seawater entry. Table includes entry date, survival, GR percentage and growth indices (thermal growth coefficient – TGC, specific growth rate (SGR)). |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Groups                          | Increasing (XL) | Increasing (I1) | Increasing (I2) | Constant (C1)  |
| Cage Name                       | T61A            | T61B            | T61C            | T61D            |
| Entry Date                      | 25-Oct-06       | 29-Nov-06       | 04-Dec-06       | 13-Dec-06       |
| Survival (%)                    | 90.37           | 87.71           | 82.98           | 80.84           |
| GR (%)                          | 3.93            | 8.07            | 10.07           | 12.34           |
| TGC (% days⁻¹)                  | 1.68            | 1.34            | 1.25            | 1.53            |
| SGR (% days⁻¹)                  | 1.40            | 1.12            | 1.06            | 1.26            |
Figure 3.12 Average daily seawater temperatures (5 m) in 2006 at Te Punga Bay (blue dots) and 12 year average (red dots). Arrows indicate the time of transfer of the groups to seawater. Fish in entry 1 (25th of October) were extra large graded (XL) reared on increasing temperature freshwater regime, entry 2 comprised the start of the second entry from the 28th November ending on the 13th December (as described in Table 3.4)
3.4 DISCUSSION

3.4.1 Growth, Morphology and Water Temperature

Metabolic processes in ectotherms are highly dependent on temperature (Clarke, et al., 1999). Temperature manifests itself throughout biological organization, controlling behaviour, biochemical reactions, cellular function and ultimately growth. Accordingly for ectothermic animals like salmonids, growth rate is highly dependent on temperature (Brett, 1979; Dwyer, et al., 1987; Elliott, et al., 1997). Average temperatures of the present study diverged with a total of 66.5 degree days separation by week 18 in mid January between constant and increasing temperatures. Being set in a production system a difficulty lay in the ability to adequately control the temperatures. The water sourced for the increasing temperature groups was dependent on ambient heating and solar radiation to warm the temperature above 12°C. Consequently this created a higher daily diel fluctuation of up to ±4 °C compared with constant temperature groups that experienced ±1 °C difference.

Within optimal metabolic ranges, diel fluctuations of temperature are suggested to have a greater positive effect on growth than constant temperature in a variety of species. Increased growth under oscillating or diel temperatures has been observed in the brown trout (Salmo trutta) (Spigarelli, et al., 1982) and large mouth bass (Micropterus salmoides Lacepede) (Diana, 1984). No difference in growth has been found in Atlantic salmon under diel fluctuations of 12-20°C and 16-20°C relative to constant temperatures of 12 and 20°C (Peterson, et al., 1989), although a remarkably similar study by Shrimpton et al (2007) reported that oscillating temperature at 12.3 °C had a significant effect on growth of smoltifying King salmon in comparison to similar constant temperatures. In the current study, fish reared in increasing temperatures were significantly larger by mid October, at which time, increased TGC and separation of less than ±5°C days from constant 12°C fish existed. This suggests that increased growth of groups is not entirely due to mean temperature and more likely due to a stimulatory effect of diel fluctuations.

Although these results showed that an increasing temperature above 12°C promoted superior growth to those reared at a constant 12°C temperature, increasing
temperatures negatively affected hypoosmoregulatory ability and gill Na\(^+\)/K\(^+\)-ATPase activity. Inhibition of gill Na\(^+\)/K\(^+\)-ATPase activity has been recorded in steelhead above water temperatures of 13°C (Adams, et al., 1975; Zaugg, 1981). Coho and King salmon held at 15°C have also been reported to have reduced osmoregulatory performance in response to seawater challenge (Clarke, et al., 1981). Wedemeyer et al (1980) and others (California Department of Water Resources, 1988) have cautioned that water temperatures be maintained below 12°C to prevent premature and inhibited smoltification in King salmon.

Within this study increased Na\(^+\)/K\(^+\)-ATPase activity did occur in both temperature groups, however, increasing temperatures above 12°C did reduce the absolute levels of Na\(^+\)/K\(^+\)-ATPase activity in comparison to fish reared on a constant 12°C. Although it remains to be seen if higher and more pronounced levels of gill Na\(^+\)/K\(^+\)-ATPase activity would occur in fish reared under a more natural springtime increase of temperature, the results do concur with the recommendation of Wedemeyer et al (1980) that temperatures be maintained below 12°C in King salmon to avoid negatively affecting Na\(^+\)/K\(^+\)-ATPase development.

### 3.4.2 Osmoregulation

Fish showed all the morphological characteristics associated with smolting with a high degree of silvering for several weeks in late November (Figure 3.2). Faint parr marks were unexpectedly regained in a minority of fish after this time. The appearance of faint marks during this time may have been augmented by increased loss of scales that is common in smoltifying and stressed salmon (NZKS, personal communication).

Survival during 24 hr hypoosmoregulatory challenges was extremely high with very little mortality illustrating a large degree of competence for at least 24 hr in 35 ‰ seawater. Smolts from the genus *Salmo* and *Oncorhynchus* that are able to adapt to seawater are expected to have plasma Na\(^+\) concentrations less than or equal to 170 mmolL\(^{-1}\) after 24 hr in full strength seawater (30-33‰) (Blackburn, et al., 1987). Peak depressions below this level were obtained for several weeks from the end of October to mid November. Although the size at which maximal hypoosmoregulatory ability was
attained (140-160 mm fork length) was significantly larger than previous studies by Franklin (1989) and Quinn (1999), the differences in smolt size cannot be compared as fish used in this study are genetically different, selected exclusively for maximum growth and high two year maturation.

All groups showed significant reductions in natural plasma Na⁺ levels until the 24th of October, concurrently with increases in, yet prior to peaks of gill Na⁺/K⁺-ATPase activity. The development of hypoosmoregulatory ability in advance of the peak gill Na⁺/K⁺-ATPase activity is in agreement with earlier studies in Atlantic salmon (Saunders, et al., 1990; Solbakken, et al., 1994). The development of elevated Na⁺/K⁺-ATPase activity and increased water permeability (Primmett, et al., 1988) during smoltification are believed to result in hydromineral imbalances. In the present study, depressions in natural Na⁺ concentrations correlated well with changes in hypoosmoregulatory ability and initial increases of Na⁺/K⁺-ATPase activity. These results support findings from an early study investigating smoltification in King salmon from New Zealand (Franklin, 1989). Depressions in natural Na⁺ concentrations during smoltification have also been reported in coho (Folmar, et al., 1981) and Atlantic salmon (Primmett, et al., 1988; Reis-Henriques, et al., 1996). Primmett et al (1988) found that maximal reductions in natural Na⁺ levels occurred just prior to the development of heightened seawater tolerance, as was the case in this study with hypoosmoregulatory ability. Primmett et al (1988) also reported that net loss of Na⁺ occurred slightly after maximal gill Na⁺/K⁺-ATPase activity. This was not seen as increases in natural Na⁺ concentrations occurred prior to peaks of gill Na⁺/K⁺-ATPase activity in late November.

Although the range of variations in natural Na⁺ levels were relatively small (0 - 20 mmolL⁻¹) in comparison to hypoosmoregulatory challenged fish (0 - 80 mmolL⁻¹) the natural fluctuations did provide strong evidence for a period of enhanced hypoosmoregulation. Measurements of fluctuating Na⁺ or alternatively Cl⁻ concentrations could in addition to other smolt analysis provide an indicator for the optimal timing of transfer to seawater. It is cautioned that natural Na⁺ levels may vary more so in wild stock depending both environmental levels and that obtainable from the diet.
3.4.3 Gill Na\(^+\)/K\(^+\)-ATPase Activity

Several studies in Atlantic salmon have shown that elevated rearing temperatures can influence the attainment of elevated gill Na\(^+\)/K\(^+\)-ATPase levels during smoltification and the rapidity of decline during desmoltification (Handeland, et al., 2004; McCormick, et al., 1999; Solbakken, et al., 1994).

All groups in this study showed increases of gill Na\(^+\)/K\(^+\)–ATPase activity during the austral spring/summer period. Smolts which were raised on an increasing temperature regime above 12°C had a lower absolute level of gill Na\(^+\)/K\(^+\)-ATPase activity relative to fish reared at a constant 12°C. Although gill Na\(^+\)/K\(^+\)-ATPase activity was variable throughout time both were observed to peak late November. Peak levels in gill Na\(^+\)/K\(^+\)-ATPase activity were comparable to a recent smoltification study of King salmon by Shrimpton et al. (2007) using the same assay developed by McCormick, (1993). However in contrast to this study Shrimpton et al. (2007) found that daily oscillations (8°C - 14°C) did not affect Na\(^+\)/K\(^+\)-ATPase activities in when compared to activities of fish reared on a constant 12.3°C.

The increase in gill Na\(^+\)/K\(^+\)-ATPase activity on the 9th of January in fish held at a constant 12°C cannot be explained (Figure 3.10). Under the assumption that elevations in gill Na\(^+\)/K\(^+\)-ATPase activity are temperature-dependent as in Atlantic salmon (McCormick, et al., 1999), peaks in Na\(^+\)/K\(^+\)-ATPase activity could be predicted by degree days. Based on the thermal sum experienced from stocking (12th September) to the first observed peak (28th November) in gill Na\(^+\)/K\(^+\)-ATPase activity, the constant temperature groups lagged approximately 31°C days behind the increasing temperature groups. This suggests that a peak in gill Na\(^+\)/K\(^+\)-ATPase activity for the constant temperature groups should be obtained in less than a week after the peak for increasing temperature groups; however, the January 9th peak occurred 5 weeks later (Figure 3.10).

The timing of elevations in Na\(^+\)/K\(^+\)-ATPase activity is in accordance with that of Franklin (1989) and Quinn (1999) who also investigated smoltification in New Zealand juvenile ocean-type King salmon. Both studies found peaks in Na\(^+\)/K\(^+\)-ATPase activity in November remaining elevated through December. Quinn (1999) used similarly bred commercial stock in 1997-98 and interestingly found several peaks in Na\(^+\)/K\(^+\)-ATPase activity from October through January. No evidence was given for the incongruity of peaks in gill Na\(^+\)/K\(^+\)-ATPase activity. Quinn (1999) did elucidate to the
possible inaccuracy of assay protocol and interactions with environmental stressors which may lead to fluctuations in \( \text{Na}^+/\text{K}^-\)-ATPase activity. It is suggested that the highly irregular development as seen in this study and that of Quinn (1999) is actual irregular development and not erroneous results as this pattern of development complements the variable migration patterns seen in King salmon (Healy, 1983; Healy, 1991; Taylor, 1990).

The reduction of gill \( \text{Na}^+/\text{K}^-\)-ATPase activity during the desmoltification process in Atlantic salmon is highly dependent on temperature, with increasing rates of decline at higher temperatures (Duston, et al., 1991; Handeland, et al., 2004; McCormick, et al., 1999). The timing of desmoltification in Atlantic salmon as measured by changes in gill \( \text{Na}^+/\text{K}^-\)-ATPase activity was suggested by McCormack (1999) to be related to degree days. For example in Atlantic salmon it is proposed that the smolt window comprises approximately 200°C days from the first observed peak in gill \( \text{Na}^+/\text{K}^-\)-ATPase, within approximately 500°C days from the first observed peak a rapid decline of should occur that reaches pre-smolt levels. In this study gill \( \text{Na}^+/\text{K}^-\)-ATPase levels of both groups were not observed to decline to pre-smolt levels which summed over 600°C days from the first peaks in activity (28\textsuperscript{th} November). These results demonstrate that the effect of degree days on \( \text{Na}^+/\text{K}^-\)-ATPase activity during smoltification and desmoltification are different in King salmon in comparison to Atlantic salmon. This highlights the dangers of extrapolating from one species to another, a common trait in the aquaculture industry.

It is often assumed that elevated gill \( \text{Na}^+/\text{K}^-\)-ATPase activity is predictive of hypoosmoregulatory ability, which for a period of time in this study was correct. The increase in gill \( \text{Na}^+/\text{K}^-\)-ATPase activity in both groups coincided with a reduction in both natural plasma \( \text{Na}^+ \) levels and levels following hypoosmoregulatory tests. \( \text{Na}^+/\text{K}^-\)-ATPase activity peaked following lowest recorded levels of natural plasma \( \text{Na}^+ \) and peak levels of hypoosmoregulatory ability. However, decreased hypoosmoregulatory ability and normalisations of natural plasma \( \text{Na}^+ \) levels occurred independently of observable or correlated decreases in gill \( \text{Na}^+/\text{K}^-\)-ATPase activity. Several studies in Atlantic salmon refer to desmoltification as involving a loss of elevated gill \( \text{Na}^+/\text{K}^-\)-ATPase activity (Handeland, et al., 2004; McCormick, et al., 1999; Stefansson, et al., 1998) which is generally assumed to result in the restoration of hyperosmoregulatory
capacity. However, this study conflicts with that reasoning as a clear dissociation between the development of hyperosmoregulation and loss of gill Na\(^+\)/K\(^+\)-ATPase activity was observed. The lack of correlation between hypoosmoregulation and gill Na\(^+\)/K\(^+\)-ATPase activity has been observed previously in Atlantic salmon (Berge, et al., 1995; Sigholt, et al., 1998; Sigholt, et al., 1995; Solbakken, et al., 1994) and rainbow trout (Madsen, et al., 1989). Several studies have also failed to find a correlation between high gill Na\(^+\)/K\(^+\)-ATPase activity prior to entry and the percent of adult returns in the wild (Bilton, et al., 1982; Ewing, et al., 1982; Ewing, et al., 1980; Ewing, et al., 1985).

As smolts gradually increase gill Na\(^+\)/K\(^+\)-ATPase activity, they are entering a state of negative Na\(^+\) balance and maladaptation to the freshwater environment. It is suggested that an alternative compensatory mechanism is occurring prior to a drop in gill Na\(^+\)/K\(^+\)-ATPase activity to normalise plasma Na\(^+\). Back-calculations showed that despite increasing gill Na\(^+\)/K\(^+\)-ATPase activity the proportion of ATP-dependent activity represented by gill Na\(^+\)/K\(^+\)-ATPase diminished significantly after several weeks (31\(^{\text{st}}\) October). This illustrates that residual ATP-dependent activity was occurring at even higher proportions to elevations in gill Na\(^+\)/K\(^+\)-ATPase activity. Initially the percentage of gill Na\(^+\)/K\(^+\)-ATPase activity reached a maximum of 62% and 56% in smolts reared in increasing and constant temperature regimes respectively. However, significant reductions were observed after the 24\(^{\text{th}}\) October. By 16\(^{\text{th}}\) of January (week 18) gill Na\(^+\)/K\(^+\)-ATPase activity accounted for approximately 40% of the overall ATP-dependent activity.

It is hypothesised that increased residual ATP-dependent activity is associated with or is directly responsible for increased natural plasma Na\(^+\) levels during desmoltification. One potential enzyme responsible for this increased activity may be the sodium potassium chloride co-transporter (NKCC). NKCC transports Na\(^+\), K\(^+\) and Cl\(^-\) into the cell in a stoichiometry of 1:1:2 respectively. It has been specifically associated with seawater adaptation in the brown trout (Salmo trutta) and Atlantic salmon (Pelis, et al., 2001; Stefansson, et al., 2007; Tipsmark, et al., 2002). Although NKCC has a specific seawater adaptive function, it has been found in gill chloride cells of freshwater adapted Atlantic salmon (Pelis, et al., 2001). NKCC has been co-localised with Na\(^+\)/K\(^+\)-ATPase in MRCs and has been shown to increase in parallel with Na\(^+\)/K\(^+\)-
ATPase during seawater acclimation (Tipsmark, et al., 2002) with synchronous surges in mRNA and protein levels (Pelis, et al., 2001). Entry of ions by the NKCC is suggested to be driven via a transepithelial electrochemical gradient established by adjacent Na\(^+\)/K\(^+\)-ATPases. Although the regulation of NKCC activity is largely unknown it is suggested to involve protein kinases and/or phosphatases (Haas, et al., 2000) which may therefore be consuming ATP. The NKCC as the candidate for increased residual activity may be justified via indirect consumption of ATP. Although its role in freshwater is unknown, under current uptake models NKCC is not involved in Na\(^+\) absorption and thus unlikely to be responsible for the normalisation of natural plasma Na\(^+\).

One enzyme that is currently recognised in Na\(^+\) absorption models is the ATP-dependent vacuolar-type proton pump (H\(^+\)-VATPase). Current models suggest that Na\(^+\) is taken up by an apical amiloride sensitive Na\(^+\) conductive channel (ENaC) electrochemically coupled to apical H\(^+\)-VATPases. The ATP dependent process of H\(^+\) extrusion through H\(^+\)-VATPase creates the required internal negative potential (-mV) for inward diffusion of Na\(^+\) though the epithelial sodium channel. On the basolateral membrane it is proposed that Na\(^+\)/K\(^+\)-ATPase works in synchrony with H\(^+\)-VATPase removing intracellular Na\(^+\) to the blood, furthermore improving the negative internal potential for apical Na\(^+\) absorption. If the residual activity is due to H\(^+\)-VATPase, the initial reduction in its activity during the first 4 weeks may be preparative action to minimise Na\(^+\) absorption in anticipation of entry to seawater, therefore facilitating a faster action. This hypothesis was also proposed by Seidelin, et al (2001) who found similar results which showed that at the peak of smoltification when both \(\alpha\) and \(\beta\) subunit mRNAs, Na\(^+\)/K\(^+\)-ATPase activity, and SW-tolerance reached their maximal levels, there was a simultaneous drop in H\(^+\)-VATPase B subunit expression and thus an increase in the Na\(^+\)/K\(^+\)-ATPase to H\(^+\)-VATPase B-subunit mRNA ratio.

A strategy of up-regulating H\(^+\)-VATPase prior to reductions in Na\(^+\)/K\(^+\)-ATPase activity may allow smolts to increase Na\(^+\) levels at a superior rate than merely a reduction of Na\(^+\)/K\(^+\)-ATPase would. It is possible that elevations in cortisol which are known to occur during smoltification (Franklin, et al., 1992) may aid this system by tightening epithelial junctions (Wood, et al., 2002), slowing the leak of accumulated Na\(^+\) on the luminal side of the basolateral membrane. The H\(^+\)-VATPase is strong candidate for
observed increases in residual activity given that its activity is ATP-dependent and is currently recognised as a primary enzyme involved in Na\(^+\) uptake (Fenwick, et al., 1999; Lin, et al., 1995; Marshall, 2002). This dynamic regulation of enzymes may explain, at least in part, the difficulty in establishing consistent relationships between hypoosmoregulatory ability gill Na\(^+\)/K\(^+\)-ATPase activity. Further studies are needed to further elucidate the enzyme(s) responsible for increased residual ATP-dependent activity and their dynamics with Na\(^+\)/K\(^+\)-ATPase during smoltification.

3.4.4 Seawater Performance

The seawater performance of stock was calculated approximately 11 months post transfer in October 2007. The performances of individual freshwater groups (increasing and constant) were unable to be assessed. This was due to study groups being combined with NZKS production stock to create cages of 180,000-200,000 fish of a similar size range (Table 3.1). Furthermore, several seawater cages were a combination of fish reared on a constant and increasing temperature rearing regime.

Although hypoosmoregulatory ability and Na\(^+\)/K\(^+\)-ATPase activity were depressed in increasing temperatures, both temperature groups followed a similar pattern of development. Taken purely on the timing of transfer, early entry stocks (XL) which were transferred on October 25\(^{\text{th}}\) 2006 performed considerably better in terms of survival, growth rate (SGR, TCG) and percent of population affected by GR. Less than 4% GR occurred in fish transferred in October, in comparison to 8 - 12% of fish transferred between the 29\(^{\text{th}}\) of November and 13\(^{\text{th}}\) of December. Although at this time XL stocks were significantly larger than other groups, no significant differences in terms of natural plasma Na\(^+\) levels and their ability to regulate Na\(^+\) on abrupt transfer were found. By late November and early December, fish from both temperature groups were transferred at a significantly larger size (74-78 g) than XL stock transferred in late October (45 g). These results contrast with a general industry (NZKS, personal communication) and published view that larger smolts show greater seawater tolerance than smaller smolts (Arnesen, et al., 1992; Duston, et al., 1995; McCormick, et al., 1987).
Chapter 3 Characterisation of Smoltification and Desmoltification in Commercial Stocks of Under-Yearling King Salmon (*Oncorhynchus tshawytscha*)

Increased temperatures are known to affect the ability of salmonids to adapt to seawater (Dickhoff, *et al.*, 1989; Handeland, *et al.*, 1998; Handeland, *et al.*, 2000). Clarke and Shelbourn (1985) examined the relationship between freshwater and seawater temperatures on the adaptability of fall King salmon. They found optimal plasma Na$^+$ regulation of 5.8 g fish transferred from 13.8°C to a seawater temperature of 10.2°C. Maximal seawater growth was found in 6 g fish transferred from 10°C to 14°C. A study by Franklin (1989) showed that King salmon that were greater than 15 g and had successfully smoltified were able to adapt to seawater temperatures of 19°C.

In this study, stock transferred late November and early December did experience an average increase of 1.3°C in seawater compared to just 0.2°C for October transfers. The possible effect of increased temperatures on seawater adaptability cannot be excluded. However, the available data suggest that higher mortality, increased GR and low growth rates are more likely to be related to lower levels of hypoosmoregulatory ability at that time. Taken together, these results give further evidence to suggest that for King salmon the timing of transfer beyond a lower critical size for smoltification is more important than a specific smolt size.

3.4.5 Summary

In summary, it should be firstly emphasised that fish used in this study were produced from strain that has been under selection for economically important traits for several generations. Due to a general lack of specificity of smoltification process in King salmon compared to other salmonids, prudent conclusions are needed if comparisons between smoltification studies in wild and other commercial strains are sought.

This study has shown that even small changes in temperatures above 12°C negatively affect the development of hypoosmoregulatory ability and amplitude of gill Na$^+$/K$^+$-ATPase activity in a commercial strain. Smolt indicators to detect elevations in hypoosmoregulatory ability for seawater transfer are critical to not only avoid high levels of GR but also improve initial seawater adaptation and growth. The present results can be used to further understand the dynamics of development during smoltification in King salmon, specifically emphasising that in some cases elevated gill Na$^+$/K$^+$-ATPase activity
alone may not be a sufficient criterion to evaluate the directional states of hypo- or hyper-osmoregulation and should only be used in combination with plasma ion analysis and hypoosmoregulatory challenges. It also brings a focus for future studies on the dynamics of branchial enzyme expression during smoltification and salinity shifts in salmonids that may improve techniques for determining \textit{in-vivo} states.
CHAPTER FOUR

Osmoregulatory Physiology of Growth Retarded and Normal Growing King salmon (*Oncorhynchus tshawytscha*) at Sea and Following Transfer to Freshwater.

4.1 INTRODUCTION


As fish do not display seawater tolerance or preference outside of a normal period of smoltification, osmoregulatory dysfunction has frequently been associated with GR. However, plasma ion profiles, specifically Na\(^+\) levels of growth retarded Coho salmon appear within normal ranges (Varnavsky, *et al.*, 1992) and in some cases slightly lower than that of normal growing cohorts (Clarke, *et al.*, 1977). However, these studies only describe the osmoregulatory status of identifiable GRd fish and not developing GRd fish. Osmoregulatory imbalances may be more dramatic during the development of GR, resulting in damage to endocrine processes. It is hypothesised that the introduction into seawater of inadequately smoltified salmonids may lead to initial osmoregulatory imbalances that directly induce terminal perturbations in the growth hormone and insulin-like growth factor-1 axis (GH/IGF-1 axis) (Sheridan, *et al.*, 1998; Very, *et al.*, 2007a; 2007b; Young, *et al.*, 1989).


For unknown reasons, GR in King salmon is often viewed differently, specifically by industry, to that observed in other species such as Coho, despite close phylogenetic relationships (Domanico, *et al.*, 1997) and life history traits (Healy, 1991). It is not currently known if GR in King salmon is reversible, but if it is, tracing
physiological and endocrinological changes during a readaptation phase may help identify processes leading to the initiation of the GR.

The initial objective of this study was to determine if freshwater adaptation can occur in GRd King salmon and if GR can be reversed, comparable to that observed in Coho and Atlantic salmon (Bjornsson, et al., 1988; Clarke, et al., 1977; Young, et al., 1989). This was achieved using a comprehensive approach to investigate changes in key osmoregulatory enzymes (branchial Na⁺/K⁺-ATPase activity and NEM-sensitive H⁺ V-ATPase), stress hormones (plasma cortisol) and changes in plasma and muscle constituents (plasma Na⁺, haematocrit and muscle water content). In order to compare rates of change and elucidate readaptation mechanisms of GRd and normal growers, staggered sampling was carried out prior to and following direct transfer to freshwater over a period of three months.

**4.2 MATERIAL AND METHODS**

**4.2.1 Fish Handling and Freshwater Transfer.**

Experiments were carried out at the NZKS Tentburn freshwater facility, Canterbury, New Zealand. Fish were obtained from Te Punga Bay sea cage located in the Marlborough Sounds. Fish were obtained from the same 2006 production year used in Chapter 3, transferred as under-yearlings during December 2006.

Grading of GRd fish from good growers at the sea cage took place between the 1st and 5th of June 2007, approximately 6 months after they were stocked. Gradings were conducted using size selective gratings and electronic pipeline counters (Vaki Aquaculture Systems Ltd. Kópavogur, Iceland). 2175 GRd fish (mass of 93.5 ± 2.6 g, range 63.9 – 168 g, and length 216 ± 1.9 mm, n = 100) were separated from normal growers in a separate holding pen. 663 normal growing fish (mass 365 ± 12.3 g, range 165 – 535 g, length 294 ± 3.1 mm, n = 100) were also isolated. Because normal growing fish were significantly larger, additional GRd fish were used to equal the rearing densities with normal growers. Both GRd and normal growing fish were allowed to recover from grading for several days prior to transport.
On the 8th June 2007, GRd fish and normal growing fish were loaded, using a brail net system into separate specially designed transportation units, filled with clean full strength seawater. The trucked units were monitored *en route* using an OxyGuard transport system (OxyGuard, International A/S, Denmark) and when necessary, supplementary oxygen was used to maintain $O_2 > 7$ mgL$^{-1}$. Transportation to the NZKS freshwater facility at Tentburn took approximately 7 hr.

**4.2.2 Experimental Protocol**

Aliquots of fish were removed and sampled at sea prior to transport, on arrival (0h) (still in seawater), 2 hr, 4 hr, 12 hr, 1, 3, 7, 14 days, 4, 8 and 16 weeks after transfer to freshwater. At time 0 hr, a group of 10 GRd and normal growing fish were sampled directly from the transport units. Fish were then immediately unloaded into a 180 m$^3$ partitioned freshwater raceway (30m x5m x1m length/width/depth).

Fish that were scheduled for sampling at 2 hr, 4 hr, 12 hr, and 1 day were placed in cages (2 m x 2 m) within the main raceway. This allowed fish to be accessed and anesthetised quickly (< 1 min) preventing any stress on them or other fish during the collection process. Mortalities were collected daily from the raceways and post mortem assessment carried out. All food was withheld for 48 hr after arrival. Following this time small amounts of feed were introduced, with full rations to satiation attained after day 7. All fish were fed using electronic spinners and hand supplementation. Fish were constantly monitored and fed accordingly, to ensure they were satiated during daylight hours. GRd fish were fed a mix of 3mm and 4mm Alitec Golden Activa (Puerto Montt, Chile). Due to their larger entry size, normal growers were fed on a mix of 4 mm and 6 mm Alitec Golden Activa. All food was withheld 24 hr prior to sampling.

**4.2.3 Sampling**

At each sampling point, 10 normal growers and 10 GRd fish were randomly selected and immediately euthanised by overdose with AQUI-S®. Fish were measured for fork length ($L_F$) to the nearest 1 mm, weight ($W_i$) to the nearest 0.1 g and external abnormalities noted. Blood was then collected by caudal puncture with ammonium
heparinised disposable syringes. All blood samples from fish were sampled within 5 min of netting to avoid a sampling-related stress response.

Haematocrit was determined from freshly drawn heparinised blood collected in micro-haematocrit tubes (Vitrex Medical A/S Denmark), followed by sealing and centrifugation (5 min 12,000 RPM) using a microhaematocrit haemofuge (Heraeus, Baxter). Haematocrit (Hct) was determined as a percentage of red blood cells to total blood volume. Plasma was obtained from remaining blood by centrifugation (7000 g, 5 min). 200 µL sample of plasma were withdrawn and immediately snap frozen in liquid N\textsubscript{2} and stored at -80°C for the subsequent determination of cortisol levels using ELISA (Lewis, et al., 1992), with modification described in Chapter (2). The remaining balance of plasma was snap frozen in liquid N\textsubscript{2} and stored at -20°C for determination of plasma Na\textsuperscript{+} (± 1 mmoL\textsuperscript{-1}) by flame photometry (Sherwood, Model 410).

For determination of gill Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and NEM sensitive gill H\textsuperscript{+} -VATPase activity, filaments from two gill arches on the left side were immediately excised and washed in ice-cold SEI buffer (150 mmoL\textsuperscript{-1} sucrose, 10 mmoL\textsuperscript{-1} EDTA, 50 mmoL\textsuperscript{-1}, imidazole pH 7.3). Following excision and washing, samples were placed in cryotubes containing fresh SEI buffer and then snap frozen in liquid N\textsubscript{2} before being stored at -80°C at the University of Canterbury. The pyloric caeca and posterior intestine of ten fish from sea cages were used for determination of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity. Gill Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and H\textsuperscript{+} -VATPase activities were measured by a modified method developed by McCormick (1993) and Lin and Randell (1993). Pyloric caeca and posterior intestine Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activities were measured by the above methods with modification described by Veillette, et al (2004). From all euthanised fish, a 2-3 g block of white muscle tissue was excised from the dorsal block and frozen for determination of total water content. Blocks of hepatic and anterior kidney tissue of ten fish from sea cages were also used to measure total tissue Na\textsuperscript{+} and K\textsuperscript{+} concentrations. Sampling and analysis for all of the above are described in detail in the General Methods section (Chapter 2).
4.2.4 Statistical Analysis

Data were handled using Excel (Microsoft Corp.) and statistical analysis was carried out using R (R-version 2.5.1) and GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego USA). Statistical significance level of 5% (α=0.05) was used. All values are expressed as means ± 1 S.E.M unless stated otherwise. Data were tested for homogeneity of variance (evaluated by residual plots) and data transformed where necessary. A two-way ANOVA was used to analyse effects of transfer to freshwater on (1) group: GRd or normal grower and (2) sampling time for differences in Na\(^+\)/K\(^+\)ATPase and H\(^+\) V-ATPase, plasma Na\(^+\), muscle water content, haematocrit and morphological changes. ANOVA was followed by a posteriori comparison (Bonferroni multiple comparison tests) between groups at a single time point and within groups from initial seawater values. Bonferroni corrections are commonly applied for multiple testing however it is argued by many researchers that the test is too conservative. However the following results were validated by a comparison to the two standard error rule based off the regression coefficients following two-way ANOVA.

4.2.5 Animal Ethics

All animal experimentation in this study was carried out with the prior approval of the University of Canterbury Animal Ethics Committee (Approval number 2007/34R)

4.3 RESULTS

4.3.1 Gill H\(^+\) -VATPase activity

Using a two-way ANOVA there was no significant difference between group H\(^+\) -VATPase activity (F\(_{1, 162}\) =1.101 p=0.296), yet there was a significant difference in activity over time (F\(_{8, 162}\)=18.02 p <0.0001) and between groups over time (F\(_{8, 162}\)=3.048 p=0.0032). A significant increase in H\(^+\) -VATPase activity was observed in normal growers after 24hr (t=2.530 p <0.05) (Figure 4.1). Significant increases in H\(^+\) -VATPase
activity were also observed at 7 (t=2.273 p <0.05) and 14 days (t=5.464 p <0.05). GRd fish also showed a similar pattern with a significant increase of activity from pre-transfer levels after 3 days (t=2.806 p<0.05) 7 days (t=2.408 p<0.05) and 14 days (t=9.355 p<0.001). Following the dramatic rise in both groups, activity levels dropped back and were not found to be significantly different from pre-transfer levels for the remainder of the trial, with the exception of a slight elevation in GRd at day 56.

4.3.2 Gill Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity

Na\textsuperscript{+}/K\textsuperscript{+}-ATPase levels prior to transfer were 3.30 ± 0.49 µmol ADP mg\textsuperscript{-1} protein hr\textsuperscript{-1} in GRd fish and 4.11 ± 0.23 µmol ADP mg\textsuperscript{-1} protein hr\textsuperscript{-1} in normal growers. Using a two-way ANOVA, there was a significant difference in Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity over time (F\textsubscript{8, 162} =54.00 p<0.0001) between normal growers and GRd fish (F\textsubscript{1, 162} =24.96 p<0.0001). There was also a significant interaction of differences over time between groups (F\textsubscript{8, 162} =2.037 p<0.05). Bonferroni corrections were used to test for significant differences in Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity between groups throughout the transfer. No differences were observed using Bonferroni corrections between groups prior to transfer (at -1) (t =2.483 p>0.05), however, they were significantly different at 12 hr (t=3.818 p<0.01) and 24 hr (t=3.227 p< 0.05) (Figure 4.1). By using a Students t-test it was shown that differences did exist between groups in seawater (at -1) (t\textsubscript{18}= 4.741 P= 0.0002).

Differences from seawater values within groups over time revealed that both groups showed significant reductions in Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity after 14 days. Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity continued to decline in both groups with over a thee-fold depression in activity by 28 days in freshwater. Subsequent to this, in both groups, Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity showed slight increases though by day 112 Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity remained significantly less than pre-transfer values of normal growers (t=4.592 p<0.001) and GRd fish (t=4.240 p<0.001). There was a significant negative relationship between specific activity of Na\textsuperscript{+}/K\textsuperscript{+} ATPase and H\textsuperscript{+}-VATPase (r =-0.8872) (Figure 4.2). Figure 4.3 shows that during periods of decreased gill Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity after transfer to freshwater, the proportion of activity attributed to H\textsuperscript{+}-VATPase increased. This was particularly obvious at 14, 28 and 56 days.
Chapter 4 Osmoregulatory Physiology of Growth Retarded and Normal Growing

King salmon (*Oncorhynchus tshawytscha*) at Sea and Following Transfer to Freshwater

Figure 4.1 Effects of transfer from seawater to freshwater water on gill Na⁺/K⁺ ATPase activity (top graph) and gill H⁺-VATPase activity in normal growing and GRd fish (bottom graph). Enzyme activity is measured in µmol ADP mg⁻¹ protein hr⁻¹. On the time axis, -1 (days) indicate levels in seawater fish obtained prior to transfer. Values at time 0 (days) are obtained from fish on arrival to the freshwater facility yet prior to freshwater entry. * indicates a significant difference (P<0.05 or better) from initial seawater values (-1 days) within groups using Bonferroni’s multiple-comparison test after two-way ANOVA. Values are means ± 1 S.E.M. (n=10 for all time points).
Figure 4.2 Spearman’s rank correlation analysis between ATP dependent specific activities (%) attributed to gill Na⁺/K⁺ ATPase and NEM sensitive H⁺ V-ATPase. Na⁺/K⁺ ATPase shows a significant negative relationship with H⁺ V-ATPase ($r = -0.8872$). Each point represents an individual fish sampled either from sea cages or during transfer to freshwater ($n = 184$).
**Figure 4.3** Changes in the percentage of specific activity attributed to gill Na⁺/K⁺ ATPase (top graph) and gill H⁺-VATPase (bottom graph) at sea and during transfer to freshwater water. On the time axis, -1 (days) indicate levels in seawater fish obtained prior to transfer. Values at time 0 (days) are obtained from fish on arrival to the freshwater facility yet prior to freshwater entry. Values are means ± 1 S.E.M. (n=10 for all time points).
4.3.3 Plasma Na⁺

There was a highly significant difference in plasma Na⁺ levels over time (F<sub>11, 216</sub> =110.8  p <0.0001) but not between groups (F<sub>1, 216</sub> =2.322  p=0.1290). The interaction between groups over time was also not significant (F<sub>11, 216</sub> 1.755  p=0.0634). Mean plasma Na⁺ levels prior to transfer were 154.63 ± 3.67 mmolL⁻¹ in GRd fish and 147.94 ± 7.30 mmolL⁻¹ in normal growers.

On arrival, after 7 hr transportation, plasma Na⁺ levels were significantly elevated from pre-transfer seawater levels reaching 190.02 ± 21.24 mmolL⁻¹ and 186.79 ± 6.11 mmolL⁻¹ for GRd and normal growing fish respectively. Upon transfer to freshwater, plasma Na⁺ rapidly declined becoming significantly depressed at 4 hr with the lowest levels attained in both groups 24 hr post-transfer. Plasma Na⁺ levels in both groups remained low to day 7 before increasing to approximately 140-145 mmolL⁻¹. No significant differences in plasma Na⁺ were seen in normal growers after 14 days post transfer normalising to within pre-transfer levels. Plasma Na⁺ remained significantly lower in GRd fish, with no significant difference to pre-transfer levels after 28 days in freshwater. However plasma Na⁺ levels were not significantly different to normal growers on 14 and 28 days. The extended depression in GRd fish is due to the higher pre-transfer levels and not due to their inability to regulate at days 14 and 28. Na⁺ levels in both groups normalised to approximately 149 mmolL⁻¹ by 112 days.
Chapter 4 Osmoregulatory Physiology of Growth Retarded and Normal Growing

King salmon (Onchorhynchus tshawytscha) at Sea and Following Transfer to Freshwater

Figure 4.4 Effects of transfer from seawater to freshwater water on plasma Na⁺ mmolL⁻¹ (top graph) and haematocrit (%) (bottom graph) in normal growing and GRd fish. On the time axis, -1 (days) indicate levels in seawater fish obtained prior to transfer. Values at time 0 (days) are obtained from fish on arrival to the freshwater facility yet prior to freshwater entry. * indicate a significant difference (P<0.05 or better) from initial seawater values (-1 days) within groups using Bonferroni’s multiple-comparison test after two-way ANOVA. Values are means ± 1 S.E.M. (n=10 for all time points).
4.3.4 Haematocrit

GRd fish were extremely anaemic in comparison to normal growing fish. Mean haematocrit levels pre-transfer were 39.25 ± 2.38 % in GRd fish in contrast to normal growing fish with a mean of 49.10 ± 5.58 %. Accordingly, there was a significant difference between groups \((F_{1, 214} \approx 381.5 \, p < 0.0001)\), over time \((F_{11, 211} \approx 10.14 \, p < 0.0001)\), and between groups over time \((F_{1, 211} \approx 3.938 \, p < 0.0001)\). There were no significant differences in haematocrit levels within each group following transportation, however, after entry into freshwater, haematocrit levels in both groups showed rapid reductions.

This reduction in haematocrit levels was transient, and within 4 hr increased back towards pre-transfer levels. By 24 hr, haematocrit increased significantly higher than pre-transfer levels in both groups of fish \((\text{GRd } t=2.613 \, p < 0.05, \, \text{normal growers } t=4.341 \, p < 0.001)\). By 3 days, normal growing fish haematocrit had returned to pre-transfer levels and remained there for the rest of the experiment. In contrast, GRd fish haematocrit remained high for at least 7 days before falling back towards pre-transfer levels. After 112 days in freshwater haematocrit levels of GRd were significantly elevated \((t=5.03 \, p < 0.001)\) from pre-transfer levels, and were equivalent to that seen in normal growing fish \((t=0.322 \, p > 0.05)\).

4.3.5 Muscle Water Levels

Mean muscle water levels of GRd fish and normal growers was 80.90 ± 1.18 % and 72.52 ± 2.89 % respectively. There were significant differences in the muscle water levels between groups \((F_{1, 216} \approx 536.6 \, p < 0.0001)\) and within groups over time \((F_{11, 216} \approx 10.22 \, p < 0.0001)\). However, there were no significant interactions between groups over time \((F_{11, 216} \approx 1.627 \, p =0.0926)\). Bonferroni corrections showed that at 3, 7 and 14 days of freshwater exposure, muscle water content was significantly higher than pre-transfer levels in normal growers \((t=3.993 \, p < 0.001, \, t=5.449 \, p < 0.001, \, t=2.633 \, p < 0.05\) respectively). Muscle water levels of normal growers normalised to pre-transfer levels with no significant differences after 56 and 112 days in freshwater. From pre-transfer values it could be concluded that there were no significant increases in initial muscle water levels of GRd fish, which shows higher water content. After 112 days, muscle water levels of GRd fish declined significantly \((t=3.892 \, p < 0.001)\) to 76.89 ± 2.05 %.
**Figure 4.5** Effects of transfer from seawater to freshwater water on muscle water content (%) (top graph) and condition factor (K) (bottom graph) in normal growing and GRd fish. On the time axis, -1 (days) indicate levels in seawater fish obtained prior to transfer. Values at time 0 (days) are obtained from fish on arrival to the freshwater facility yet prior to freshwater entry. * indicate a significant difference (P<0.05 or better) from initial seawater values (-1 days) within groups using Bonferroni’s multiple-comparison test after two-way ANOVA. Values are means ± 1 S.E.M. (n=10 for all time points).
4.3.6 Condition Factor

Condition factors of larger samples \((n=100)\) prior to transfer and after 112 days are shown in Table 4.1. Pre-transfer, GRd fish had a significantly lower condition factor of \(0.89 \pm 0.029\) in comparison to \(1.34 \pm 0.031\) in normal growing fish \((t=8.726\ p<0.001)\). Changes in condition factors throughout sampling points \((n=10)\) are shown in Figure 4.5. Using a two way ANOVA, there was a significant effect of time \((F_{11, 216} = 7.304\ p<0.0001)\) group \((F_{1, 216} = 754.4\ p<0.0001)\), and their interaction \((F_{11, 216} = 8.062\ p<0.0001)\). Condition factor increased significantly in normal growers at 24 hr and 3 days \((t=2.618\ p<0.05,\ t=2.472\ p<0.05)\) before decreasing below pre-transfer levels at day 7. Transfer of GRd to freshwater resulted significant increases in condition after 56 and 112 days \((t=4.707\ p<0.001,\ t=7.847\ p<0.001)\) with an improved final condition factor of \(1.29 \pm 0.15\). There was no significant difference between groups condition factor at 112 days \((t=1.309\ p>0.05)\). A larger sample of 100 fish measured immediately after 112 days revealed similar results with a final condition factor of \(1.181 \pm 0.018\) and \(1.37 \pm 0.109\) in GRd and normal growers respectively.

4.3.7 Plasma Cortisol

Plasma cortisol levels at sea prior to transfer were \(39.82 \pm 28.81\ \text{nmolL}^{-1}\) and \(37.60 \pm 32.95\ \text{nmolL}^{-1}\) in GRd fish and normal growers respectively. As shown in Figure 4.6, transportation initiated significant increases in plasma cortisol levels \((t=3.457\ p<0.01)\) in GRd fish and small non-significant increase in normal growers. After freshwater transfer, normal growing fish showed dramatic and significant increases in plasma cortisol at 12 hr, 24 hr, 7 and 28 days post-transfer \((t=9.789\ p<0.01,\ t=8.520\ p<0.001,\ t=6.495\ p<0.001\) and \(t=3.992\ p<0.001\) respectively). Significant elevations in plasma cortisol of GRd fish were found at 12 hr, 24 hr and 7 days \((t=4.739\ p<0.001,\ t=4.965\ p<0.001,\ t=3.526\ p<0.01)\). At 28 days, plasma cortisol levels of GRd fish were not significantly different from pre-transfer levels \((t=1.999\ p>0.05)\). Further, multi-comparison analyses between groups throughout time revealed significant differences in cortisol levels in normal growers compared to GRd fish at 12 hr, 24 hr and 7 days post transfer \((t=5.031\ p<0.001,\ t=3.538\ p<0.01\) and \(t=2.951\ p<0.05\) respectively).
Figure 4.6 Effects of transfer from seawater to freshwater water on plasma cortisol levels (nmol/L) in normal growing and GRd fish. On the time axis, -1 (days) indicate levels in seawater fish obtained prior to transfer. Values at time 0 (days) are obtained from fish on arrival to the freshwater facility yet prior to freshwater entry. * indicate a significant difference (P<0.05 or better) from initial seawater values (-1 days) within groups using Bonferroni’s multiple-comparison test after two-way ANOVA. Values are means ± 1 S.E.M. (n=10 for all time points).

4.3.8 Mortality

No mortality occurred during loading or transportation to the freshwater facility. As Figure 4.7 shows, mortality started occurring in both groups after 24 hr in freshwater. Post-mortem dissections of initial mortalities (> 48 hr) revealed both GRd, yet more so normal growers to have gastric dilation air sacculitis (GDAS). The stomach cavity was severely dilated with a lack of smooth muscle tone, and mucosal infiltrate with some but not all fish showing minor amounts of fluid in the swim bladder. These symptoms were not as apparent in mortalities after 7 days. Maximal daily mortality rates occurred on day five with 2.89 % and 6.47 % in GRd and normal growers respectively.
Approximately 12 days post-transfer, many GRd fish displayed severe fungal infection around the gill integument and eyes that was later classified as one of the ubiquitous species of \textit{Saprolegnia}. In response to this, the entire raceway was immediately treated with Halimid (chlorine based substance) at 50ppm for 1 hr for three consecutive days. Treatments of fungal or bacterial infections were conducted in accordance with established husbandry practices for New Zealand King Salmon Co Ltd. This did cause a small mortality spike in physiologically vulnerable fish but it proved effective at eliminating infections. In spite of this, the final % mortality for GRd fish was substantially lower at 13.3% compared with normal growers at 22.7%.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.7.png}
\caption{Cumulative mortality from direct freshwater entry in normal growing and GRd fish. Values at time 0 (days) are following freshwater entry. Values are calculated as cumulative % of population.}
\end{figure}
Table. 4.1 Summary of inventory, morphological parameters, survival and growth rate in normal growers and GRd fish before and after 112 days in freshwater. Values are mean ± 1 SEM. Morphological parameters n = 100 fish.

<table>
<thead>
<tr>
<th></th>
<th>GRd</th>
<th>Normal Grower</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting Inventory</td>
<td>2175</td>
<td>663</td>
</tr>
<tr>
<td>Starting weight (g)</td>
<td>93.48 (± 2.6)</td>
<td>365.07 (± 12.30)</td>
</tr>
<tr>
<td>Final weight (g)</td>
<td>237.10 (± 9.1)</td>
<td>595.53 (± 13.7)</td>
</tr>
<tr>
<td>Starting Length (mm)</td>
<td>216 (± 1.9)</td>
<td>294.13 (± 3.08)</td>
</tr>
<tr>
<td>Final Length (mm)</td>
<td>266.48 (± 3.2)</td>
<td>349.47 (± 2.21)</td>
</tr>
<tr>
<td>Starting Condition Factor (K)</td>
<td>0.925 (± 0.015)</td>
<td>1.400 (± 0.015)</td>
</tr>
<tr>
<td>Final Condition Factor (K)</td>
<td>1.181 (± 0.018)</td>
<td>1.37 (± 0.109)</td>
</tr>
<tr>
<td>Specific Growth Rate (SGR) % days⁻¹</td>
<td>0.814</td>
<td>0.428</td>
</tr>
<tr>
<td>Thermal Growth Coefficient (TGC) % days⁻¹</td>
<td>1.689</td>
<td>1.296</td>
</tr>
<tr>
<td>Total Mortality (%)</td>
<td>13.3</td>
<td>22.7</td>
</tr>
</tbody>
</table>

4.3.9 Growth and Condition

Approximately 100 fish were measured prior to transfer and again after 112 days in freshwater to more accurately determine changes in body weights, lengths and growth rates (Table 4.1). GRd fish were smaller than normal growers on entry. Final mean body weight and length of GRd fish were significantly lower than normal growers when compared using a two-tailed Students t-test ($t_{198}$=21.73 $p<0.0001$ and $t_{198}$=21.68 $p<0.0001$ respectively), despite growth rates (TGC and SGR) for the 112 days of the experiment being appreciably higher. Although GRd fish had a lower final condition factor and size in comparison to normal growers ($t_{198}$ =8.854 $p<0.0001$), they did showed a considerable increase above that of normal growers from pre-transfer values.
4.3.10 Tissue Ion Concentrations

Table 4.2 illustrates the results of total Na\(^+\) and K\(^+\) concentrations in hepatic and anterior portions of kidney tissue. Total Na\(^+\) levels were significant elevated in both anterior portions of kidney and hepatic tissue. However, K\(^+\) was only found to be elevated in hepatic tissue. Even though this analysis was conducted using whole tissue segments, differences in ion concentrations were not reciprocal with changes in blood plasma and therefore most likely represents cellular concentrations.

<table>
<thead>
<tr>
<th>Table 4.2 Total Na(^+) and K(^+) content of liver and kidney tissue of GRd and normal growing fish in seawater and 112 days after transfer to freshwater.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Group</strong></td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td><strong>Seawater</strong></td>
</tr>
<tr>
<td>Normal Grower</td>
</tr>
<tr>
<td>GRd</td>
</tr>
<tr>
<td>Normal Grower</td>
</tr>
<tr>
<td>GRd</td>
</tr>
<tr>
<td><strong>Freshwater</strong></td>
</tr>
<tr>
<td>Normal Grower</td>
</tr>
<tr>
<td>GRd</td>
</tr>
<tr>
<td>Normal Grower</td>
</tr>
<tr>
<td>GRd</td>
</tr>
</tbody>
</table>

* and ** denote and significantly higher than normal growing fish \(P < 0.05\) and \(P < 0.01\) respectively by Students two-tailed t-test. All values are means ± 1 S.E.M. \((n=10)\), except normal grower GRd liver values \((n=8)\).
4.3.11 Pyloric Caeca and Posterior Intestine $\text{Na}^+/\text{K}^+$ ATPase Activity

Figure 4.8 shows the results of $\text{Na}^+/\text{K}^+$ ATPase activity of the pyloric caeca and posterior intestine of normal growing and GRd fish at sea. $\text{Na}^+/\text{K}^+$ ATPase activity of GRd fish pyloric caeca were significantly lower of the ($t_{14}=2.348 \ p=0.0341$) than normal growing fish. Conversely, $\text{Na}^+/\text{K}^+$ ATPase activity was much higher in the posterior intestine of GRd fish ($t_{14}=3.363 \ p=0.0046$) in contrast to normal growers.

![Graph](image)

**Figure 4.8** $\text{Na}^+/\text{K}^+$ ATPase activity of pyloric caeca (P.C) and posterior intestine (P.I) of normal growing (NG) and GRd fish. Enzyme activity is measured in $\mu$mol ADP mg-1 protein hr$^{-1}$. $^*$ denotes and significantly higher activity $P <0.05$ of the same tissue between GRd and normal growing fish using a Students two-tailed t-test. Values are means ± 1 S.E.M.
4.4 DISCUSSION

Whilst kept in seawater GRd Atlantic and Coho salmon fail to feed and typically die within a year of entry. Over a six month period in seawater GRd fish grew approximately 24% in contrast to a 387% increase in normal growing fish. Clarke *et al* (1977), Young *et al* (1989) and Björnsson *et al* (1988) have shown that GRd Atlantic and Coho salmon transferred back to freshwater survive, and often continue normal growth patterns. In this study, the transfer of GRd King salmon to freshwater also results in a restoration of appetite, growth and condition, moreover in terms of growth rate and survival on transfer, GRd fish outperform normal growing cohorts.

4.4.1 Feeding and Survival

In the present study, GRd fish started feeding shortly after transfer (48 hours) to freshwater, which is in marked contrast to that in seawater, where they do not feed at all on commercial pellets, instead passively consuming organic debris and flotsam. The initiation of feeding once in freshwater clearly shows that GRd fish are prevented from feeding in seawater as a result of the hypersaline environment.

After a week in freshwater, GRd fish fed voraciously whilst normal growers took over two weeks to start feeding actively. The dichotomy of feeding behaviours on entry to freshwater may be justified by the relative availability of stored energy and possibly, *via* an inhibitory effect of chronic cortisol levels (Gregory, *et al.*, 1999; Peterson, *et al.*, 2005). During acclimation to either seawater or freshwater, the gill is undergoing vast changes in protein biosynthesis, of which the preferred oxidative substrate for gill metabolism is glucose (Mommsen, 1984; Mommsen, *et al.*, 1999) and as a result, the nutritional state of the individual is critical. Liver, oxidative (red) and glycolytic (white) muscle tissues store appreciable levels of glycogen. Under normal situations, blood glucose levels are maintained *via* the degradation and formation of glycogen by glycogenesis in the liver (Dabrowski, *et al.*, 2002). During times of starvation and/or stress, where energy is critical, levels are maintained by a further process known as gluconeogenesis, in which glucose is formed from non-carbohydrate sources *via* peripheral proteolysis. Non-carbohydrate sources include gluconeogenic
amino acids, lipid, lactate, pyruvate and glycerol, some of which may be sourced at the liver but in the long term the majority is supplied from muscle tissue. Glucogenic amino acids from muscle tissue are generally last to be mobilised during starvation (Dabrowski, et al., 2002). The composition of GRd muscle and lipid tissue reflect this selective breakdown with lower levels of glycogen and lipid yet higher muscle protein levels (Timothy, et al., 2004; Woo, et al., 1978). Normal growing fish have an abundant supply of lipid and glycogen present in muscle tissue, which lets them to abstain from food for prolonged periods. GRd fish are able to feed as a result of transfer to freshwater; their highly voracious feeding behaviour in contrast to normal growers is likely to be a response to attain adequate energy to sustain metabolic processes during adaptation.

Transfer of normal growing and GRd sub-adult King salmon to freshwater resulted in mortality, which contrasts with a similar study (Talbot, et al., 1989) which was able to transfer sub-adult Atlantic salmon directly to freshwater without mortality. Rates of mortality were markedly different between the two groups, with GRd fish performing significantly better overall (13.3 %) in contrast to normal growers (22.7 %). This is robust evidence to show that GRd fish are able to readapt back to freshwater in a superior fashion compared with normal growing sub-adult King salmon.

Post-mortem analysis of initial mortalities (< 48 hr) revealed GDAS-like characteristics – the majority of which did in fact occur in normal growers. Examination revealed flaccidity of the stomach and large volumes of viscous mucus. GDAS in King salmon has recently been described in New Zealand seawater culture (Forgan, et al., 2007; Lumsden, et al., 2002). Low-cohesion feeds and osmoregulatory stress have been described as epidemiological co-factors in the development of GDAS in seawater adapted fish (Forgan, et al., 2007). It was also shown that neither factor (diet cohesion or osmoregulatory stress) alone was sufficient to trigger GDAS. In this study feed was withheld from both groups of fish for a week prior to and several days following transfer to freshwater, furthermore feed which was given had been routinely checked for cohesiveness using the NZKS pellet cohesion test. These findings conflict with that of Forgan et al (2007) and suggest that GDAS-like characteristics can occur by osmoregulatory stress alone. Such a rapid occurrence suggests active drinking did not cease immediately on transfer to freshwater, which was also indicated by the rapid rise
in condition factor. Initial consumption of freshwater coupled with an influx across the body surfaces would have led to many fish reaching an unregulatable internal water level that resulted in severe hydromineral imbalances and ultimately death.

The lower incidence of GDAS related deaths in GRd fish suggests that the GRd fish did not drink on entry to freshwater; this may also indicate that GRd fish may already have a lower drinking rate and absorption level at sea. Solute and water transport as determined by Na\(^+\) dependent proline influx (\(J_{\text{max}}\)) and half-saturation constants (\(K_t\)) were shown by Collie 1985 to be significantly lower in GRd fish and in fish that were transferred late in the smoltification window in comparison to normal growing seawater smolts. Not surprisingly, solute and water transport across the intestinal epithelium is coupled to the co-transport of Na\(^+\) by the basolaterally located Na\(^+\)/K\(^+\)-ATPase enzyme. Therefore, lower \(J_{\text{max}}\) and \(K_t\) values of GRd fish suggest that intestinal nutrient absorption and osmoregulatory ability are impaired. This is supported in the present work by enzyme analysis of pyloric caecal extracts that show that GRd fish had significantly lower gill Na\(^+\)/K\(^+\)-ATPase activity when compared to normal growers (see appendix 2). Moreover, lower gill Na\(^+\)/K\(^+\)-ATPase activity in GRd fish as Figure 4.1 shows suggest a lower capacity to excrete excess ions. This suggests that not only do GRd fish have a lower intestinal absorption capacity for nutrients and water uptake, but that they have a lower capacity to excrete ions at the gill. Drinking and feeding behaviours in GRd fish may therefore be restricted to a maintenance level to avoid a possibly fatal hyper-ionic state. This result concurs with findings by Collie (1985), supporting compromised intestinal uptake in GRd fish. The lower occurrence of GDAS-associated deaths and overall survival during transfer to freshwater of GRd fish may be related to a lowered drinking activity and intestinal absorption capacity. It is currently not known why GRd fish at sea showed higher Na\(^+\)/K\(^+\)-ATPase activity in the posterior intestine (Figure 4.8) despite a lower activity in pyloric caeca. Further work, particularly related to intestinal osmoregulatory ability in GRd fish is necessary to elucidate the significance of these findings.

Mortality of normal growers effectively ceased after day seven, yet mortality of GRd fish continued due to the contraction of a gill infection. Postmortem analysis concluded it to be one of ubiquitous fungal species within the genus *Saprolegnia*. Saprolegnian fungi can act as primary invaders in immunologically-compromised fish,
typically creating initial dermal mycoses of the gill integument and fin margins. The hyphae create extensive necrosis of focal tissues that eventually left untreated results in cellular infiltration, bacterial contamination, and death (Iwama, et al., 1996.). Fish in this study predominantly displayed dense hyphal permeation of the gills and necrosis of filaments, which severely reduced the surface area essential for gas and ion exchange.

With few normal growers infected, it strongly indicates that GRd fish were immunologically compromised at the point of transfer. Although no direct measurements of the immune system components were made, haematocrit levels taken during the study indicate a high degree of erythrocytic anaemia in GRd fish. The erythrocytic anaemia in GRd fish may be a result of the poor nutritional status (Halver, 1957) as it was shown that haematocrit levels normalised and were not significantly different to normal growing cohorts by the end of the study.

### 4.4.2 Plasma Cortisol

It is well known that in the face of a perceived threat to homeostasis salmonids can initiate a severe stress response, which primarily includes a dramatic increase in plasma cortisol. Following transportation, GRd King salmon showed a substantial increase in cortisol levels, higher, although not significantly above that of normal growers (Figure 4.6). After entry to freshwater, normal growers exhibited a sustained and significant rise in cortisol levels at 12 hr, 24 hr and 7 days, which did not appear as predominant in GRd fish (Figure 4.6). The partitioning of variation by two-way ANOVA reduced the ability to detect significant differences specifically between seawater and 12 hr values, higher sample sizes were required to do so. Nevertheless at 12 hr, normal growers peak cortisol levels were 33.8 times resting levels (peak level 1271.2 ± 116.8 nmolL⁻¹) and at 24 hr GRd fish were 16.7 times resting levels (peak level 665.6 ± 82.6 nmolL⁻¹). Peak cortisol levels were higher than many previous studies investigating the effects of acute stressors in salmonids (Iversen, et al., 2005; Pickering, et al., 1982; Waring, et al., 2004; Wilson, et al., 2002). However, the amplitude of the stress response will vary depending the severity of the stressor given,
developmental stage, species, metabolic scope (Mommsen, et al., 1999) and temperature (Perez-Casanova, et al., 2008).

Furthermore, differences in cortisol determinations between studies may exist. In this study, an ELISA was modified to use homologous plasma standards (Chapter 2) which generates far greater sensitivity and accuracy than many fish studies using human or other mammalian plasma standards.

The disparity between group mean cortisol levels is argued to result from the inability of GRd fish to produce and/or release cortisol and less likely to be related to a lowered state of stress. The kidney of teleosts is a highly important organ and quite different from that of the mammalian structure, because aside from its excretory function, it is a major site for haemopoietic, reticuloendothelial and endocrine systems. The main corticosteroid cortisol is secreted from adrenocortical cells located in the anterior portion of the tissue (referred to as the anterior kidney or head kidney) specifically populating the dorsal posterior cardinal veins and their branches.

Several reports have shown signs of dysfunction in the anterior kidney of GRd salmon (Kerstetter, et al., 1989; Marini, et al., 1982; Nishioka, et al., 1982; Young, et al., 1989). This has been demonstrated in-vitro by reduced responsiveness to ACTH, the principal secretagogue of cortisol release (Young, et al., 1989). Unpublished work, as cited in Young, et al (1989), reported that the transfer to freshwater results in the restoration of internal ACTH responsiveness, suggesting that the hyperosmotic environment inhibits or mediates inhibition of cortisol production and/or release. The present study has revealed high areas of necrosis, and ionic imbalance with Na\(^+\) levels significantly higher in anterior portions of kidney tissue and significantly higher levels of both Na\(^+\) and K\(^+\) in hepatic tissue of GRd fish in seawater (see Appendix). It was also shown that after 112 days in freshwater there were no differences in intracellular Na\(^+\) or K\(^+\) of hepatic and anterior kidney tissue between GRd and normal growing fish. GRd fish showed an 11.7% elevation in hepatic K\(^+\) levels relative to normal growers which is similar the 10% elevation described by Marini et al (1982) in GRd Coho salmon. Marini et al (1982) could not provide a clear explanation for differences in whole tissue K\(^+\) levels, initially hypothesising that disruptions may result from the steady flux of ion rich blood that the hepatic portal system receives from the intestine, which seems likely considering absorptive dysfunction (Collie, 1985). Kerstetter et al. (1989), as cited by
Young et al (1989) went on to suggest that $K^+$ loading may result from increased hepatic $Na^+/K^+$-ATPase activity. $K^+$ loading had been previously shown in euthyroid rat hepatic and diaphragm tissues treated with T$_3$ (Ismail-Beigi, et al., 1970). It was also shown T$_3$ treatment reduced intracellular $Na^+$ levels as would be expected during elevated $Na^+/K^+$-ATPase activity. This was confirmed in a subsequent paper (Ismail-Beigi, et al., 1971) showing that hepatic plasma membrane fractions showed a 69% increase in $Na^+/K^+$-ATPase activity after T$_3$ treatment. Comparison of $Na^+/K^+$-ATPase activity and intracellular ion levels of GRd hepatic tissue are required to clarify this. However if elevated $Na^+/K^+$-ATPase activity in hepatic tissues are responsible for $K^+$ loading, reductions in $Na^+$ should be noticeable which they are not. Furthermore, circulating T$_3$ which is shown to stimulate activity are reduced in GRd salmon (Folmar, et al., 1982; Nishioka, et al., 1982). Although further investigations are warranted, it is more than likely that the lowered cortisol response in GRd fish is a result of ionic dysfunction and necrosis in kidney tissues.

Besides differences between groups, the pattern of cortisol levels diverges from a typical pronounced and transient rise as shown in some transfer studies (Wilson, et al., 2002) by remaining elevated for several weeks – specifically in normal growers (Figure 4.6). Although cortisol analysis was not carried out past day 28, it is probable that the downward trend would have continued and would have normalised to within pre-transfer levels. Due to the multifunctional relationship of cortisol with several metabolic processes, it is difficult to accurately describe causally related changes, especially in the long term. In this study, the prolonged chronic elevations after transfer are unusual and may be explained both in terms of its mineralocorticoid and glucocorticoid functioning. Cortisol is considered to promote hyperglycaemia and elevations may be assisting gluconeogenesis to fuel enhanced metabolic rates required to make acclimatory changes (Mommsen, et al., 1999). This may be particularly so in normal growers, where feed intake was minimal during initial weeks. The prolonged elevations may also be involved in the continued stimulation of $Na^+/K^+$-ATPase (McCormick, 1995) and $H^+\text{-VATPase}$ (Lin, et al., 1993; Lin, et al., 1995). Lin et al (1993) showed that chronic cortisol infusion in the freshwater adapted rainbow trout (Oncorhynchus mykiss) resulted in a 30% increase in gill NEM sensitive $H^+\text{-VATPase}$ activity and increased $Na^+$ uptake. It is currently believed that the primary regulator of $H^+\text{-VATPase}$ activity is
external Na\(^+\) concentration and the activity of H\(^+\)-VATPase is primarily mediated through the increase in plasma cortisol potentated via an increase in prolactin (Agustsson, et al., 2003; McCormick, 2001). The elevated plasma cortisol levels in the long term cannot be accurately partitioned by mineralocorticoid or glucocorticoidal actions, it is more than likely the observed long term elevations are a response of both.

### 4.4.3 Osmoregulation

A well known secondary effect of stress in teleosts is the disruption to hydromineral balance, which in seawater results in a net gain of ions and a net loss of water, resulting in a concentration of body fluids. This is largely due to the effect of cortisol, which acts on the intestinal membrane to increase paracellular permeability (Sundell, et al., 2003) and intestinal fluid transport (Veillette, et al., 1993; Veillette, et al., 1995). In the present study, hydromineral imbalance was evidenced by a dramatic rise in plasma Na\(^+\) following transportation (Figure 4.4) concomitant with increases in cortisol levels. In freshwater, cortisol has an adaptive effect on gill membranes that has been shown in-vitro to decrease electrical characteristics (transepithelial potential, transepithelial resistance) and epithelial permeability as measured by the use of \[^{3}H\] polyethylene glycol-4000 (PEG-4000) (Wood, et al., 2002). These reductions during cortisol exposure seem to be a result of junctional tightening, which effectively slows ionic efflux. The effect of junctional tightening appears to be an important freshwater adaptive feature that may also aid in ion conservation during smoltification and during freshwater entry when Na\(^+/K\(^+\)-ATPase is elevated, slowing the possible leak of Na\(^+\) which would accumulate on the luminal side of the basolateral membrane. Whether increased cortisol has a significant in-vivo effect during salinity shifts remains to be seen – isolated whole-gill studies measuring loss of plasma Na\(^+\) during cortisol administration may provide an insight into the significance of junctional tightening.

In this study, the dramatic increase in plasma Na\(^+\) levels in both groups after transportation was transitory, as entry to freshwater resulted in a dramatic reduction in plasma Na\(^+\). Plasma Na\(^+\) levels persisted at low levels for over a week post-transfer and was concomitant with changes in haematocrit and muscle water content. Given the low
concentrations of plasma Na\(^+\) levels, the initial reductions in haematocrit levels (<2 hr) are likely due to a shift in plasma volume from an influx of water. The subsequent rise in haematocrit levels appears to be osmotically induced erythrocyte swelling. However, it is also possible that a stress related induction of catecholamine release increased erythrocyte numbers via splenic contraction (Pearson, et al., 1991; Pearson, et al., 1992; Yamamoto, et al., 1983). Furthermore, circulating catecholamines may directly induce swelling of erythrocytes by activation of \(\beta_1\) adrenergic receptors which are coupled to a Na\(^+\)/H\(^+\) antiporter (Reid, et al., 1991).

The high water content of muscle tissue as seen prior to transportation in GRd fish (Figure 4.5) is not considered to be due to a disruption of water balance, but an artefact of nutritional state as a distinct inverse relationship exists between percent of water and lipid in muscle (Shearer, 1994). Although occurring slightly slower to changes in blood constituents, significant increases in the water content of muscle tissue occurred at 3, 7 and 14 days in normal growers. This was not as evident in GRd fish, which is most likely a result of lower drinking rates on transfer. It may also be due to the fact that water content in GRd fish was already elevated over 80% prior to transfer, indicating that muscle tissue is unable to tolerate much more.

### 4.4.4 Gill Na\(^+\)/K\(^+\)-ATPase Activity and NEM-sensitive H\(^+\)-VATPase Activity

An important aspect of the present study was the analysis of the rapidity of changes in branchial Na\(^+\)/K\(^+\)-ATPase and NEM-sensitive H\(^+\)-VATPase activity during a forced acclimation directly to freshwater. Both GRd and normal growing cohorts transferred back to freshwater showed a similar pattern of change, with a significant depression in gill Na\(^+\)/K\(^+\)-ATPase activity after 2 weeks and a three-fold depression in activity levels after 4 weeks from values measured in seawater (Figure 4.1). A consistent observation of GRd fish was a lower level of gill Na\(^+\)/K\(^+\)-ATPase activity in comparison to normal growers; this appears to have facilitated a slightly faster adaptation by reducing initial Na\(^+\) loss (Figure 4.4). Concomitant with reductions in gill Na\(^+\)/K\(^+\)-ATPase activity was a significant increase in NEM-sensitive gill H\(^+\)-VATPase activity.
activity in both normal growers and GRd fish. Significant increases in NEM-sensitive gill H⁺-VATPase occurred after just 24 hr in normal growers and 3 days in GRd fish.

Whilst NEM-sensitive H⁺-VATPase did decline at 28 and 56 days, the proportion of its activity in gill homogenates remained elevated due to the concurrent depression in Na⁺/K⁺-ATPase activity. The initial decrease in gill Na⁺/K⁺-ATPase activity and increased NEM-sensitive H⁺-VATPase activity occurred simultaneously with the correction of plasma Na⁺ levels. Correlation analysis illustrated this further with a highly significant inverse linear relationship between Na⁺/K⁺-ATPase and NEM-sensitive H⁺-VATPase specific activities over the study period. This showed that Na⁺/K⁺-ATPase activity declines in close correlation to increasing NEM-sensitive H⁺-VATPase activity. The dynamic relationship of activity between these two enzymes and correction of plasma Na⁺ levels is obvious and provides strong evidence for the major significance in the branchial uptake of Na⁺ in freshwater.

The timing of changes in NEM-sensitive H⁺-VATPase activity contrasts with a related study (Wilson, et al., 2002) which investigated the short-term exposure of coho smolts to a gradual increase in salinity over 2 days (0‰–32‰). It was shown that whilst both a dramatic increase in Na⁺/K⁺-ATPase activity and decreased H⁺-VATPase abundance as detected by changes in immunoreactivity occurred, decreases in NEM-sensitive H⁺-VATPase were not significantly lower over 8 days. They concluded that NEM-sensitive H⁺-VATPase is less responsive than Na⁺/K⁺-ATPase activities during seawater transfer and that a general lack of change in NEM-sensitive H⁺-VATPase activity may reflect a slower down-regulation of mRNA levels in individual cells and functional activity of enzymes in the gill. In contradiction to that of Wilson, et al (2002), this study showed that that gill NEM-sensitive H⁺-VATPase activity was highly responsive on transfer to freshwater, with significant increases in activity by 24 hr. Lin, et al (1993) also showed rapid responsiveness of H⁺-VATPase within 6 hr of hypercapnia treatment of freshwater-adapted rainbow trout. Moreover, on freshwater transfer in this study, it was shown that Na⁺/K⁺-ATPase lagged after changes in H⁺-VATPase, taking two weeks to show significant depressions in activity. This is opposite to that observed by Wilson, et al (2002) during transfer to seawater, which suggests that these enzymes are differentially regulated depending on the salinity change. However, it
hypothesised that this dynamic regulation of enzyme activity during freshwater transfer occurs to optimise ionic homeostasis.

Kirschner, (2004) modeled data taken from the frog skin (Civan, et al., 1983) to suggest that Na\(^+\) uptake may be able to be carried out by Na\(^+\)/K\(^+\)-ATPase alone without the requirement of H\(^+\)-VATPase to create the required internal negative gradient for Na\(^+\) uptake. It is hypothesised that in this study the rapid upregulation of apical NEM-sensitive H\(^+\)-VATPase, coupled with a high Na\(^+\)/K\(^+\)-ATPase improves the negative internal gradient for apical Na\(^+\) uptake, more so than if Na\(^+\)/K\(^+\)-ATPase was immediately reduced on entry to freshwater. Elevated cortisol levels during this time may have an adaptive function to improve the efficiency of the system by tightening epithelial junctions (Wood, et al., 2002), slowing the loss of accumulated Na\(^+\) on luminal side of the basolateral membrane from high Na\(^+\)/K\(^+\)-ATPase activity.

Thus, the lag of changes in Na\(^+\)/K\(^+\)-ATPase in contrast to NEM-sensitive H\(^+\)-VATPase activity may not necessarily represent delay in mRNA down-regulation or lack of responsiveness; instead by remaining elevated, it may optimise Na\(^+\) uptake during freshwater transfer. The lack of significant depressions in NEM-sensitive H\(^+\)-VATPase activity as seen by Wilson, et al (2002) is unclear, yet again may not reflect a lack of responsiveness, instead they may represent basolateral H\(^+\)-VATPase activity responsible for the counteraction of high blood alkalosis (Tresguerres, et al., 2007) that is known to occur during transfer (Madsen, et al., 1996). The lack of significant depressions may also be due to the reduction in H\(^+\)-VATPase that has shown to occur during smoltification prior to seawater entry (Seidelin, et al., 2001). The transfer of fish outside of a smoltification period may result in much larger depressions.

Interestingly, both groups showed an increase in Na\(^+\)/K\(^+\)-ATPase activity at 112 days (Figure 4.1). The noted increase in Na\(^+\)/K\(^+\)-ATPase activity towards the end of the study is not considered a methodological error, as assays were always validated prior to testing. An increase in Na\(^+\)/K\(^+\)-ATPase activity, such as that seen during smoltification would result in a detectable reduction in plasma Na\(^+\). Because Na\(^+\) levels continued to rise during this time, an alternative explanation for elevations may be a result of the upregulation of the freshwater-specific Na\(^+\)/K\(^+\)-ATPase isoform (α1a). The Na\(^+\)/K\(^+\)-ATPase itself consists of two protein subunits which create a αβ heterodimer. The two subunits, which join in a 1:1 ratio, are a catalytic α subunit and a glycoprotein β subunit.
(Sweedner, 1989). It has been shown recently that several isoforms of Na⁺/K⁺-ATPase are differentially regulated during acclimation to seawater and freshwater (D’Cotta, et al., 2000; Richards, et al., 2003). Richards et al (2003) showed that during seawater acclimation, mRNA expression of Na⁺/K⁺-ATPase isoform-α1b is increased whilst isoform-α1a declines. During freshwater acclimation, a partial reversal occurs with increases in mRNA expression of a freshwater specific Na⁺/K⁺-ATPase isoform-α1a yet not isoform-α1b. However isoform-α1b was shown to reduce in maturing fish—presumably due to a higher osmotic disruption during spawning (Richards, et al., 2003; Shrimpton, et al., 2005). Although outside the scope of this thesis, the development of a rapid assay capable of determining isoform ratios may provide a more accurate measure of branchial enzyme dynamics during development and salinity shifts.

4.4.5 Summary

To summarise, the results of this study show that the process of GR in King salmon is reversible and comparable to that of Coho and Atlantic salmon. These results are congruent with several prior studies (Bjornsson, et al., 1988; Clarke, et al., 1977; Young, et al., 1989) and indicate that the hyperosmotic environment most likely through osmoregulatory dysfunction in the intestinal, hepatic and renal portal system obstructs seawater adaptation. Further detailed examination of key enzyme expression and isoform ratios in the gill and intestinal tissue of GRd fish are required to provide answers to the findings of ionic imbalances leading to endocrine dysfunction.
CHAPTER FIVE
General Discussion

5.1 INTRODUCTION

The development of elevated hypoosmoregulatory ability during smoltification pre-adapts anadromous salmonids like King salmon to life in the marine environment. It is currently believed that transfer of Coho and Atlantic salmon to seawater outside of a period of elevated hypoosmoregulatory ability results in GR in a minority of the population. GR in King salmon is often view differently, specifically by industry, to that already described in Coho, despite their close phylogenetic relationships and life history traits, one of the major reasons for which, is that smoltification and seawater adaptation of King salmon is poorly understood and has received little scientific attention.

The aim of this thesis was set out in Chapter 1, which firstly sought to describe the austral spring time smoltification period of King salmon in a commercial environment (Chapter 3). This was carried out using current physiological indicators of elevated hypoosmoregulatory ability in salmonids. This was further expanded to investigate the effects of elevated temperatures commonly used in hatcheries on the development of hypoosmoregulatory ability. Chapter 4 was undertaken in light of several previous reports (Bjornsson, et al., 1988; Clarke, et al., 1977; Young, et al., 1989) which have shown that the transfer of GRd Coho and Atlantic salmon back into freshwater results in a restoration of appetite, growth, condition and long term survival. It was unknown if this also occurs in King salmon.

The current chapter discusses the development of hypoosmoregulatory ability in King salmon. It also discusses the ability of GRd King Salmon to readapt to freshwater and proposes a model for the development of GR in salmonids in general. Implications for industry and how GR may be remedied or mitigated is also discussed.
5.1.1 Indicators for Smoltification and Seawater Adaptability

It was clearly shown in Chapter 3 that King salmon do undergo a detectable and relatively short period of elevated hypoosmoregulatory ability as determined by hypoosmoregulatory tests in seawater and enzymatic assays of gill Na\(^+/K^+\)-ATPase. However, these parameters were significantly affected by water temperatures above 12°C in contrast to a constant 12°C. Alterations in the body morphology were observed over the study period, with minor reduction or stabilisation of condition factor during the initial smoltification period. This was not reciprocal with changes in SGR or TGC, which was found to be highly variable. Interestingly, body silvering and the regaining of faint parr markings were congruent with changes in hypoosmoregulatory ability. It is cautioned however that the use of body silvering as a measure of development is crude, subjective, and in no way a measure of the level of hypoosmoregulatory ability.

Gill Na\(^+/K^+\)-ATPase activity was found to increase steadily during spring, peaking in late November with no appreciable or consistent reduction seen for December and January which agrees with several similar studies (Dougan, 1993; Franklin, 1989; Quinn, 1999). Consequently, there was a lack of correlation of changes in freshwater smolt plasma Na\(^+\) levels and hypoosmoregulatory ability of smolt challenged to seawater. Interestingly, it was discovered that despite increasing gill Na\(^+/K^+\)-ATPase activity the percent of ATP dependent activity in gill homogenates as determined by the use of a specific Na\(^+/K^+\)-ATPase inhibitor decreased markedly four weeks prior to the attainment peak gill Na\(^+/K^+\)-ATPase activity. The reduction in the percent of activity was a result of increased residual activity from an undetermined ATP-dependent enzyme (Figure 3.11). The marked increase of this enzyme correlated well with the normalisation of plasma Na\(^+\) levels and reduction in hypoosmoregulatory ability and was hypothesised to be result of the upregulation of H\(^+/\)VATPase activity. It is suggested that an upregulation of H\(^+/\)VATPase activity is an adaptive desmoltification mechanism that minimises and eventually reverses the reduction in hyperosmoregulatory ability (Figure 3.8, 3.9). This occurs prior to reductions in Na\(^+/K^+\)-ATPase which presumably augments the uptake of Na\(^+\) by enhancing the internal negative potential (-mV) for inward diffusion of Na\(^+\) through the epithelial Na\(^+\) conductive channel. This hypothesis was also supported by work in Chapter 4 that
showed gill $\text{H}^+\text{-VATPase}$ activity increasing prior to a reduction in gill $\text{Na}^+/\text{K}^+$-ATPase activity.

These results also emphasise the importance of using multiple predictors, as elevated gill $\text{Na}^+/\text{K}^+$-ATPase activity alone specifically in King salmon may not accurately determine periods of elevated hypoosmoregulatory ability. The dynamic regulation of gill $\text{Na}^+/\text{K}^+$-ATPase may provide the answer to why some studies have failed to find a correlation between high gill $\text{Na}^+/\text{K}^+$-ATPase activity prior to seawater entry and the percent of adult returns in the wild (Bilton, et al., 1982; Ewing, et al., 1982; Ewing, et al., 1980; Ewing, et al., 1985).

### 5.1.2 Temperature and Smoltification

A second objective of the thesis was to investigate possible interactions between physiological processes during the development of hypoosmoregulatory ability and growth under elevated temperature regimes of under-yearling production stock. The thermal regime that fish are exposed to is important and is known to affect the timing and duration of smoltification (Clarke, et al., 1981; McCormick, et al., 1987). Several studies have investigated the effect of elevated temperatures (Duston, et al., 1991; Handeland, et al., 2004; McCormick, et al., 1999) and very recently Shrimpton, et al (2007) reported very similar findings to those presented here.

There was very little difference in the number of degree days between groups and there were not major temperature changes. However, it was shown that the use of rearing temperatures fluctuating above $12^\circ\text{C}$ in contrast to a constant $12^\circ\text{C}$, enhanced growth, yet negatively affected hypoosmoregulatory processes. $\text{Na}^+/\text{K}^+$-ATPase activity was clearly reduced at higher temperatures (Figure 3.10). The number of degree days has been shown to be intimately linked to hypoosmoregulatory and behavioural changes during smoltification. Initiation and termination of downstream movements have been linked to number of degree days on several occasions in Atlantic salmon reared under different temperature regimes (Handeland, et al., 2004; Zydlewski, et al., 2005). This study suggests however, that although degree days experienced may determine the timing of development, elevated and diel fluctuations in temperature, in contrast to
elevated constant temperature can negatively affect hypoosmoregulatory processes despite similar degree day exposure. This study agrees with several recommendations (California Department of Water Resources, 1988; Wedemeyer, et al., 1980) that state 12°C exists as an upper limit that temperatures should be maintained below during smoltification in King salmon.

5.1.3 Seawater Performance

Logistical and time constraints meant that groups monitored in the freshwater phase could not be individually followed once transferred to seawater. Nevertheless, it was observed that significant temporal differences in the levels of GR exist. October transfers were made within a period of elevated hypoosmoregulatory ability as determined by hypoosmoregulatory tests and plasma Na\textsuperscript{+} levels. The long term performance of the stock was far superior to December transfers with a significant reduction in GR levels, increased growth rate and higher survival rate (see table 3.4). Fish transferred during October were significantly smaller than December. These results contrast with a general industry (NZKS, personal communication) and published view that larger smolts show greater seawater tolerance than smaller smolts (Arnesen, et al., 1992; Duston, et al., 1995; McCormick, et al., 1987). Although further monitoring using progressive transfers and long term performance measures are desirable, these results do clearly show that GR in King salmon is similar to that described in Coho and Atlantic salmon, resulting from an inability to adequate adapt to the hyperosmotic environment transferred outside a period of elevated hypoosmoregulatory ability.

5.1.4 Freshwater Transfer of Growth Retarded and Normal Growers

The transfer of GRd King salmon to freshwater initiated a voracious feeding response, increased condition, compensatory growth and long term survival, which is comparable to that observed in Coho and Atlantic salmon (Bjornsson, et al., 1988; Clarke, et al., 1977; Young, et al., 1989). Moreover, the results presented in Chapter 4
show that GRd fish are able to readapt back to freshwater with higher survival and growth rates compared to the transfer of normal growing sub-adult King salmon. This suggests that GRd King salmon are more adapted to freshwater, providing further evidence to show that GR is a result of the inability to adapt to the marine environment.

Changes in the key osmoregulatory enzymes (gill Na\(^+/K^+\)-ATPase activity and NEM-sensitive H\(^+\)-VATPase), stress hormones (plasma cortisol) and plasma and muscle constituents (plasma Na\(^+\), haematocrit and muscle water content) were monitored during readaptation to freshwater. Plasma Na\(^+\) levels of GRd fish in seawater were within normal ranges despite finding gill and pyloric caeca Na\(^+/K^+\)-ATPase activity being significantly reduced. However, it is hypothesised that GRd fish are able to achieve osmotic homeostasis by a restriction of feeding and drinking activity to a maintenance level. The lower levels of GDAS-like symptoms found in GRd fish immediately after transfer may indirectly reflect a lowered drinking behaviour. Further investigations are needed to elucidate if in fact the drinking levels GRd fish are lower in comparison to normal growers.

There were no significant differences in rapidity of changes in gill Na\(^+/K^+\)-ATPase and H\(^+\)-VATPase activity between the two groups. However their regulation is far more dynamic than first expected. Wilson, et al., (2002) described the changes in activity of both enzymes in response to seawater transfer and described a lag, or lack of responsiveness to changes in H\(^+\)-VATPase mRNA to functional activity. Although this study was not backed by molecular data, the transfer to freshwater resulted in the complete opposite, with a rapid activation of H\(^+\)-VATPase activity and lag in depression of Na\(^+/K^+\)-ATPase activity. It is suggested that Na\(^+/K^+\)-ATPase remains elevated to enhance the electrochemical gradient for Na\(^+\) uptake when coupled with H\(^+\)-VATPase. Elevated cortisol levels may be formative in the efficiency of the system by tightening epithelial junctions (Wood, et al., 2002), slowing the leak which would otherwise occur from accumulated Na\(^+\) on the luminal side of the basolateral membrane.
5.1.5 **The Proposed Somatogenic Mechanism of Growth Retardation in Salmonids**

GR is considered a degenerative state that is a result of terminal perturbations in the growth hormone and insulin like growth factor-1 (GH/IGF-I) axis as a result of initial osmoregulatory dysfunction in physiologically unprepared fish transferred to seawater. A model of for the initiation and maintenance of GR is described here.

The GH/IGF-I axis is an important endocrine pathway of growth and metabolism in vertebrates. In the process of GR it is suggested that a disruption in this pathway induces a complex resistance to GH that is mediated by starvation coupled with, but, initiated and controlled by osmoregulatory dysfunction which amplifies the GR state to inhibit seawater adaptation. The transfer of GRd fish to freshwater results in the release of this inhibition by allowing GRd fish to consume feed or drink without the risk of critical ionic loading that would otherwise occur in seawater.

It has been established that GRd Coho and Atlantic salmon both display high GH secretory activity and circulating levels with low levels of hepatic IGF-I mRNA and plasma insulin (Björnsson, *et al.*, 1988; Bolton, 1987; Duan, *et al.*, 1995; Varnavsky, *et al.*, 1995; Young, *et al.*, 1989). This is significant because GH is a primary secretagogue of IGF-I (Mommsen, 2001) which suggests that that GRd fish are GH-resistant.

It is well known that starvation increases GH levels (Kakisawa, *et al.*, 1995; Sumpter, *et al.*, 1991; Varnavsky, *et al.*, 1995). Starvation is also known to result in a decrease in circulating IGF-I levels (Moriyama, *et al.*, 1994) which may itself result in an initial increase in GH due to decreased negative-feedback inhibition by IGF-I (Blaise, *et al.*, 1995). The increase in GH is also known to have stimulatory effects on lipid mobilisation and protein accretion (Björnsson, 1997). Fatty acid and glycerol release from fasted rainbow trout liver slices have been shown to increase under heterologous (ovine) and homologous (salmon) GH stimulation *in-vitro* (O'Connor, *et al.*, 1993) presumably *via* stimulation of triacylglycerol lipase activity (Björnsson, 1997). GH is also known to have a prominent stimulatory effect on skeletal growth which, coupled with catabolic effects, often results in a decrease in condition factor (Björnsson, 1997) which has even been seen during starvation (Johnsson, *et al.*, 1994).

In juveniles transferred to seawater outside a period of elevated hypoosmoregulatory ability, the exposure to seawater results dramatic hydromineral
disruption. It is likely that fish exhibiting low hypoosmoregulatory ability lack the initial capacity to efficiently take up seawater and extrude excess ions from the gill and kidney. This study which showed that GRd fish have a lower Na\(^+\)/K\(^+\)-ATPase activity in both the pyloric caeca and gill in comparison to normal growers. It appears that even though GRd fish have a lower gill Na\(^+\)/K\(^+\)-ATPase activity compared to normal growers, they are able to maintain plasma Na\(^+\) within normal levels, which has been also shown by other studies (Varnavsky, et al., 1992). It is hypothesised that drinking and feeding may be initially restricted and minimally maintained during seawater residence as a result of an inability to handle the resultant flux of seawater.

Despite plasma ion levels being within normal ranges, hepatic and anterior kidney tissue of GRd fish display ionic dysfunction (see Table 4.2) and necrosis (see Appendix). Fryer et al (1987) revealed that GRd Coho salmon have a decreased ability to bind GH, illustrated by a deficit of \(^{125}\)I-labelled GH-binding sites in hepatic and gill tissue. The decreased ability to bind GH was suggested by Young, et al (1989a) to be a result of ionic loading. GRd hepatocyte K\(^+\) levels in this study were elevated as also described by Marini, et al (1982), however, Na\(^+\) levels were also found to be elevated in hepatic and anterior kidney tissue. It is unclear to what extent ionic loading may reduce hepatic GH receptor numbers. The fact that transfer of GRd fish to freshwater results in a reduction of elevated GH levels (Young, et al., 1989) coupled with the normalisation of intracellular ion levels in hepatic and kidney tissue suggest that ionic dysfunction is still involved in inhibition at sea.

Somatostatins (SRIFs) specifically SRIF-14 has been shown to inhibit the release of GH (Very, et al., 2007a; 2007b; Very, et al., 2001). In-vivo it has been shown that SRIF-14 implantation in rainbow trout results in reduced food intake and GR relative to controls (Very, et al., 2001). Interestingly SRIF variants SRIF-25 and SRIF-14 have been shown to be elevated in conjunction with high GH levels in GRd Coho salmon (Sheridan, et al., 1998). The lack of inhibitory effect of SRIF-14 on pituitary GH release suggests a pituitary GH resistance (Sheridan, et al., 1998). SRIF-14 is now known to act in an extra-pituitary manner to reduce target organ sensitivity to GH by inhibiting steady-state levels of GH receptor mRNA in isolated hepatocytes (Very, et al., 2007a). It is suggested that SRIFs regulate hepatic GH sensitivity by increasing GH receptor internalisation and expression (Very, et al., 2007a; 2007b). However, at the
level of the pituitary, it seems that SRIFs work primarily to inhibit basal GH secretion (Lin, *et al.*, 1999) as pituitary GH mRNA levels are unaffected (Lin, *et al.*, 2001).

Outside of the pituitary, SRIF-14 appears to affect target organs reducing the sensitivity to IGF-I by reducing the expression of IGF-I receptor mRNA, including \([^{125}\text{I}]\)-IGF-I binding *in-vitro* (Very, *et al.*, 2007b). A reduction in IGF-I may have dire consequences not only for growth but for osmoregulation, as IGF-I has been shown *in-vitro* to stimulate \(\text{Na}^+/\text{K}^+\)-ATPase activity (McCormick, 1996) and possibly mediate the seawater adaptation mechanism of GH (Sakamoto, *et al.*, 1993). GH through IGF-I is also known to stimulate \[^{35}\text{S}\]\ incorporation into gill cartilage; however again, this action has been shown to be inhibited by SRIF-14 and SRIF-25-II treatment *in-vivo* and *in-vitro* (Very, *et al.*, 2001). Increased SRIF levels may also act to reduce nutrient uptake by inhibiting the release of gastrointestinal peptides which include: secretin, ghrelin, cholecystokinin, vasoactive intestinal polypeptide, gastric inhibitory peptide, motilin, enteroglucagon, neurotensin, gastric acid, peptide intrinsic factor, bile and colonic fluids (Barnett, 2003; Nelson, *et al.*, 2005).

The changes in the GH/IGF-I axis are complex and similar to that observed in starved fish – however GR is mediated by transfer to seawater. It is suggested therefore that the degenerative GR state is initiated firstly by ionic dysfunction, the inability to feed effectively without a terminal flux ions may result in fish becoming starved augmenting the increase of GH and initiating the increase in SRIF-14 production. SRIF-14 acts to down-regulate GH-receptor mRNA creating a GH-resistance, this is potentiated further by increased SRIF-14 which down-regulates IGF-I-receptor mRNA biosynthesis and IGF-I binding. SRIF-14 may also act to reduce intestinal absorption and potentially hypoosmoregulatory adaptation.
5.1.6 Future Work

There are several research questions that need to be answered regarding the initial development and maintenance of GR in salmonids. Individual farming companies are unlikely to be able to carry out such research. However companies and joint industry bodies such as the New Zealand Salmon Farmers Association Incorporated (NZSFA) should consider collaborative efforts with university researchers and/or commissioning independent studies allowing for publication. The most relevant research considered appropriate to the physiological understanding of GR and seawater adaptation will be highlighted here.

A major impediment to the study of GR development in seawater is the inability to determine which fish is GR fated. The mapping of physiological and endocrinological reversal of the GR state during transfer to freshwater, as was performed in this study, may be highly informative to the sequence of events leading to GR, specifically surrounding the disruption of the GH/IGF-I axis. It would also be highly informative to characterise temporal changes of their degeneration from the onset of GR to their death in seawater. Immediate isolation and progressive sampling of SRIF-14, SRIF-25, GH, and IGF-I in moribund GRd fish would be helpful to gain further insight into the disruption of the GH/IGF-I axis. This could also include mapping time course changes of osmoregulatory and gastrointestinal physiology in GRd fish in seawater and changes in energy stores. The lower pyloric caeca epithelial Na\(^{+}\)/K\(^{+}\)-ATPase activity as reported in this study may be expanded and correlated with nutrient and fluid transport in \textit{in-vitro} sac preparations (Veillette, \textit{et al.}, 1993; Veillette, \textit{et al.}, 2005).

Indisputably GR arises in Coho, Atlantic and King salmon transferred outside of a period of elevated hypoosmoregulatory ability and therefore equal research efforts should also focus on physiological development in freshwater and during salinity changes to optimise seawater adaptability. With regards to smoltification and the adequate development of hypoosmoregulatory ability, one of the most important environmental factors is water temperature. The influence of absolute water temperatures and thermal regimes on smoltification is understudied. It was shown in this study that even small changes in thermal regimes can have dramatic effects on
hypoosmoregulatory development. It is critical that further, more comparative research be conducted to develop freshwater species-specific thermal standards, which can be used for both commercial aquaculture and the management of wild stocks. Furthermore, increasing evidence suggests that smoltification in hatchery fish is less pronounced than that of wild stock; studies need to reflect this and avoid the use of overly broad conclusions relating to smoltification. Smoltification indicators need to be further refined using higher resolution proteomic studies, namely Na\(^+/K^+\)-ATPase, and H\(^+\)-VATPase enzymes to improve determination of directional states of hypo- or hyperosmoregulation. The development of rapid assays capable of determining Na\(^+/K^+\)-ATPase isoforms during smoltification may also be highly informative.

This thesis provided a new perspective into the regulation of key osmoregulatory enzymes during salinity shifts and smoltification in freshwater. The determination of the enzyme responsible for the increased residual ATPase-dependent activity during smoltification (Chapter 3) is required. With regards to the dynamic regulation of Na\(^+/K^+\)-ATPase and H\(^+\)-VATPase during salinity shifts, it may be highly informative to characterise changes in Na\(^+/K^+\)-ATPase and H\(^+\)-VATPase mRNA using quantitative PCR techniques coupled with enzyme kinetics to investigate changes in regulation during salinity shifts.

### 5.1.8 Commercial Implications: Prevention and Mitigation

In an attempt to clarify the preceding evidence for commercial producers affected by GR, several potential areas for focus exist. Whilst the mechanisms of the GR state are still to be fully characterised the occurrence of the phenomenon in Coho, Atlantic and King salmon is evidently dependent on adequate development of hypoosmoregulatory ability and timing of transfer. It is believed therefore that GR can be reduced to economically acceptable levels by focusing on hypoosmoregulatory development and seawater transfer. However, achieving this is highly dependent on the level of implementation.

There are various processes that can be readily implemented with little capital investment for operators looking improve current husbandry practices in freshwater for
smolt determination and seawater transfer. The return in investments will be reflective in improved initial seawater growth, health and reduced GR. However, changes to husbandry practices should be made ethically to improve the welfare of stock and should not be based solely on financial gains.

Without a change in husbandry processes costs may be recovered from the resultant GRd sea-cage stock. As shown in Chapter 4, GRd fish can be graded, isolated and transferred to freshwater for further grow-out. Cost recovery may also include the selling of GRd fish for stocking select public and private freshwater water bodies for recreational angling. A cost-benefit analysis is required to determine if freshwater GR grow-out would be a viable alternative for commercial producers.

For companies wishing to implement practices to prevent, or dramatically lessen the impact of GR, the main areas which deserve concentration are the use of 1) proper freshwater thermal regimes 1) hypoosmoregulatory indicators and 2) salinity gradients. The latter, which has not been covered by this thesis, refers to the use of gradual salinity changes over several days and weeks to minimise osmotic disturbances and avoid the loss of hypoosmoregulatory capacity during extended freshwater residency. The production cycle of salmonids tends to conform to the natural lifecycle, yet many commercial operators opt to transfer directly into seawater pens/cages. For smolt, even during a period of elevated hypoosmoregulatory ability abrupt transfer results in osmoregulatory stress as seen by initial dehydration of tissues and loading of ions. It is recommended that gradual salinity changes are used prior to transfer to seawater pens/cages to help alleviate osmotic disturbances. For optimal transfer a gradual increase of external salinity should occur over approximately 7-10 days. The determination of duration is based on molecular and enzymatic activity data that show that key osmoregulatory enzymes become functionally active after this period of exposure. Part of this was detailed in Chapter 4, which showed that on transfer to freshwater, normalisation of the osmoregulatory imbalance occurred after 7 days. This is congruent with studies in Coho showing gill Na\(^+/K\(^+\)-ATPase activity responsiveness between 4-8 days (Wilson, et al., 2002). Salinity gradients would lessen osmoregulatory imbalances following transfer further improving initial seawater growth. This practise is not currently employed by the New Zealand salmon farming industry.
In terms of hypoosmoregulatory predictors it was recommended in Chapter 3 that several be used for determining and predicting the period for elevated hypoosmoregulatory ability. For on-site monitoring there are several physiological measures that could be conducted. Enzymatic assays such as that used for determining gill Na\(^+\)/K\(^+\)-ATPase activities are complex and relatively expensive for smaller operators. The results of this study also draw caution to the use of gill Na\(^+\)/K\(^+\)-ATPase activity alone as a predictor for determining hypoosmoregulatory ability. It was revealed that elevations of gill Na\(^+\)/K\(^+\)-ATPase were not always representative of elevated hypoosmoregulatory ability as determined by hypoosmoregulatory seawater tests. Nevertheless, its use is invaluable in determining directional states of hypo- or hyperosmoregulation when coupled with hypoosmoregulation tests and plasma ion profiles.

Hypoosmoregulatory testing in seawater is a relatively inexpensive and straightforward task that can be carried out by trained technicians. The acclimatory pattern to seawater can be influenced by many factors. It is recommended that hypoosmoregulatory testing be standardised between weeks to minimise intra-assay variation. Due to their being high survival during testing at 35‰ for 24 hr, it is recommended to they be carried out using elevated salinities >40‰ to further improve the detection level. The use of this technique appears relatively accurate alone; however detection of hypoosmoregulatory ability would be further enhanced by measurement of plasma ion levels in freshwater controls. On-farm analysis by trained technicians can be carried out immediately with the use of a chloride titrator or flame photometer.
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Altiora Peto
APPENDIX

Histopathology of Hepatic and Kidney Tissue of Growth Retarded and Normal Growing King Salmon

Hepatic and kidney sections of GRd and normal growing King salmon in seacages were processed routinely; hematoxylin and eosin (H&E) stained (Leica Autostainer XL, Wetzlar, Germany) and analysed by light microscopy for necrosis, apoptosis, and other deformities. The processing of tissue samples was carried out by New Zealand Veterinary Pathology Ltd IVABS Building, 1st Floor Tennent Drive, Massey University, Palmerston North. The microscopy analysis of slides was carried out by Alistair Brown, Aquatic Veterinary Services, 69 Harrison St East Brunswick, 3057, Victoria, Australia. Figures A.1 and A.2 show sections taken from normal growing salmon in seawater exhibiting a healthy glomerulus and hepatic tissues. Figures A.3 to A.15 are tissue sections taken from GRd salmon in seawater. In contrast to the well-vascularised glomerulus seen in normal growers, the tubules of GRd fish are atrophic. GRd fish also show focal necrosis and increased apoptotic cells in both hepatic and kidney tissue.

![Figure A.1](image)

**Figure A.1** Photomicrograph of H&E transverse stained section of a relatively normal glomerulus of normally growing King salmon. Magnification is 400x.
Figure A.2 Photomicrograph of H&E transverse stained section of healthy hepatic tissue of normally growing King salmon. Magnification is 400x.

Figure A.3 Photomicrograph of H&E transverse stained section of hepatic tissue of a GRd King salmon showing hepatocyte necrosis. Magnification is 100x.
Figure A.4 Photomicrograph of H&E transverse stained section of hepatic tissue of a GRd King salmon showing hepatocyte necrosis. Magnification is 400x.

Figure A.5 Photomicrograph of H&E transverse stained kidney section of a GRd King salmon showing tubular necrosis. Magnification is 100x.
Figure A.6 Photomicrograph of H&E transverse stained kidney tissue section of a GRd King salmon showing atrophied glomeruli. Magnification is 400x.

Figure A.7 Photomicrograph of H&E transverse stained kidney tissue section of a GRd King salmon showing glomerular atrophy. Magnification is 400x.
**Figure A.8** Photomicrograph of H&E transverse stained kidney tissue section of a GRd King salmon showing tubular necrosis. Magnification is 400x.

**Figure A.9** Photomicrograph of H&E transverse stained kidney tissue section of a GRd King salmon showing tubular necrosis. Magnification is 400x.
Figure A.10 Photomicrograph of H&E transverse stained hepatic tissue section of a GRd King salmon exhibiting an apoptotic hepatocyte. Magnification is 400x.

Figure A.11 Photomicrograph of H&E transverse stained hepatic tissue section of a GRd King salmon exhibiting an apoptotic hepatocyte. Magnification is 1000x.
**Figure A.12** Photomicrograph of H&E transverse stained hepatic tissue section of a GRd King salmon showing mononuclear inflammatory cells infiltrating around a duct. Magnification is 400x.

**Figure A.13** Photomicrograph of H&E transverse stained hepatic tissue section of a GRd King salmon showing mononuclear inflammatory cells infiltrating around a duct. Magnification is 1000x.
Figure A.14 Photomicrograph of H&E transverse stained kidney tissue section of atrophied kidney glomerular of a GRd King salmon. Magnification is 400x.

Figure A.15 Photomicrograph of H&E transverse stained kidney tissue section of a GRd King salmon showing atrophied glomerulus and necrotic tubules. Magnification is 400x.