DOES THE MINERALOCORTICOID RECEPTOR HAVE A FUNCTIONAL ROLE IN THE TELEOST GILL?

by

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ABSTRACT

Cortisol has been shown to have a glucocorticoid as well as a mineralocorticoid role in teleosts. The dual role of cortisol, however, was thought to be through a single class of receptor, the glucocorticoid receptor (GR) in teleosts until the recent discovery of the gene for a mineralocorticoid receptor (MR) in this group of fishes. My research objective was to determine if MR has a functional role in ionoregulation and if the changes in MR expression were related to biochemical responses when fish move among waters that vary in salinity. I addressed this research question in two ways. In my first experiment, I transferred rainbow trout from fresh- to ion-poor or salt water. An increase in gill Na^+, K^+-ATPase (NKA) activity in salt water was associated with higher NKA α1b subunit mRNA. In contrast, there was little change in gill NKA activity following the transfer to ion-poor water, but mRNA for the α1a subunit was significantly elevated. Gill GR and MR mRNA showed little change when fish were transferred to either treatment, but prolactin receptor (PrlR) and growth hormone 1 receptor (GH1R) mRNA showed significant changes. In my second experiment, I examined adult sockeye salmon migrating from the ocean to spawning grounds. Gill NKA activity declined consistently during migration, accompanied by significant increases in the gill NKA α1a isoform. Generally, mRNA for gill MR and GR declined during migration to fresh water, but gill PrlR and GH1R increased – particularly after the fish were in fresh water. These findings suggest that cortisol may have a more limited role in ionoregulation than previously described.

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PROLOGUE

The mechanisms that fish use to maintain ion concentrations in either fresh- or seawater differ considerably between the two environments, but many species of fish are capable of exploiting both fresh- and seawater. The ability to maintain constant plasma ion levels despite large differences in the external salt concentration is essential for these euryhaline species of fish to survive (McCormick, 2001). A large number of species migrate between fresh- and saltwater habitats at specific times in the year. For example, anadromous fish undergo seasonal preparatory changes that allow them to migrate and adapt to a new environment before they encounter it. Prior to entering seawater, anadromous salmonids undergo a distinct springtime transformation known as smolting. Smolting is a coordinated process involving several physiological, biochemical and behavioural changes over a very limited time period (McCormick and Saunders, 1987; Hoar, 1988; Clarke *et al.*, 1995). Additionally, recent work has shown that adult salmon migrating in the ocean towards fresh water undergo physiological changes prior to entering the river (Shrimpton *et al.*, 2005).

This prominent life history characteristic shows a wide range of variability within and among species of salmonids and is intimately linked with migratory behaviour (McCormick, 1994). For example, some species such as pink salmon (*Oncorhynchus gorbuscha*) and chum salmon (*O. keta*) are able to immediately migrate into seawater after emerging from the gravel as fry, while other species like brown trout (*Salmo trutta*), Artic char (*Salvelinus alpinus*) and brook trout (*S. fontinalis*) require three or more years in fresh water before they migrate and are able to tolerate the increased salinity levels (Hoar, 1988; McCormick, 1994.). Other salmonids such as Atlantic salmon (*Salmo salar*) are able to acclimate to seawater after 96 hours compared to rainbow trout (*O. mykiss*), which require 4-5 days (Bystriansky *et al.*,

2006). The "degree of anadromy" also varies among and within a single salmonid species and depends on several specific characteristics of the anadromous life cycle (McCormick, 1994). These characteristics include geographical length or distance of migration, time or duration spent at sea, the state of maturity at sea, spawning habits, mortality after spawning (i.e. semelparity versus iteroparity) and the occurrence of strictly freshwater forms (McCormick, 1994). The interaction between animal and the environment is also important and changes in latitude can affect the degree of anadromy.

During their early life history, salmon live in a hypo-osmotic freshwater environment and continuously lose ions to the environment and gain water through passive diffusion (Moyle and Cech, 2004). In contrast, salmon in a hyper-osmotic marine environment constantly gain ions and lose water. Fish are able to cope with these ionic challenges through physiological functions in the gill, intestine, urinary bladder and kidney (Hoar, 1988). For freshwater acclimated fish, these adjustments comprise of the gill actively taking up ions across the gill epithelium, a reduction in ingestion and intestinal absorption of water and the production of very dilute urine in the urinary bladder to expel the excess water gained from diffusion (Hoar, 1988; Bern and Madsen, 1992). In the marine environment, seawater is ingested to replace water that has diffused into the environment and ions are absorbed by the intestine. Excess ions are actively excreted across the gill through a process of selective secretion and paracellular conductance by a leaky gill epithelium (Hoar, 1988; Moyle and Cech, 2004).

Although numerous organs are involved in ionoregulation, the gill plays a prominent role in both ion uptake and ion excretion (Evans *et al.*, 2005). The gill epithelium is composed of four different cell types: pavement cells, mucus cells, neuro-epithelial cells and

chloride cells (Perry, 1997). Chloride cells are highly specialized cells that secrete excess sodium and chloride ions in seawater adapted fish and actively take up ions in freshwater adapted fish (Hoar, 1988). Affiliated with the chloride cell is the enzyme Na^+, K^+-ATPase (NKA). NKA is essential for ion uptake and ion secretion, as it maintains Na^+ and K^+ gradients across the basolateral membrane (Bystriansky *et al.*, 2006). These gradients indirectly energize the active movement of ions into or out of the fish (Evans *et al.*, 2005). Therefore, the action of gill NKA is important for ion homeostasis, regardless of the environment fish inhabit. NKA is composed of two essential subunits (α and β) and several different α isoforms (α1a, α1b, α1c, α3) have been identified to change in rainbow trout transferred among waters of different salinity (Richards *et al.,* 2003). The expression pattern and distribution of these α isoforms switches depending on the environment and is thought to play different roles in acclimation. The mRNA expression of NKA α1b increases when fish are acclimating to seawater in contrast to fish transferring from seawater to fresh water, which causes an increase of NKA α1a mRNA (Richards *et al.,* 2003).

The physiological responses required for fish to acclimate to different environments are maintained through the synthesis and release of hormones (McCormick, 2001). These hormones are carried in the blood to other tissues, where they act as messengers to regulate the function of the target tissue or organ (Nelson *et al.,* 2000). One of the major hormones shown to be important for ionoregulation is cortisol, a major corticosteroid in teleosts. Cortisol appears to have a glucocorticoid function, affecting metabolism and growth, but also a mineralocorticoid function, which regulates the movement of ions and water (McCormick *et al.,* 2008). Complicating things further, cortisol has been shown to be important for ion uptake and ion excretion and is considered by some to be a dual regulator of chloride cell

differentiation and function (Kiilerich *et al.,* 2007a). During smolting and acclimation to seawater, cortisol has been shown to stimulate proliferation and differentiation of ion-transporting chloride cells (Madsen, 1990a) and increase NKA enzyme activity in the gill to improve salinity tolerance (McCormick and Bern, 1989; Kiilerich *et al.,* 2007a). Several studies focusing on increasing cortisol levels in response to salinity levels and cortisol treatments provide evidence that cortisol has a considerable physiological impact on ion uptake (McCormick, 2001) and has been shown to synergistically interact with growth hormone (GH) to stimulate chloride cell proliferation (Madsen, 1990b). Conversely, it has also been suggested that cortisol primarily facilitates freshwater acclimation by increasing the number of chloride cells on the lamellae to promote an influx of gill Na^+ and Cl^- ions (McCormick, 2001; Laurent *et al.,* 1990). The role of cortisol in freshwater acclimation appears to be in conjunction with prolactin (Prl) to maintain ion-osmotic homeostasis (Kiilerich *et al.,* 2007b).

The biological effect of cortisol, however, will not only depend on circulating concentration of the hormone, but number and affinity of the intracellular receptors for this hormone in the gills (Shrimpton and McCormick, 1999; Marsigliante *et al.*, 2000). High-affinity corticosteroid receptors (CR) are present in the gills and the responsiveness of cells has been shown to be dependent on the receptor concentration (Shrimpton and McCormick, 1999). CRs have been detected and characterized in several salmonids species including coho salmon (Maule and Schreck, 1990), rainbow trout (Pottinger, 1991), brook trout (Chakraborti *et al.*, 1987), and Atlantic salmon (Shrimpton and McCormick, 1999). Until recently, only a single class of receptor for cortisol was known in fish and the protein composition and affinity of the receptor for cortisol suggested it belonged to the family of glucocorticoid

receptors (GR). Two GR have been identified and Bury *et al.* (2003) provide evidence that the two GR have distinct functions in teleost fish. With the discovery of a mineralocorticoid receptor (MR) in teleosts (Sturm *et al.*, 2005), however, the possibility for cortisol to function in multiple roles could be related to action through different receptors. The two different classes of hormone receptors for cortisol that play a prominent role in ionoregulation are the GR, subtypes GR1 and GR2, and the MR. There is an increase in GR protein during smolting when saltwater tolerance develops and the receptor increase occurs prior to or simultaneously with an increase in plasma cortisol levels (Shrimpton and McCormick, 1998a; Shrimpton *et al.*, 2000). More recently, Nilsen *et al.* (2008) found that GR mRNA levels were elevated at the time of peak smoltification in anadromous Atlantic salmon. In contrast, MR is suggested to have a role in freshwater osmoregulation and is coupled with hyper-osmoregulation (Kiilerich *et al.,* 2007b).

The mechanisms of action for corticosteroid signalling initially involves the secretion of cortisol from the interrenal cells and the binding of the hormone to a specific cytosolic receptor, either GR or MR. The hormone receptor complex is then translocated into the nucleus where it binds directly to specific DNA regions of the promoter, the hormone response element (HRE), initiating changes in gene expression that are translated into a cellular response (Shrimpton and Randall, 1994). Sathiyaa and Vijayan (2003) hypothesized that CR signalling is auto regulated by a negative feedback loop in rainbow trout hepatocytes. These authors found that cortisol significantly elevated GR mRNA content over a 24 hour period; however, cortisol also significantly decreased GR protein content, suggesting that post-transcriptional modifications may affect mRNA stability. Consequently, lower GR protein content may be regulating GR mRNA abundance (Sathiyaa and Vijayan,

2003). An experimental approach to assess a functional role for cortisol, therefore, is to examine changes in expression of receptors for this hormone, either a GR or an MR, in a tissue such as the gill that performs a specific physiological function.

The objective of my research was to determine if MR plays a role when fish move between waters of different salinities and if the response was related to known biochemical changes important for ionoregulation. I addressed this research question in two ways. In my first experiment, molecular changes were assessed when rainbow trout were moved from fresh water to either an ion-poor treatment or a 75% saltwater experimental treatment. This experiment was designed to assess if the response by the fish to a decrease or an increase in environmental salinity was by cortisol through a glucocorticoid or mineralocorticoid class of receptor. In my second experiment, I analyzed gill tissue from adult sockeye salmon naturally migrating in the ocean and upriver to the spawning grounds in fresh water. This experiment allowed me to assess whether preparatory physiological changes for freshwater entry were linked to cortisol and a mineralocorticoid class of receptor. Endocrine signals associated with the physiological changes in the gill were assessed with specific mRNA probes for each class of receptor using quantitative real-time polymerase chain reaction (qRT-PCR).

CHAPTER 1

Hormonal signals regulating the physiological response of rainbow trout, *Oncorhynchus mykiss*, transferred from fresh water to ion-poor or salt water: evidence from expression of growth hormone, prolactin, glucocorticoid and mineralocorticoid receptors in the gill*

ABSTRACT

To understand the physiological and molecular endocrine changes that occur in response to a salinity challenge, we transferred rainbow trout from fresh water to an ion-poor or 75% saltwater treatment group for 14 days. An increase in gill Na^+, K^+-ATPase (NKA) activity in salt water was associated with higher mRNA expression for the NKA α1b subunit. In contrast, there was little change in gill NKA activity following transfer to ion-poor water, but the mRNA expression of NKA α1a was significantly elevated. Endocrine signals were assessed by quantifying changes in mRNA extracted from the gill for glucocorticoid receptors 1 and 2 (GR1 and GR2), mineralocorticoid receptor (MR), growth hormone 1 receptor (GH1R), and prolactin receptor (PrlR). Both GR and MR mRNA in the gill showed little change when fish were transferred to either treatment group; however, mRNA for PrlR and GH1R in the gill showed significant changes. PrlR mRNA was significantly higher when fish were transferred to the ion-poor water and GH1R mRNA was elevated during the 75% saltwater challenge. Theses findings suggest that cortisol may have a more limited role in freshwater ionoregulation and that prolactin and growth hormone may be more important for

* Throughout this chapter I use the first person plural to acknowledge the contribution of others to this work, which will be submitted for publication with the authorship of A.-M. Flores and J.M. Shrimpton

promoting physiological change in the gills when fish are subjected to an ionoregulatory challenge.

INTRODUCTION

The ability to maintain plasma ion concentrations within a narrow tolerance range is essential for fish to tolerate salinity changes in different surroundings (McCormick, 2001). Ionoregulation and salinity tolerance are especially important for euryhaline fish such as salmonids that are able to survive in saline environments, but can also survive in environments that approach the osmolarity of distilled water (Perry and Laurent, 1989). Independent of the external salinity, however, fish maintain nearly constant internal ion concentrations (Evans *et al.*, 2005).

The gill is the primary organ used for ionoregulation and maintenance of homeostasis in both freshwater and seawater acclimated fish (Evans *et al.*, 2005). For freshwater acclimated fish, the gill actively transports Na^+ and Cl^- from the dilute fresh water into the blood to match the passive loss of these ions (Marshall, 2002). In contrast, seawater acclimated fish must counteract the passive gain of ions and loss of water by drinking seawater and secreting the excess Na^+ and Cl^- ions across the gills. The movement of fish between seawater and freshwater environments requires considerable remodeling of the gill for the animal to maintain homeostasis. In anadromous species, physiological changes enabling the fish to tolerate a change in salinity are preparatory and seasonal. Prior to seaward migration, anadromous salmonids undergo a distinct springtime transformation to tolerate the increase in salinity known as smolting (McCormick and Saunders, 1987; Hoar, 1988). Smolting involves numerous behavioral, morphological and physiological changes

(Clarke *et al.*, 1995). Physiological changes in adult salmon migrating in the ocean towards fresh water have also been show to occur before entering the river (Shrimpton *et al.*, 2005; Chapter 2). The principle enzyme in ion-absorption and ion-secretion is Na^+, K^+-ATPase (NKA) (McCormick, 2001; Marshall, 2002). NKA is composed of two essential subunits (α and β) and two isoforms of the α-subunit (α1a and α1b) have been identified to change in rainbow trout transferred among waters of different salinity (Richards *et al.*, 2003). The abundance and localization of α1a and α1b change depending on the external salinity (McCormick *et al.*, 2009). In fresh water, NKA α1a is the abundant isoform (Richards *et al.*, 2003) present in lamellar chloride cells in the gills (McCormick *et al.*, 2009), whereas NKA α1b becomes the dominant isoform after seawater acclimation (Richards *et al.*, 2003) and is found primarily in filamental chloride cells (McCormick *et al.*, 2009).

The physiological responses required for fish to adapt and survive in extreme environments are maintained by the neuroendocrine system through the synthesis and release of hormones (McCormick, 2001). Hormones are carried in the blood to their target tissues, where they act as messengers to regulate the function of the tissue or organ (Nelson *et al.*, 2000). The sensitivity of the target cells to a specific hormone depends on the stability of the hormone receptor complex, the amount of receptors specific for the hormone available and the affinity of the hormone to its receptor (Shrimpton and McCormick, 1999). Numerous hormones including the thyroid hormones, cortisol, growth hormone, and insulin-like growth factor 1 have been shown to seasonally increase during smolting and have been suggested to play a role in preparing the animals to tolerate the increased salinity of seawater (McCormick and Saunder, 1987; Hoar, 1988). Similarly, a complex suite of hormones, such as cortisol, prolactin, testosterone, estradiol, have been observed to increase when fish move from

seawater to fresh water or are transferred from fresh water to ion-poor water (Sower and Schreck, 1982; Sakamoto *et al.*, 1991; Perry and Laurent 1989). Evidence also suggests that cortisol interacts with other hormones, including growth hormone (GH) and prolactin (Prl), to promote a physiological response (Madsen, 1990b; Shrimpton *et al.*, 1995; Shrimpton and McCormick, 1998b). Our current understanding is that cortisol and GH are important for promoting seawater acclimation, while an increase in cortisol and Prl promotes acclimation to fresh water, but how cortisol can stimulate physiological mechanisms involved in both ion uptake and ion excretion is not clear. It was long thought, however, that the function of cortisol was through a single class of receptor; the glucocorticoid receptor (GR) (Ducouret *et al.*, 1995). Recently a second class of receptor for cortisol, the mineralocorticoid receptor, has been identified (Colombe *et al.*, 2000; Greenwood *et al.*, 2003). Potentially, through these two classes of steroid receptors, cortisol may function in both fresh- and seawater ionoregulation in concert with Prl or GH.

To investigate what hormones play a role in stimulating physiological modifications following a change in salinity, rainbow trout were transferred from fresh water to ion-poor water (1/3 fresh water and 2/3 distilled water) or 75% (24 ‰) salt water for up to 14 days. Changes in gill NKA activity, plasma [cortisol], plasma [chloride], and mRNA in the gill for NKA α1a and α1b were measured to assess the response of the fish to the challenge. To elucidate what endocrine signals may be associated with the physiological changes in the gill, mRNA from the gill was measured using quantitative real-time polymerase chain reaction (qRT-PCR) for changes in expression of hormone receptors. We measured mRNA for the glucocorticoid receptor 1 and 2 (GR1 and GR2), the mineralocorticoid receptor (MR), the

growth hormone 1 receptor (GH1R) and the prolactin receptor (PrlR) to assess the relative importance of each hormone for ionoregulation in the gill.

MATERIALS AND METHODS

Animals

Two strains of rainbow trout, *Oncorhynchus mykiss*, Pennask (P) and Blackwater (BW) from the Fraser Valley Hatchery (FVH, Abbotsford, British Columbia, Canada) were used to examine their response to transfer in ion-poor or 75% seawater. Pennask rainbow trout originated in the Thompson Region of British Columbia (Pennask Lake, BC) and are a relatively small, slow growing fish that are highly resilient against diseases (Freshwater Fisheries of BC, 2004). This particular strain of trout is indigenous to monoculture lakes and shows poor growth in lakes with other non-salmonid species. Furthermore, Pennask has a lower survival rate in waters with extreme environments, i.e. alkaline lakes. Blackwater rainbow trout originated from British Columbia's Cariboo Region (Blackwater River, BC) and are fast-growing fish that are very aggressive and competitive in lakes stocked with other fish species. Based on its riverine origin, this strain of rainbow trout prefers shallow shoal areas in lakes that imitate river conditions. Unlike Pennask, however, this strain has been shown to be highly susceptible to diseases (Freshwater Fisheries of BC, 2004). Use of these two strains made it possible to assess if there was an effect of size on ionoregulatory response to the salinity challenges.

The Blackwater strain were spawned from FVH brood stock on June 1, 2007 and the Pennask strain were spawned from FVH brood stock on June 21, 2007. Fish were reared at the FVH in separate tanks supplied with flowing, aerated well water at approximately 10 ^{o}C

under a natural photoperiod regime. To identify the two different strains for this study, all Pennask fish were adipose fin clipped. Trout were fed to satiation daily with commercial trout feed, but fasted throughout the experiment. All experimental procedures were approved by the University of Northern British Columbia Animal Care and Use Committee.

Experimental Set-up

Fish were initially held in 60 x 100 cm oval fiberglass tanks (volume 135L) supplied with FVH well water. To determine the physiological response of fish to changes in salinity, rainbow trout were transferred to one of three treatments; flow through freshwater control, recirculating salt water (24‰), or re-circulating low ion strength (ion-poor) water. The control tank utilized a flow through system that maintained a mean temperature of 10.6 ± 0.002 °C. The concentration of sodium was 0.35 ± 0.01 mmol • L^{-1} and calcium was 0.87 ± 0.01 mmol • L^{-1} in the well water.

Experimental salt water was prepared by mixing 25 g of *Instant Ocean Mix* (Aquarium Systems Inc, Mentor, OH) per liter of FVH well water and allowed to dissolve in a recirculating temperature controlled system for 24 hours. A salinity of approximately 24‰ was targeted, since lower salt concentrations may not cause a measurable effect on biochemical changes and higher salinity levels can cause fish mortality. Water was pumped from the rearing tank through a filter and into a 200 L reservoir equipped with a refrigeration unit and aeration. Water was gravity fed back into the rearing tank. Salinity was monitored daily using a handheld refractometer to ensure that salinity levels did not change appreciably over the duration of the experiment and water samples were collected to measure ion

concentrations. The concentration of sodium was 300.1 ± 2.6 mmol • L^{-1} and calcium was 6.93 ± 0.07 mmol • L^{-1} for the salt water. Mean temperature was 11.3 ± 0.02 °C.

Ion-poor water was made by combining 4 parts distilled water to one part FVH water. The recirculation system was the same as described above. Water samples were also collected throughout the experiment; the concentration of sodium was 0.14 ± 0.01 mmol • L^{-1} and calcium was 0.27 ± 0.01 mmol • L^{-1} in the ion-poor treatment. Mean temperature was 10.5 ± 0.02 °C.

On July 15, 2008, 30 fish of each strain were transferred to the control, ion-poor or saltwater tanks; 60 fish in total per tank. Fish were sampled on days 1, 2, 3, 6 and 14 after transfer. At each time interval, 12 fish (6 from each strain) were randomly sampled per treatment.

Tissue Sampling

For sampling, fish were captured with a dip net from the experimental tank and rapidly transferred to a bucket containing 200 mg • L^{-1} MS-222 (tricaine methanesulfonate) buffered with 400 mg • L^{-1} sodium bicarbonate. This concentration of anesthetic has been shown to limit the increase in cortisol due to the stress of handling (Sumpter *et al.* 1990). Once fish were anesthetized, fork length to the nearest mm and weight to the nearest 0.1 g of each fish was recorded. Blood samples were taken by severing the caudal peduncle and collected in heparinized capillary tubes. Plasma was separated from the red blood cells through centrifugation and quickly frozen for later analysis of plasma cortisol and chloride concentrations. A gill biopsy (six to eight primary gill filaments) was taken to determine gill NKA activity and placed in 100 µL of cold SEI buffer (150 mmol • L^{-1} sucrose, 10 mmol • L^{-}

[1] Na_2EDTA, 50mmol • L^{-1} imidazole, pH 7.3) and was then frozen at -20 °C for up to 7 days before transferring to -80 °C. The second and third gill arches were then removed and placed in *RNAlater* for 24 hrs at 4 °C before transfer to -20 °C for up to 6 days before transfer to -80 °C. All tissue samples were collected within 5 minutes following first disturbance of the fish.

Plasma Analysis

Plasma chloride concentration was determined using a Labconco digital chloridometer. All assays were done in duplicate if sufficient plasma was available. Plasma chloride was measured in mmol • L^{-1}. Plasma cortisol concentrations were quantified with an Enzyme Immunoassay (EIA), using methods outlined by Carey and McCormick (1998). Microtitre plates (Costar high binding polystyrene EIA plates, Corning, NY) were coated with rabbit anticortisol antibody and left to incubate for 3 hours at 37 °C. The plates were then washed with a solution of 0.15 M NaCl and 0.05% Tween 20. A blocking solution of EIA buffer 0.1 M phosphate, 0.15 M NaCl, pH 7.0, with 0.1% BSA was added and then aspirated after blocking for 15 minutes at room temperature. Another 150 μL of EIA buffer was then added, in addition to 2.5 μL of standard or sample and 100 μL of cortisol-horseradish peroxidase conjugate. Plates were incubated overnight at 25 °C, washed and 200 μL of 3,3',5,5' - tetramethylbenzidine containing 0.01% hydrogen peroxide TMB was added to each well. Plates were read on a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA) for approximately 5 minutes to assess color development. The plate reader was then stopped and 50 μL of 0.5 M HCl was added to each well to stop color development and an endpoint reading was taken at 450 nm. Sensitivity, defined by the dose- response curve, was measured from 1 ng • mL^{-1} to 200 ng • mL^{-1}.

Gill Na^+, K^+-ATPase activity

Gill NKA activity was measured according to the microassay protocol of McCormick (1993). In brief, gill filaments were homogenized in SEI buffer containing 0.1 % sodium deoxycholate (SEID). After centrifugation (5000 g for 30 seconds), the supernatant was used to determine the activity by relating ATP hydrolysis to the oxidation of NADH, measured at 340 nm for 10 minutes at 25°C in the presence and absence of 0.5 mmol • L^{-1} ouabain on a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Protein content was then measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Specific activities were expressed as μmol ADP • mg^{-1} of protein • h^{-1}.

Purification of total RNA from Gill Extractions and cDNA synthesis

Gill tissue (approximately 20-50 mg) was extracted for total RNA using *RNeasy Mini Kit* (Qiagen, Mississauga, ON). Samples were homogenized in a Geno/Grinder 2000 (BT&C, Inc., Burlington, ON). Isolated RNA was dissolved in 30 μL RNase-free deionized water. The concentration and purity of each RNA sample was determined on a 1% agarose gel and also by measuring optical absorbance at 260 and 280 nM on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Purified RNA was converted to cDNA using Qiagen's *QuantiTect Reverse Transcription Kit* (Protocol: Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR).

Primers and Real-Time PCR

Messenger RNA (mRNA) copies for two gill NKA α-subunits (α1a and α1b) were measured using quantitative real-time PCR (qRT-PCR). The primers used to amplify the

NKA α1b-subunit were designed for *O. mykiss* by Richards *et al.* (2003). We used the primers designed by Madsen *et al.* (2008) for the NKA α1a-subunit of rainbow trout because they found it to be more specific, as it was designed for α1a near the 3' terminus of the complete mRNA sequence to achieve maximum isoform specificity. The mRNA copies were normalized to β-actin and expressed relative to the gill samples from the control fish. Primers designed for rainbow trout β-actin were used (Sathiyaa and Vijayan, 2003).

To assess hormonal responses to the salinity manipulations, mRNA expression of receptors for cortisol, growth hormone and prolactin were measured. Receptor mRNA examined for cortisol were GR1, GR2and MR. Primer sequences were from Sathiyaa and Vijayan (2003) for GR1 and from McCormick *et al.* (2008) for GR2 and MR. For the GH1R and PrlR, primers were designed with the NCBI Primer Designing Tool (http://www.ncbi.nlm.nih.gov/tools/ primer-blast/) for rainbow trout sequences (GenBank Accession Nos.: NM_001124535 and AF229197, respectively) to amplify an approximately 80 bp product. Sequences for GH1R forward primer were 5' CGA GCT GGA CAT GGA GGA GC 3', for GH1R reverse primer were 5' GGG GAC AGT CTG AGG AGG CA 3', for PrlR forward primer 5' CAT GGG AAC CGC CCC GTA AG 3', and for PrlR reverse primer 5' CGC CTC CTC CAG TTT GAC CC 3'.

To verify that the primers designed for GH1R and PrlR amplified the gene of interest, rainbow trout cDNA was amplified by polymerase chain reaction (PCR) in a PTC-100 thermocycler (MJ Research, Watertown, MA) using *Taq* DNA polymerase (Invitrogen, Burlington, ON) and cDNA isolated from the above gill tissues. One microliter of the reverse transcribed reaction was used as template for PCR amplification and a 40-cycle PCR was performed with each cycle consisting of 30 seconds at 95°C (denaturation), 1 minute at 58°C

(annealing) and 5 seconds at 72°C (extension). In the last cycle, the extension time was increased to 5 minutes. PCR products were electrophoresed on 2% agarose gel containing ethidium bromide to verify a single product and also tested for quality by measuring optical absorbance at 260 and 280 nM on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). A single band was resolved and amplicon size was approximately 80 bp. PCR products for both hormone receptors were then cleaned using the QIA quick PCR purification kit (Qiagen, Mississauga, ON) and sequenced in both directions on an Applied Biosystems 3130XL DNA Analyzer (Carlsbad, CA) at the University of British Columbia – Okanagan (Kelowna, BC, Canada). Sequence assembly and analysis were performed using Sequencer (Gene Codes Corporation, Ann Arbor, MI) and all sequences were compared with published sequences in GenBank. The GH1R sequence showed 100% sequence similarity to *O. mykiss* GH1R (GenBank Accession No.NM001124535.1) For PrlR, the sequence showed 100% similarity to *O. mykiss* PrlR (GenBank Accession No. NM001124599.1).

All qRT-PCR reactions contained 1 μL of cDNA template, 4 pmoles of each isoform specific primer and Universal SYBR green master mix (Applied Biosystems Inc., Carlsbad, CA). All qRT-PCR reactions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute using an Applied Biosystems Inc. 7300 Real-Time PCR System (Carlsbad, CA). Melt curve analysis was performed after each reaction to confirm the presence of only a single product of the reaction. RNA controls were also performed for a selection of samples using RNA samples that were not reverse transcribed to test for the possible presence of genomic contamination. Genomic DNA contamination was present in all samples, but it was never was more than 1:4920 starting copies for NKA α1a-isoform, 1:1150 starting copies for NKA α1b-isoform, 1:7820 starting

copies for β-actin, 1:43500 for GR1, 1: 1590 starting copies for GR2, 1: 6390 starting copies for MR, 1:3410 copies for GH1R and 1:1250 starting copies for PrlR. Genomic DNA, therefore, was found to be negligible.

Randomly selected samples were serially diluted to develop a standard curve relating threshold cycle to cDNA amount for each primer set. The slopes were linear and similar for all genes, suggesting that the amplification efficiency in the qRT-PCR reactions did not differ between genes. Therefore, the relative expression of the target genes could be normalized to a reference gene by utilizing the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Gene expression levels measured in qRT-PCR assays were normalized to the mRNA level of the reference gene, β-actin. Normalization with a reference gene corrects for variation in reverse transcription (RT) efficiency and it is expected that variation in RT efficiency will affect target and reference genes equally. The mRNA amounts were expressed relative to the gill samples collected from control fish sampled on the same day. All samples were run in duplicate and the coefficient of variation between duplicate samples never exceeded 1%.

Statistical Analysis

A three-way analysis of variance (ANOVA) was used to determine differences between treatments over time and the effect of strain; interaction effects were examined between time and group. If significant differences were found, a Bonferroni test was conducted to determine differences between treatment groups, time and strain. Statistical significance was taken at a level of $P < 0.05$. All values are expressed as means ± 1 standard error (se).

RESULTS

The sizes of fish sampled on each day ($p > 0.1$) and for each treatment ($p > 0.1$) did not differ significantly. The two strains of rainbow trout, Pennask (P) and Blackwater (BW), however, differed significantly in length ($p < 0.001$) and weight ($p < 0.001$). The mean length and weight for the Blackwater and Pennask strains were 15.9 ± 0.3 cm and 43.6 ± 2.0 g, and 10.8 ± 0.2 cm and 12.4 ± 0.8 g, respectively. However, with the exception of the gill NKA isoforms, strain did not statistically affect any of the other variables.

Gill NKA activity differed significantly due to day ($p < 0.001$), treatment ($p < 0.001$), and there was a significant interaction effect between day and treatment ($p < 0.001$), but fish strain was not significant ($p = 0.147$). NKA activity showed little change in the control group or the ion-poor group (Figure 1.1a). There was also little change in the saltwater group until day 14, when enzyme activity in the saltwater fish was significantly greater than either the control or ion-poor fish. Plasma chloride also showed a significant effect of day ($p < 0.005$) and treatment ($p < 0.001$), but there was no interaction effect ($p = 0.39$) and no effect of fish strain ($p = 0.30$). For the saltwater group, plasma chloride concentration increased and remained elevated until day 6, when it was significantly greater than the control and ion-poor group, and then declined by day 14 (Figure 1.1b). Plasma chloride concentration was lower in the ion-poor group than the controls on day 1, but the difference was not significant (Figure 1.1b). From day 2 to the end of the study, there was little difference between the ion-poor group and controls for plasma chloride. Plasma cortisol differed significantly due to day ($p < 0.001$), treatment ($p < 0.001$), and there was a significant interaction effect between day and treatment ($p < 0.005$), but fish strain was not significant ($p = 0.85$). Cortisol concentrations were high one day after transfer to the experimental tanks, but the magnitude

differed among the treatment groups as cortisol levels in the ion-poor groups were significantly greater than the control fish (Figure 1.1c). By day 2, plasma cortisol levels in the ion-poor group decreased significantly from day 1 and did not differ from the control fish. In contrast, the saltwater group had elevated plasma cortisol levels throughout the experiment, but the values did not differ significantly from control values for the same day.

Gill NKA α1a differed significantly due to day ($p < 0.001$), treatment ($p < 0.001$) and strain of fish ($p < 0.05$); however, there was no significant interaction effect between day and treatment ($p = 0.08$). A post hoc test on the effect of treatment indicated that the ion-poor treatment had significantly higher gill NKA α1a mRNA than controls and the saltwater treatment had significantly lower gill NKA α1a mRNA than controls. All possible day by treatment comparisons, however, revealed that only on day 14 did the saltwater group differ from the control (Figure 1.2a). The effect of strain was further examined with a 2-way ANOVA using treatment group and strain as the independent variables for each day and indicated that BW and P fish did not differ for control, ion-poor or saltwater groups on any given day ($p > 0.05$).

There were significant differences for gill NKA α1b due to day ($p < 0.005$), treatment ($p < 0.001$), and strain of fish ($p < 0.01$), as well as interaction effects between day and treatment ($p < 0.005$). A post hoc test on the effect of treatment indicated that gill NKA α1b mRNA was significantly higher in salt water than the control fish and the ion-poor treatment did not differ from the control. The mRNA expression of gill NKA α1b showed a statistically significant increase in fish transferred to salt water on days 3, 6 and 14 ($p < 0.05$) (Figure 1.2b). When analyzing the effect of strain, a significant difference was present in the saltwater treatment group ($p < 0.05$) for day 2, where BW fish had higher mRNA compared

to P fish (Figure 1.2b). No effect of strain was seen for either the control ($p > 0.05$) or ion-poor group ($p > 0.05$).

Based on the results from RT-PCR, there were no statistically significant differences due to treatment, day or strain observed for the corticosteroid receptors: GR1 and GR2 (Figures 1.3a and 1.3b) or MR (Figure 1.3c). When compared to the control group, an increase in relative mRNA was seen on the first and second day for both experimental treatments. This was followed by a decline in mRNA expression, until the levels were lower than the controls on day 6 and day 14.

For GH1R, copies of mRNA showed significant changes due to the experimental treatment ($p < 0.05$) and day ($p < 0.001$); however, there were no interaction effects and the response of the two strains of rainbow trout did not differ significantly ($p = 0.10$). A similar mRNA expression pattern was observed for both the ion-poor and salt water treatments: mRNA expression was high on day 1, which was followed by a decrease in mRNA expression for the remainder of the experiment (Figure 1.4a). GH1R levels continually declined until mRNA expression was lower than the controls, although it was not significantly different. A comparison among the groups indicated that the two experimental groups did not differ significantly from the controls, but that the saltwater treatment differed significantly from the ion-poor group ($p < 0.05$). For PrlR, there were significant differences due to day ($p < 0.001$), treatment ($p < 0.05$) and an interaction effect was seen between day and group ($p < 0.05$). There was no significant effect due to the different strains of rainbow trout ($p = 0.85$). Compared to the control group, fish in the ion-poor water treatment had a significant increase in PrlR mRNA on Day 3, followed by a drop in expression (Figure 1.4b). Throughout the experiment, fish transferred to salt water showed a decrease in PrlR mRNA

expression, although it did not differ significantly from the controls, but the saltwater and ion-poor treatment groups differed significantly ($p < 0.05$).

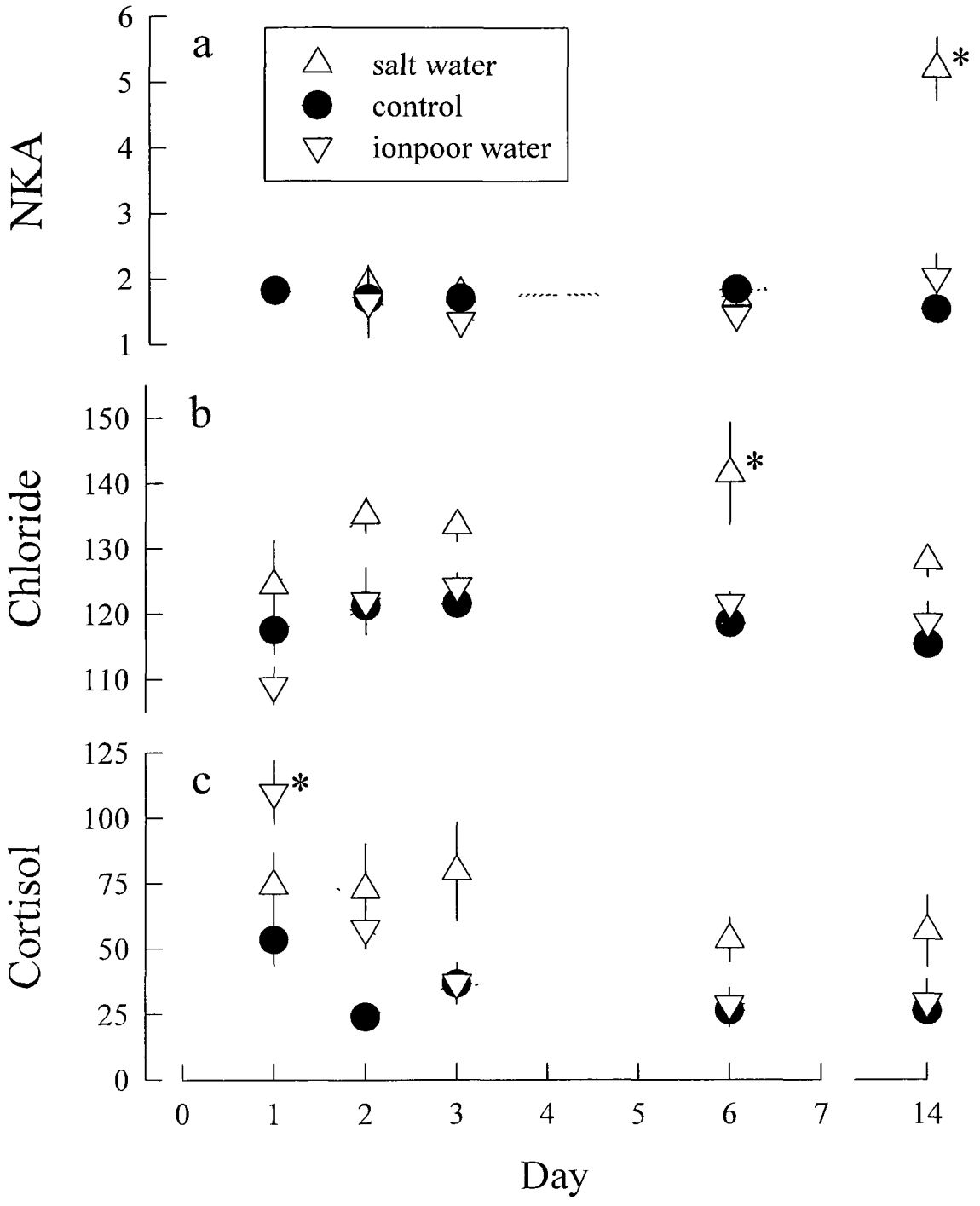

Figure 1 1: (a) Gill Na^+,K^+-ATPase (NKA) activity (μmol • ADP mg^{-1} protein • h^{-1}), (b) plasma [chloride] (mmol • L^{-1}) and (c) plasma [cortisol] (ng • mL^{-1}) for rainbow trout transferred to 3 different experimental treatment groups: control, ion-poor and salt water. Values are means ± se. Asterisks denote significant difference ($p < 0.05$) between treatment group and control for the same day.

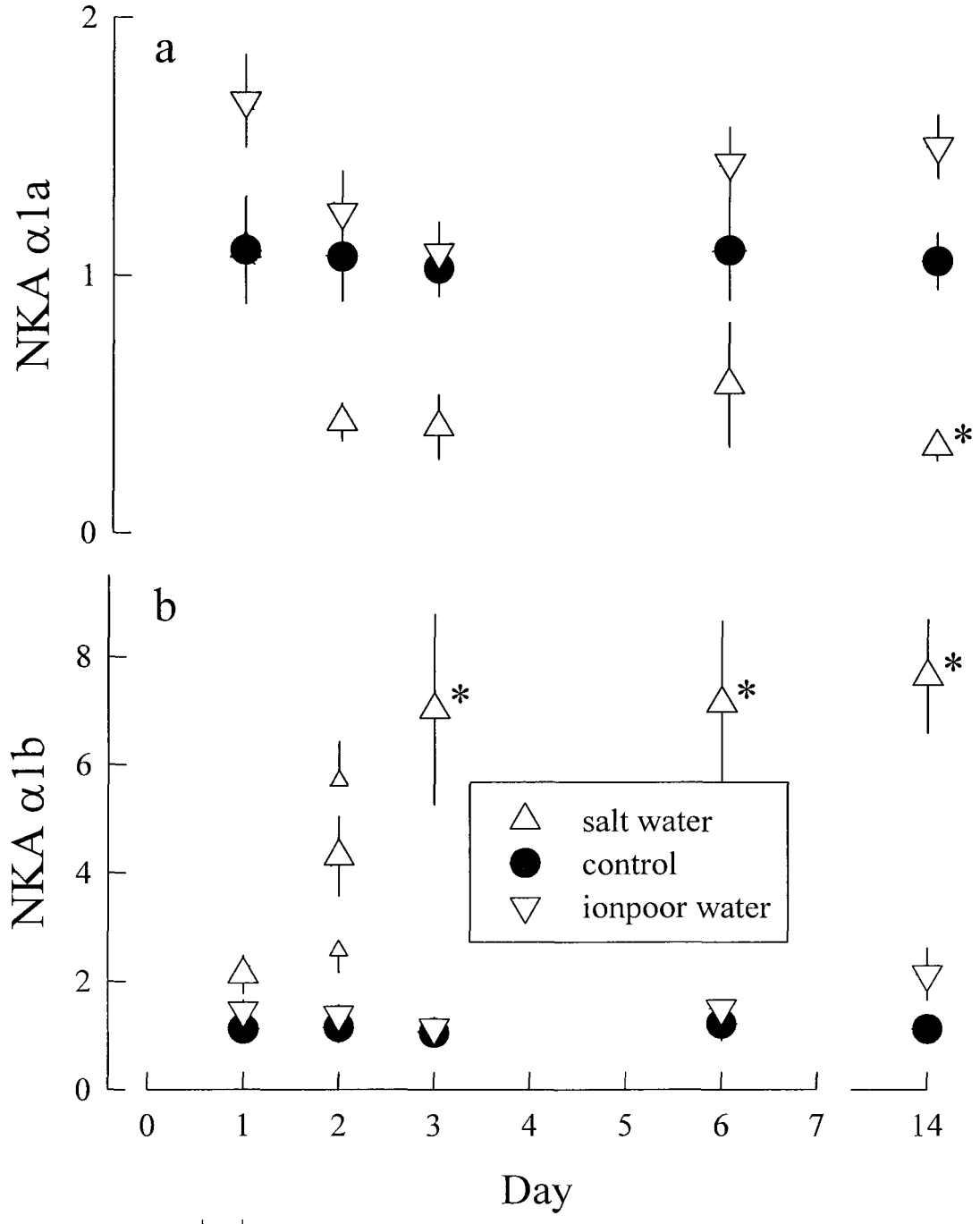

Figure 1 2: (a) Gill Na^+,K^+-ATPase (NKA) α1a expression (relative to β-actin) and (b) gill NKA α1b expression (relative to β-actin) for fish in 3 experimental treatment groups: control, ion-poor and salt water. Sample size ranged from 8 to 12. Values are means ± se. Asterisks denote significant difference ($p < 0.05$) between treatment group and control on the same day. Small triangles on day 2 for the saltwater group denote significant difference ($p < 0.05$) between the Blackwater (BW) and Pennask (P) fish

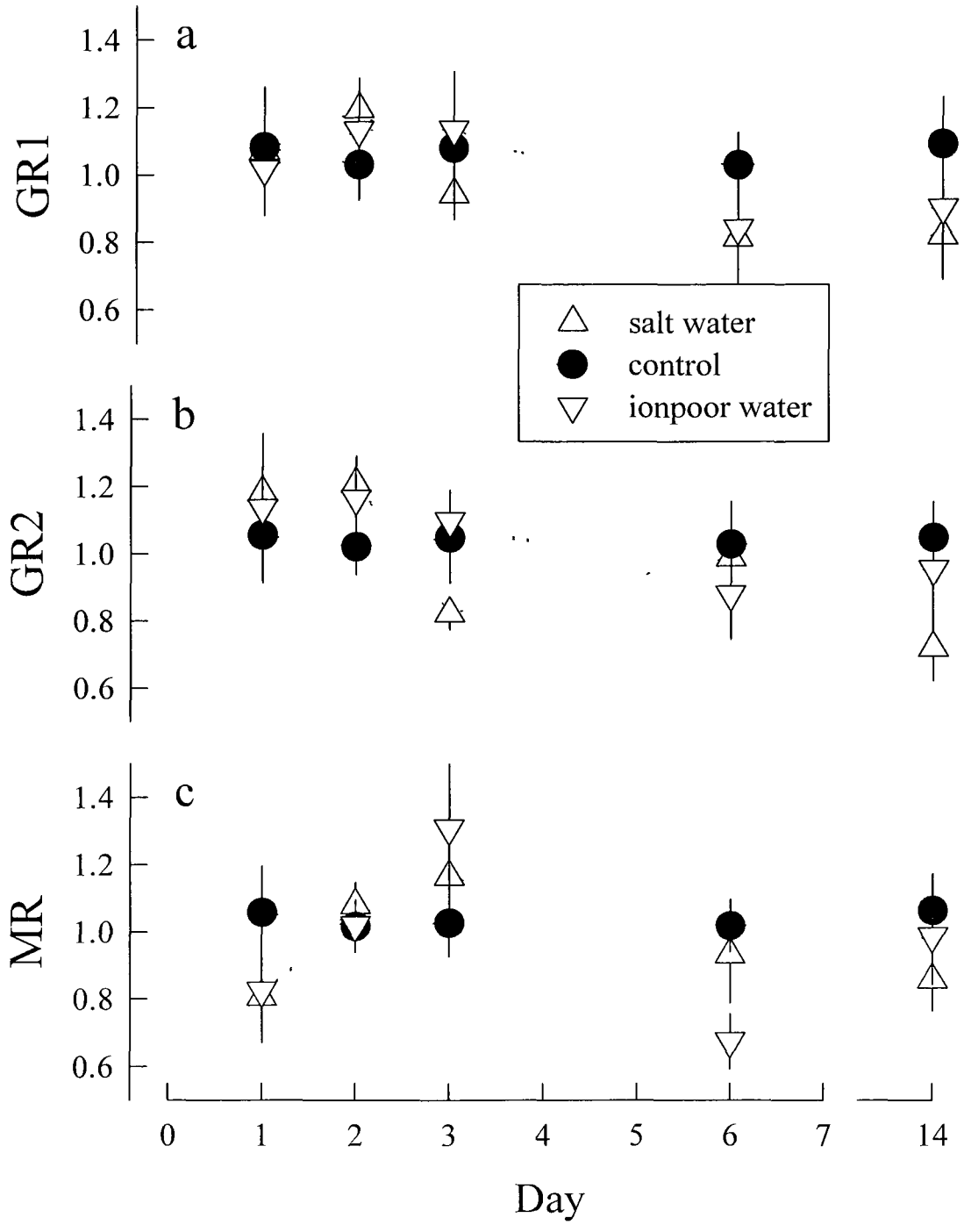

Figure 1.3: Changes in (a) gill glucocorticoid receptor 1 (GR1) mRNA expression (relative to β-actin), (b) gill glucocorticoid receptor 2 (GR2) mRNA expression (relative to β-actin), and (c) gill mineralocorticoid receptor (MR) mRNA expression (relative to β-actin) in rainbow trout during transfer to 3 different experimental groups. control, ion-poor and salt water. Sample size ranged from 8-12. Values are means ± se.

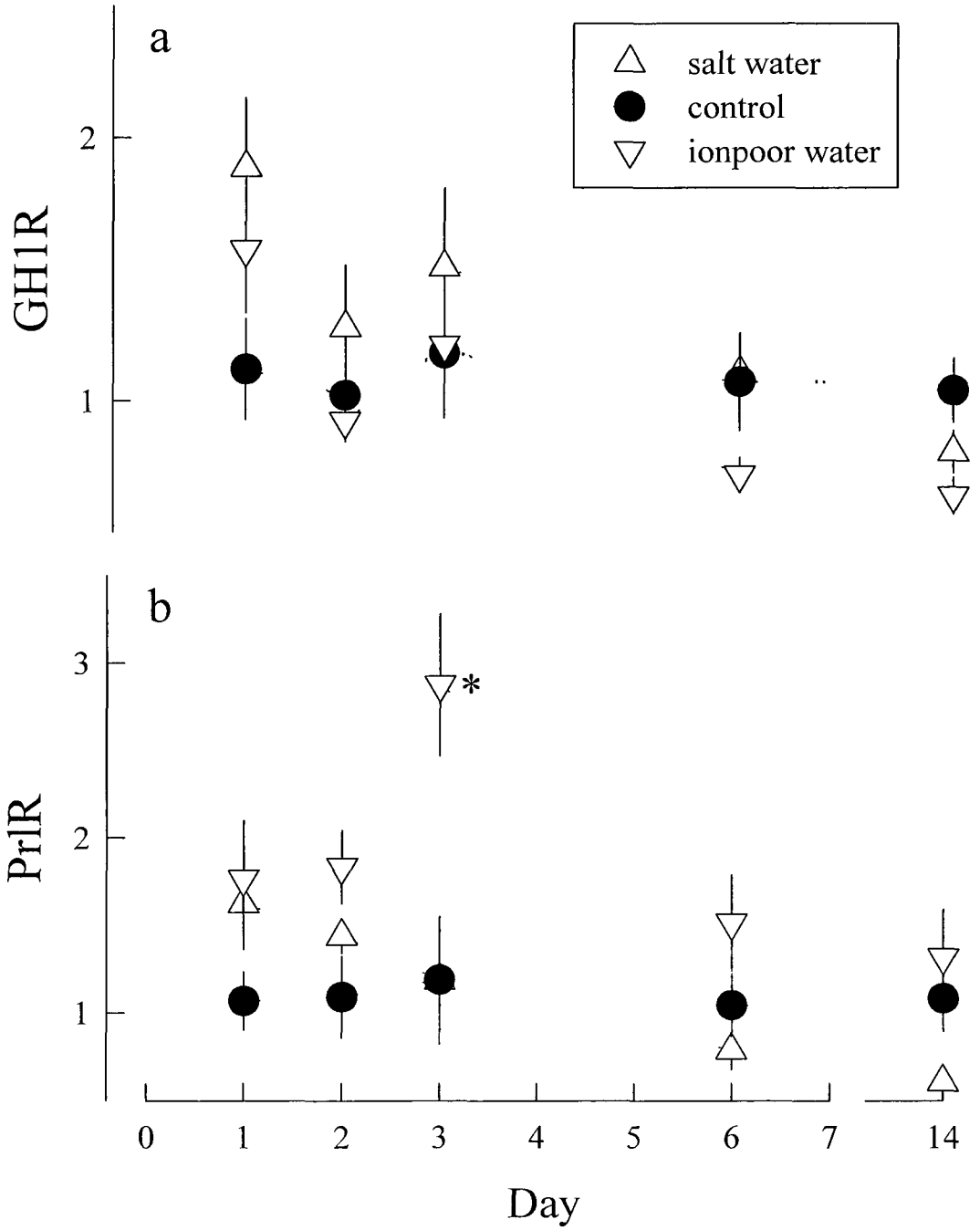

Figure 1.4: Changes in (a) gill growth hormone 1 receptor (GH1R) relative mRNA expression (relative to β-actin) and (b) gill prolactin receptor (PrlR) mRNA expression (relative to to β-actin) for rainbow trout transferred to 3 treatment groups: control, ion-poor and salt water. Sample size ranged from 8-12. Values are means ± se. Asterisks denote significant difference ($p < 0.05$) between treatment group and control for the same day.

DISCUSSION

Abrupt transfer of rainbow trout from fresh water to 24 ‰ salt water or ion-poor water resulted in significant physiological and endocrine changes as the fish adapted to the higher or lower salinity. Plasma chloride showed perturbations in both treatment groups that reflected the salinity challenge; rebounding by day 2 in the ion-poor group, but after day 6 in the saltwater group. Temporary perturbations in plasma ion concentration are consistent with work previously shown by Blackburn and Clarke (1978) during hyper-osmotic challenge and by Perry and Laurent (1989) during hypo-osmotic challenge. The difference in time to rebound in our study may be due to the magnitude of the challenge experienced by the two different treatments; suggesting the saltwater treatment was a greater ionoregulatory challenge than the ion-poor treatment. Additionally, greater time is likely to be required for the gill of a freshwater fish to become ion secreting and for the animal to adapt to the saltwater treatment. The higher plasma cortisol concentrations in the saltwater group and especially the ion-poor group on day 1 compared to the control fish suggest that the change in salinity was stressful (Barton and Iwama, 1991), although an increase in plasma cortisol has been linked to an ionoregulatory role following salinity transfer (Perry and Laurent, 1989; Shrimpton *et al.,* 1994a).

Previous work has shown that seawater exposure resulted in a significant increase in gill NKA activity after approximately 7 to 14 days (McCormick and Saunders, 1987). In contrast, little difference in gill NKA activity was observed for fish transferred to lower salinity levels (ion-poor treatment). Sloman *et al.* (2001) found that ion-poor water had no effect on gill NKA activity despite a dramatic increase in chloride cell density on the secondary lamellae. Despite the lack of a change in NKA activity in the gill, changes in the

NKA α-isoform mRNA in the gill indicated that rainbow trout responded physiologically to not only the increased salinity, but also the decreased salinity. Saltwater exposure significantly increased gill NKA α1b mRNA and decreased gill NKA α1a mRNA (Richards *et al.,* 2003); a finding confirmed by our study. The α1b isoform has also been shown to seasonally increase in juvenile Atlantic salmon smolts as they prepare to migrate downstream to the ocean (Nilsen *et al.*, 2007). The reciprocal treatment of moving saltwater rainbow trout to fresh water resulted in an increase in gill NKA α1a mRNA, but little change in gill NKA α1b mRNA (Richards *et al.*, 2003). Our study showed that even the transfer of fish from fresh water to ion-poor water resulted in increased gill NKA α1a mRNA. Migration of adult sockeye salmon from the ocean to the freshwater environment was accompanied by a significant increase in the α1a isoform (Shrimpton *et al.*, 2005). The different roles of the two isoforms have been further clarified by McCormick *et al.* (2009) who showed in the gills of Atlantic salmon that the two different α1-isoforms are present in distinct chloride cells.

It is interesting to note that we saw no difference in the response of the Blackwater or Pennask rainbow trout strains due to the experimental treatment except for a significant effect on the NKA α1b isoform. Both strains originated from non anadromous populations in the interior of British Columbia, so a differential response to increased salinity was surprising. Size differs considerably between the two strains with the Blackwater fish being significantly larger than the Pennask; Blackwater also had higher gill NKA α1b mRNA on day 2. Several species of juvenile salmon, trout and char have been shown to become progressively more tolerant of salt water as they grow older and larger (Hoar, 1988). Therefore, the more rapid response of the NKA α1b mRNA from the larger Blackwater

rainbow trout may be due to size or growth rate and enabled them to tolerate salt water better than the smaller Pennask strain.

The overall changes in gill NKA activity looked similar to the mRNA expression of the NKA α1b-isoform. The microassay method of McCormick (1993) used to measure gill NKA activity does not differentiate among the different α-isoforms. There does not appear to be an overall effect on gill NKA activity in response to ion-poor water, even though α1a is being up regulated to cope with the change in salinity. The lack of a change in gill NKA activity instead of an increase when fish were exposed to ion-poor water may be because the assay measures more of the ion-secreting enzyme (α1b) compared to the ion-absorbing enzyme (α1a). The activity per molecule of NKA measured in the microplate assay may be greater in seawater than fresh water or the method may favour measurement of α1b activity over that of α1a activity (McCormick *et al.*, 2009). Consequently, the present enzymatic gill NKA assay should only be used as a static measurement that reflects the difference between protein being degraded and synthesized (Madsen *et al.*, 2008).

What signals cause changes during abrupt salinity transfer?

There is considerable evidence that the modifications and regulation of gill NKA for fish acclimation to changes in salinity are primarily mediated through changes in circulating hormones, particularly cortisol (McCormick, 1995). Cortisol is the major corticosteroid in fish that has long been hypothesized to be the saltwater adapting hormone. The higher plasma cortisol levels in the ion-poor group on day 1 and the saltwater group throughout the experiment may, therefore, serve an ionoregulatory role. Cortisol has been shown to enhance ionoregulation in salt water by stimulating cellular differentiation of chloride cells

(McCormick, 1990) and increase NKA activity (Madsen, 1990a; McCormick *et al.*, 1991) in the gills of juvenile salmonids. Additionally, McCormick *et al.* (1991) found that elevated [cortisol] increased NKA activity *in vitro* for gill tissue from coho salmon (*Oncorhynchus kisutch*). Moreover, these authors did not find prolactin (Prl), growth hormone (GH) or insulin-like growth factor I (IGF-I) to have a direct effect individually or in combination with cortisol on gill NKA activity in *vitro* (McCormick *et al.*, 1991). Cortisol has also been suggested to play a role in freshwater ionoregulation. Laurent *et al.* (1994) found that rainbow trout injected with cortisol and transferred to an ion-poor environment showed enhanced whole body Na^+ and Cl^- influxes through the differentiation and proliferation of branchial chloride cells.

Despite the substantial evidence supporting the role of cortisol in fresh- and seawater ionoregulation (McCormick, 1995), our findings do not strongly support a role for cortisol in ion secretion or ion uptake. When compared to the control fish, rainbow trout transferred to 24 ‰ salt water had elevated plasma cortisol levels over the duration of the study, but the post-hoc test did not reveal differences from controls for each day of the experiment. Shepherd *et al.* (2005) also found that plasma cortisol levels only had a minor change when rainbow trout were initially transferred to 66% seawater. Richards *et al.* (2003) also showed that rainbow trout transferred to 80% seawater did not significantly affect plasma [cortisol] at any time, although there was a salinity-dependent increase in plasma [cortisol] 1 day post-transfer. Rainbow trout transferred to ion-poor water had a significant increase in plasma [cortisol] on day 1, but after this plasma [cortisol] declined steadily and did not differ from the control fish. It seems unclear whether the increase observed in gill NKA α1a mRNA can be attributed to the brief higher cortisol levels at the start of the experiment.

The biological effect of cortisol, however, will not only depend on circulating concentration of the hormone, but number and affinity of the intracellular receptors in the gills (Shrimpton and McCormick, 1999; Marsigliante *et al.*, 2000). High-affinity corticosteroid receptors (CR) are present in the gills and the responsiveness of cells is dependent of the receptor concentration (Shrimpton and McCormick, 1999). In the present study, we measured two different classes of hormone receptors for cortisol, GR, subtypes GR1 and GR2, and MR. GR cDNAs has been previously identified in different teleost species including rainbow trout (Ducouret *et al.*, 1995), Japanese flounder, *Paralichthys olivaceus* (Takeo *et al.*, 1996), and cichlid, *Haplochromis burtoni* (Greenwood *et al.*, 2003) and tilapia, *Oreochromis mossambicus* (Tagawa *et al.*, 1997). Experimental evidence suggests that GR is important for the transition from fresh water to seawater. For example, an increase in GR abundance was detected during seawater adaption for the euryhaline teleost, *Anguilla anguilla* (Marsigliante *et al.*, 2000), rainbow trout (Bury *et al.*, 2003) and Atlantic salmon (Kiilerich *et al.*, 2007b). Conversely, it has been suggested that cortisol may act through an MR in freshwater acclimation (Colombe *et al.*, 2000; Sloman *et al.*, 2001). When spironolactone, the MR antagonist, was injected into rainbow trout and fish transferred to ion-deficient fresh water, the proliferation of chloride cells normally associated with acclimation to ion-deficient water was blocked (Sloman *et al.*, 2001). While these studies suggest that MR is functional in fish, the biochemical characterization, localization and function has not been established or very well understood (McCormick *et al.*, 2008). The lack of a significant change in gill GR1, GR2 or MR mRNA in the present study, however, suggests that cortisol is not interacting with any of these receptors in the gill.

Given the support in the literature for cortisol as an important hormone for ionoregulation, the lack of an effect of cortisol in our experiment is puzzling. Cortisol has repeatedly been shown to increase in salmon smolts (McCormick and Saunders, 1987) and there is a strong correlation between plasma cortisol and gill NKA activity (Shrimpton and McCormick, 1998a), but such changes are seasonal. Gill responsiveness to cortisol has also been shown to change seasonally. Prior to the parr-smolt transformation, Atlantic salmon and coho salmon showed increased sensitivity to *in vitro* cortisol treatment exhibited by an upregulation of gill NKA activity (McCormick *et al.*, 1991). However, at the peak of the parr-smolt transformation, when gill NKA activity was highest, the gill tissue was unresponsive to the cortisol treatment. Responsiveness to cortisol increased in the gill when gill NKA activity declined after smolting. The timing of the experiment may have contributed to the lack of changes we observed in cortisol receptor mRNA.

Rainbow trout are also not typically anadromous and it is unclear if they even undergo seasonal changes that resemble smoltification. This non-migratory salmonid is often used in experiments to understand endocrine and physiological adjustments inherent with salinity acclimation, where preparative changes that could alter the acclimation process to higher salinity should not occur (Shepherd *et al.*, 2005). However, despite this advantage, there are differences observed in the endocrine response for rainbow trout transferred to abrupt salinity challenges (Sakamoto and Hirano, 1991). Further, a study conducted by McLeese *et al.* (1994) found that plasma cortisol levels did not change significantly for steelhead-rainbow trout hybrids, but there was a significant rise in steelheads from March to May. The osmoregulatory physiology of rainbow trout has also been shown to vary depending on size, stock of fish and state of maturity (Bern and Madsen, 1992).

The lack of any change in steroid receptor mRNAs indicates that other endocrine factors are likely involved. There is evidence that the two closely related pituitary hormones, Prl and GH, are involved in the control of ionoregulation (Hoar, 1988). It is generally accepted that Prl is important for freshwater ionoregulation, while GH is important for seawater ionoregulation in a large and phylogenetically diverse number of teleosts (McCormick, 2001). During freshwater acclimation, pituitary and plasma Prl levels increase to regulate hydromineral balance by decreasing water uptake and increasing ion retention (Manzon, 2002). Earlier studies have examined the effects of Prl injection on the expression of NKA α –subunits (Seidelin and Madsen, 1999; Deane *et al.*, 1999). Unfortunately, the protocol did not distinguish between the different α –isoforms and more recent work has shown that they are differentially expressed during freshwater and seawater acclimation (Richards *et al.*, 2003), so we do not know if GH and Prl differentially regulate the NKA α1a and α1b subunits. A correlation between mRNA for the gill PrlR and gill NKA α1a was recently shown in adult migratory sockeye salmon (Chapter 2). PrlR are heavily expressed within the gill (Kiilerich *et al.*, 2007b) and changes in PrlR expression have been detected in response to changes in environmental salinity (Manzon, 2002; Tomy *et al.*, 2009). Tomy *et al.* (2009) found that PrlR gene expression was higher for freshwater adaptation in the euryhaline black porgy (*Acanthopagrus schlegeli)*. For our study, mRNA for PrlR significantly increased in the gills of rainbow trout transferred to ion-poor water and levels peaked on Day 3. Ayson *et al.* (1993) and Auperin *et al.* (1994) working on tilapia also found that pituitary PrlR mRNA expression was up-regulated resulting in higher circulating prolactin levels present in fresh water. Sandra *et al.* (2000) and Shiraishi *et al.* (1999) also found that an increase in environmental salinity resulted in a decrease in the expression of

PrlR in the gills of adult *O. niloticus* and *O. mossambicus*; a finding consistent with our gill PrlR mRNA data for rainbow trout transferred to salt water.

The physiological changes that occurred when fish were transferred to the saltwater treatment also do not appear to be regulated by cortisol. If cortisol was stimulating ionoregulation and the changes in NKA α1b, we would have expected to see a greater magnitude of change in GR during the first few days of salinity transfer. Instead, the increases in gill GH1R mRNA indicate that GH initiated the physiological changes in the gills; a finding supported by Yao *et al.* (1991) and Very *et al.* (2005). Changes in GH1R expression have also been found in smolting Atlantic salmon (Nilsen *et al.*, 2008). These authors found that plasma GH and gill GH1R mRNA levels increased during the spring during the smolting period in anadromous Atlantic salmon, whereas no changes in GH were detected for landlocked Atlantic salmon.

Based on plasma [cortisol] and the mRNA levels for both receptors of cortisol, our findings suggest that cortisol is not the primary hormone that induces a physiological response to an ionoregulatory challenge. Instead, this study is the first to demonstrate an increase in gill PrlR mRNA that parallels the changes in gill NKA α1a when rainbow trout were transferred to a lower salinity level. Furthermore, the increase in gill GH1R mRNA further supports the importance of GH for seawater acclimation by increasing the expression of gill NKA α1b, the saltwater isoform.

CHAPTER 2

Physiological and molecular endocrine changes in maturing wild sockeye salmon, *Oncorhynchus nerka*, during ocean and river migration*

ABSTRACT

Maturing adult sockeye salmon *Oncorhynchus nerka* were intercepted while migrating in the ocean and upstream in fresh water to determine physiological and endocrine changes associated with ionoregulation. Sockeye were caught in the ocean from up to 850 km away from the river mouth, throughout the freshwater migration upstream and on the spawning grounds, a distance of almost 500 km. Sockeye migrating in seawater and fresh water showed a consistent decline in gill Na^+, K^+-ATPase (NKA) activity, plasma osmolality and plasma chloride concentration. In contrast, plasma sodium concentrations increased in seawater as fish approached the river mouth and then declined after entering the river. Accompanying the movement from seawater to fresh water was a significant increase in mRNA for the α1a subunit of NKA in the gill, with little change in the α1b subunit in the gill. Potential endocrine signals stimulating the physiological changes during migration were assessed by quantifying mRNA extracted from the gill for glucocorticoid receptors 1 and 2 (GR1 and GR2), mineralocorticoid receptor (MR), growth hormone 1 receptor (GH1R), and prolactin receptor (PrlR). Generally, the mRNA expression for GR and MR declined as the fish were migrating, particularly after entering fresh water. In contrast, PrlR mRNA

* Throughout this chapter I use the first person plural to acknowledge the contribution of others to this work, which will be submitted for publication with the authorship of A.-M. Flores, D.A. Patterson, J.A. Hills, S.J. Cooke, S.G. Hinch, A.P. Farrell, and J.M. Shrimpton.

increased throughout migration and the changes were highly significant as sockeye approached the spawning grounds. A highly significant relationship existed between gill PrlR mRNA and gill NKA α1a mRNA. GH1R mRNA also increased significantly after migrating beyond tidal influence in the river and just before the fish reached the spawning grounds. These findings suggest that prolactin is the principle hormone for stimulating ionoregulation in the gill as sockeye salmon adapt to fresh water and that cortisol may not have a functional role in freshwater ionoregulation.

INTRODUCTION

Migration is one of the most energetically demanding and physiologically challenging phases of an animal's life history and represents a complex interplay between behaviour and physiology (Hinch *et al.*, 2005; Cooke *et al.*, 2006a). This is evident for anadromous Pacific salmon (*Oncorhynchus* spp.) during their reproductive migration to natal streams. The majority of Pacific salmon are semelparous and failure to migrate to the spawning grounds in fresh water and reproduce means a fish does not contribute genes to future generations, resulting in no lifetime fitness (Dingle, 1980). Maturing adults that are leaving the ocean for fresh water undergo physiological transformations to maintain osmotic and ionic balance (Wagner *et al.*, 2005; Shrimpton *et al.*, 2005). This ability is dependent on the regulation of water and ions by the gills, intestine, urinary bladder and kidney; however, the gills play the primary role in the maintenance of ion balance in both freshwater- and seawater-acclimated fish (Marshall, 2002; Evans *et al.*, 2005). In fresh water, fish actively take up Na^+ and Cl^- ions in dilute fresh water to maintain a hyper-osmotic state, whereas in seawater, fish

maintain a hypo-osmotic state by actively drinking and excreting excess ions (Hoar, 1988; Evans *et al.*, 2005).

The principle enzyme involved in ion-absorption and ion-secretion is Na^+, K^+-ATPase (NKA) (McCormick, 2001; Marshall, 2002). Increases in gill NKA activity are seen in juvenile salmon in fresh water preparing to migrate to the ocean, a higher ionic concentration (McCormick and Saunders, 1987) and a comparable decrease in gill NKA enzyme activity also occurs for maturing adult salmon in seawater returning to fresh water (Shrimpton *et al.*, 2005). The decline in NKA activity may promote a higher tolerance to the lower levels of salinity in fresh water, analogous to the parr-smolt transformation. Furthermore, two isoforms of the NKA α-subunit (α1a and α1b) have been identified to change in rainbow trout transferred among waters of different salinity (Richards *et al.*, 2003). In fresh water, NKA α1a is the abundant isoform (Richards *et al.*, 2003) present in lamellar chloride cells in the gills (McCormick *et al.*, 2009). NKA α1b becomes the dominant isoform after seawater acclimation (Richards *et al.*, 2003) and is found primarily in filamental chloride cells (McCormick *et al.*, 2009).

The neuroendocrine system is the primary link between a changing environment and physiological adaptations; therefore, the hormonal control of NKA is critical for maintaining homeostasis in both fresh water and seawater (McCormick 1995; McCormick, 2001). The sensitivity of the target cells to a specific hormone depends on the stability of the hormone receptor complex, the amount of receptors specific for the hormone available and the affinity of the hormone to its receptor (Shrimpton and McCormick, 1999). Cortisol and prolactin (Prl) have been observed to increase when fish move from seawater to fresh water or are transferred from fresh water to ion-poor water (Manzon, 2002; Perry and Laurent 1989) and

appear to promote acclimation to fresh water, but cortisol has also been linked to seawater acclimation (McCormick and Saunders, 1987; Hoar, 1988). It is unclear how cortisol can stimulate physiological mechanisms involved in both ion uptake and ion excretion. It was long thought that the function of cortisol was through a single class of receptor; the glucocorticoid receptor (GR) (Ducouret *et al.*, 1995). Recently, a second class of receptor for cortisol, the mineralocorticoid receptor, has been identified (Greenwood *et al.*, 2003). Potentially, cortisol may function through an MR in freshwater ionoregulation in concert with Prl.

In the present study, we investigated which hormones have a role in stimulating physiological modifications in wild adult sockeye salmon that were intercepted migrating in the ocean and in fresh water to their natal spawning grounds in the Fraser River watershed, British Columbia, Canada. Changes in gill NKA activity, osmolality, plasma [chloride], plasma [sodium] and mRNA in the gill for NKA α1a and α1b were measured to assess the response of the fish to changes in salinity. To elucidate what endocrine signals may be associated with the physiological changes in the gill, mRNA from the gill was measured for changes in expression of hormone receptors. We measured mRNA for the glucocorticoid receptor 1 and 2 (GR1 and GR2), the mineralocorticoid receptor (MR), the growth hormone 1 receptor (GH1R) and the prolactin receptor (PrlR) to assess the relative importance of each hormone for ionoregulation in the gill.

MATERIALS AND METHODS

Fish capture and sampling

Migratory adult sockeye salmon *Oncorhynchus nerka* Walbaum were sampled in the coastal waters of British Columbia and at several locations during their upstream migration through the Fraser River system in 2003 and 2006. Although multiple spawning populations of sockeye salmon were caught at each sampling location, there is a considerable amount of information on the run timing for the Lower Shuswap stock and sampling was timed to intercept the peak of the run. Fish were identified as belonging to the Lower Shuswap stock by variation in genetic markers as outlined by Beacham *et al.* (1995). Since stock identification was determined *a posteriori*, however, sample size varied among locations, and also between the years.

Sockeye salmon were captured during their marine migration off the Queen Charlotte Islands (QCI, ~850 km; the mouth of the Fraser River is 0 km – initial site for freshwater entry), through the inside passage of Vancouver Island off Port Hardy (PH, ~375 km), in Johnstone Strait (JS, ~200 km) and in Georgia Strait (GS, ~25 km) (Figure 2.1). Salinities off the north coast of British Columbia are greater than 32‰. In Johnstone Strait, salinity levels fluctuate seasonally from the river run-off in the spring, but levels usually exceed 27‰ in August when sockeye are migrating through the region (Shrimpton *et al.*, 2005). For Georgia Strait, the sea surface salinity is also greater than 27‰ during August and September, when sockeye are migrating through this area. In Georgia Strait close to the mouth of the Fraser River, however, surface water salinities are lower and fluctuate with the tide. In this area, sockeye preferred to be below the halocline when they were migrating (Shrimpton *et al.*, 2005). For their upstream migration, sockeye were caught in the lower Fraser River at

Cottonwood (C, ~11 km), an estuarine site, and at Whonnock (W, ~50 km), the first sampling site beyond the saltwater intrusion. Mid-Fraser sockeye of the Late Shuswap stock were then sampled at Yale (Y, ~170 km), Lytton (L, ~255 km), Savona (S, ~369 km), and at one of the main spawning areas for the Lower Shuswap, the Adams River (AR, 484 km). Fish were sampled twice on the spawning grounds; when they had just arrived as healthy gravid fish and when they had been on the spawning grounds approximately two weeks. These sexually mature fish have been offset on the data figures to prevent overlap of the data points. The methods used to collect fish were dependent on each sampling location; trolling off the west coast of the Queen Charlotte Islands and in Georgia Strait, purse seine off the coast of Vancouver Island near Port Hardy and in Johnstone Strait, gill netting near the mouth of the Fraser River at Cottonwood and at Whonnock, and beach seining to capture sockeye in freshwater sites and on the spawning grounds.

Tissue sampling

Most of the fish sampled were part of normal test fisheries or stock assessment operations and the type of treatment for each fish was dependant on gear type. Effort was made to eliminate time between capture and tissue sampling. Fish that were caught using seine nets remained in the ocean constrained by the seine net until they could be individually dip-netted out for sampling. For instances involving capture by troll line, fish were landed and sampled within minutes. When using gill nets, the soak time was reduced to less than 15 minutes when possible and only fish that were still moving vigorously at capture were sampled. All fish were killed with a single blow to the head and both blood and gill tissue samples were taken. Blood was collected from the caudal vasculature using a vacutainer

syringe (1.5", 21 gauge) and a section of a gill arch was taken to determine NKA activity and also for RNA extraction. The gill tissue and centrifuged plasma samples were kept on dry ice for several days before being transferred to a -80 °C freezer, where they were held until analysis. Fork length (cm) was measured and an adipose fin clip was removed for DNA stock identification.

Analysis of gill Na^+, K^+-ATPase activity

Gill NKA activity was measured using the microassay protocol of McCormick (1993). In brief, gill filaments were homogenized in SEI buffer containing 0.1 % sodium deoxycholate (SEID). After centrifugation (5000 g for 30 seconds), the supernatant was used to determine the activity by relating ATP hydrolysis to the oxidation of NADH, measured at 340 nm for 10 minutes at 25°C in the presence and absence of 0.5 mmol • L^{-1} ouabain on a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA). Protein content was then measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Specific activities were expressed as μmol ADP • mg^{-1} of protein • h^{-1}.

Plasma Analysis

Plasma samples were thawed, vortexed and centrifuged for 5 minutes before analysed. Osmolality was measured in duplicate using a model 5500 Wescor Vapour Pressure meter (Logan, UT). Plasma sodium was measured using a model 410 Cole-Parmer single channel flame photometer (Montreal, PQ). Plasma samples were diluted 1:500 for analysis; values were checked against a standard approximately every ten samples. Plasma chloride was measured using a 4425000 Haake Buchler digital chloridometer (Kansas City,

MO); values were checked against a chloride standard before and after approximately 10 duplicates.

Purification of total RNA from gill tissue and cDNA synthesis

Gill tissue (approximately 20-50 mg) was extracted for total RNA using *RNeasy Mini Kit* (Qiagen, Mississauga, ON). Samples were homogenized in a Geno/Grinder 2000 (BT&C, Inc., Burlington, ON). Isolated RNA was dissolved in 30 μL RNase-free deionized water. The concentration and purity of each RNA sample was determined on a 1% agarose gel and also by measuring optical absorbance at 260 and 280 nM on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Purified RNA was converted to cDNA using Qiagen's *QuantiTect Reverse Transcription Kit* (Protocol: Reverse Transcription with Elimination of Genomic DNA for Quantitative, Real-Time PCR).

Primers and Real-Time PCR

Messenger RNA (mRNA) was measured for genes involved in ion regulation using quantitative real-time PCR (qRT-PCR). Earlier work showed that gill NKA α subunits (α1a and α1b) are differentially expressed for fish in fresh water and salt water (Richards *et al.*, 2003). The primers used to amplify the NKA α1b-isoform were designed for *O. mykiss* by Richards *et al.* (2003). We used the primers designed by Madsen *et al.* (2008), however, for the α1a-isoform of rainbow trout as they were shown to be more specific; they were designed for α1a near the 3' terminus of the complete mRNA sequence to achieve maximum isoform specificity. mRNA copies were normalized to β-actin and expressed relative to the fish

samples caught from the Queen Charlotte Islands. β-actin primers designed by Sathiyaa *et al.* (2003) for rainbow trout were used.

To assess the role of hormones in response to migration and changes in salinity, receptor mRNA for cortisol, growth hormone and prolactin were measured. Three cortisol receptors have been identified in fish; GR1, GR2, and MR. Primers designed by Sathiyaa and Vijayan (2003) for GR1 in *O. mykiss* and by McCormick *et al.* (2008) for GR2 and MR for *O. mykiss* were used. Growth Hormone 1 Receptor (GH1R) primers were designed with *O. mykiss* GH1R as template by Very *et al.* (2005) and the Prolactin primers (PrlR) were designed with *O. mykiss* PrlR as template by Kiilerich *et al.* (2007b).

To verify that all of the primers designed amplified the particular gene of interest in sockeye salmon gill tissue, sockeye cDNA was amplified by polymerase chain reaction (PCR) in a PTC-100 thermocycler (MJ Research, Watertown, MA) using *Taq* DNA polymerase (Invitrogen, Burlington, ON). One microliter of reverse transcribed reaction product was used as template for PCR amplification and a 40-cycle PCR was performed with each cycle consisting of 30 seconds at 95°C (denaturation), 1 minute at 58°C (annealing) and 5 seconds at 72°C (extension). In the last cycle, the extension time was increased to 5 minutes. PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide to verify a single amplicon and also tested for quality by measuring optical absorbance at 260 and 280 nM on a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE).

For both of the α1-isoforms, a single band was resolved and an amplicon size of ~90 bp was seen. PCR products were then cleaned using an ethanol precipitation and sequenced in both directions on an Applied Biosystems 3130XL DNA Analyzer (Carlsbad, CA)

University of British Columbia – Okanagan (Kelowna, BC). Sequence assembly and analysis were performed using Sequencer (Gene Codes Corporation, Ann Arbor, MI) and all sequences were compared with published sequences in GenBank. The α1a subunit sequence showed 100% sequence similarity to the *O. mykiss* NKA α1a subunit (GenBank Accession No. NM_001124461.1). The α1b subunit showed the highest similarity to the *O. mykiss* NKA α1b subunit, 89% (GenBank Accession No. NM_001124460.1).

For the peptide and steroid hormone receptors, the bands of interest were cut and isolated from a 2% agarose gel using a QIAquick Gel Extraction Kit (Qiagen, Mississauga, ON). The resulting PCR products were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI). The purified plasmids (QIAprep, Miniprep Kit; Qiagen, Mississauga, ON) obtained were then sequenced using the Beckman-Coulter CEQ 8000 (Mississauga, ON) at the University of Northern British Columbia (Prince George, BC). Sequences were analyzed using Sequencer (Gene Codes Corporation, Ann Arbor, MI) and compared with published sequences in GenBank.

All of the steroid receptors for cortisol showed 100% similarity with each of the *O. mykiss* receptor sequences (GR1, GenBank Accession No. NM_001124730.1; GR2, GenBank Accession No. NM_001124482.1; MR, GenBank Accession No. NM_001124740.1 and NM_001124483.1). For the peptide receptors, PrlR showed 100% similarity to the *O. mykiss* PrlR receptor (GenBank Accession Nos. EU084744.1, EU084743.1 and NM_001124599.1). For the GH1R receptor, aligning the sequence with the *O. mykiss* GH1R sequence designed by Very *et al.* (2005), revealed a 95% similarity (GenBank Accession No. AY861675.1).

All qRT-PCR reactions contained 1 μL of cDNA template, 4 pmoles of each isoform specific primer and Universal SYBR green master mix (Applied Biosystems Inc., Warrington, UK). All qRT-PCR reactions were 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Melt curve analysis was performed after each reaction to confirm the presence of only a single product of the reaction. RNA controls were also performed for a selection of samples using RNA samples that were not reverse transcribed to test for the possible presence of genomic contamination. Genomic DNA contamination was present in all samples, but it was never was more than 1:1,000,000 starting copies for the NKA α1a isoform, 1:190,000 starting copies for NKA α1b isoform, 1:7820 starting copies for β-actin, 1:1660 starting copies for GR1, 1:345 starting copies for GR2, 1: 22,200 starting copies for MR, 1:1800 copies for GH1R, and 1:3600 starting copies for PrlR. Genomic DNA contamination, therefore, was considered to be negligible.

Randomly selected control samples were serially diluted to develop a standard curve relating threshold cycle to cDNA amount for each primer set. The slopes were linear and similar for all genes, suggesting that the amplification efficiency in the qRT-PCR reactions did not differ between genes. Therefore, the relative expression of the target genes could be normalized to a reference gene by utilizing the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Gene expression levels measured were normalized relative to the reference gene, β-actin. Relative expressions of mRNA were related to the gill samples collected from the QCI location, the farthest seawater sampling location. All samples were run in duplicate and the coefficient of variation between duplicate samples was always <1%.

Statistical Analysis

Sockeye collected at the peak of the run were compared for each variable measured using a one-way analysis of variance (ANOVA) to determine whether location in seawater had a significant effect for the 2003 and 2006 samples. The analysis was repeated for just the freshwater sampling locations. We also used a one-way ANOVA to compare across all sampling locations in seawater and fresh water to determine at what point in the migration significant changes in the variables occurred. Sampling was more extensive in 2006 than 2003 and data were not directly compared between the 2 years. When significant differences were found with the one-way ANOVA, a Bonferroni test was conducted to determine differences between sample locations. Linear regression analysis was used to test for relationships between relative mRNA levels for the different genes examined. Statistical significance was taken at a level of $P < 0.05$. All values are expressed as means ± 1 standard error (se).

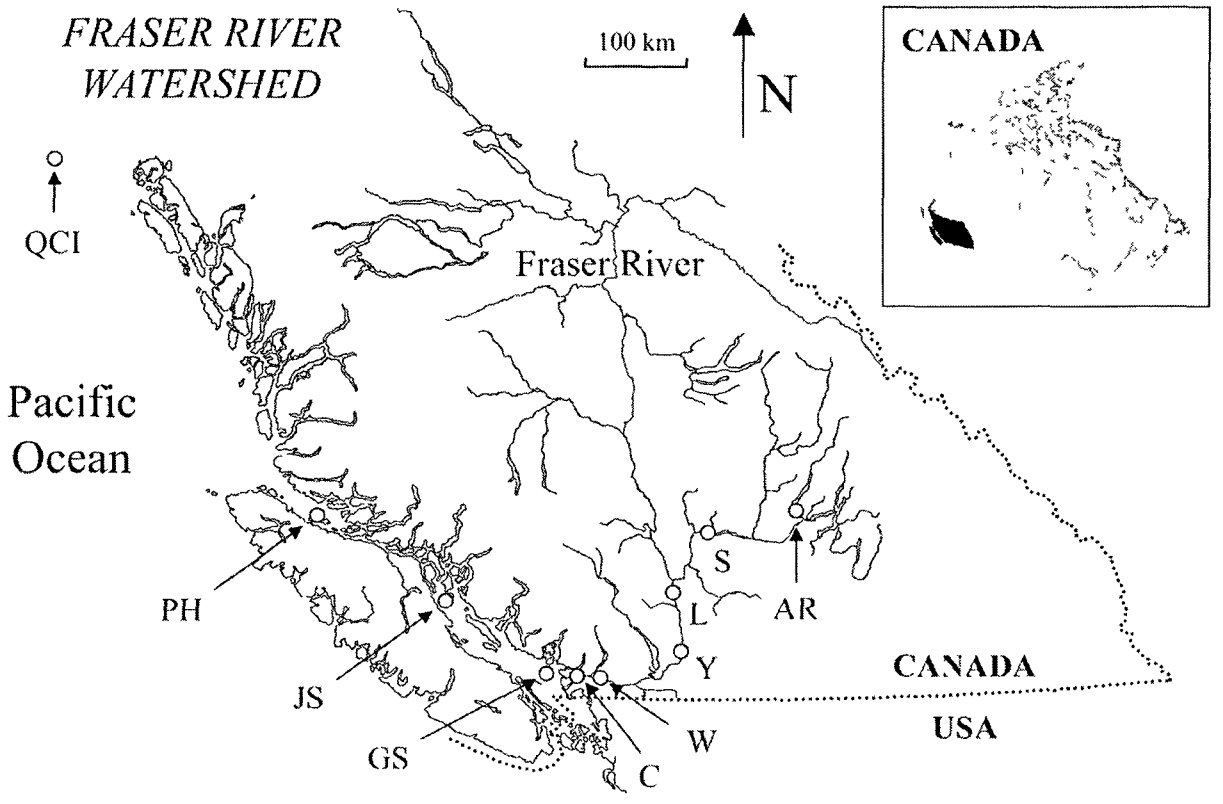

Figure 2.1: Map of the Fraser River watershed and coastal British Columbia, Canada showing locations where Late Shuswap stock were intercepted during migration. Samples were collected off the Queen Charlotte Islands, (QCI), Port Hardy (PH), Johnstone Strait (JS), and Georgia Strait (GS) in seawater. Sample locations within the Fraser River were Cottonwood (C), Whonnock (W), Yale (Y), Lytton (L), and Savona (S). Spawning sockeye were sampled on the spawning grounds in the Adams River (AR).

RESULTS

Adult sockeye salmon from the Late Shuswap stock exhibited a number of physiological changes as they migrated from the ocean back to their natal spawning grounds. For fish captured in seawater, a number of the variables examined showed significant changes among locations as the fish migrated from the Queen Charlotte Islands to Georgia Strait. Variables that changed significantly during seawater migration in one or both years were gill NKA, plasma ion concentrations, gill NKA α1a mRNA, and gill PrlR mRNA (Table 2.1). For fish captured in fresh water, the number of physiological variables that changed significantly during migration were even greater; all variables examined showed significant differences among freshwater locations in one or both years.

Our statistical analysis across all seawater and freshwater sample sites revealed locations in the migration where significant changes in the variables occurred. There were significant changes in gill NKA activity between seawater and freshwater locations in 2003 ($F_{6,48} = 7.73$; $p < 0.001$) and 2006 ($F_{3,21} = 13.08$; $p < 0.001$). Enzyme activities were initially high in seawater for both years, but declined significantly before fish entered fresh water and remained low while the fish were migrating up river (Figure 2.2a). Measurements of plasma variables also showed significant changes with location in migrating sockeye salmon. Plasma osmolality (Figure 2.2b) showed significant declines throughout migration in 2003 ($F_{6,48} = 17.9$, $p < 0.001$) and 2006 ($F_{10,75} = 13.5$; $p < 0.001$). In 2003, osmolality showed significant declines prior to freshwater entry and continued declines during up river migration. In contrast, changes in osmolality in 2006 were less marked and a significant decline was not seen until after freshwater entry. Plasma [sodium] also showed significant changes during migration ($F_{10,76} = 6.71$; $p < 0.001$), but the pattern differed markedly from that of osmolality

(Figure 2.2c). Plasma [sodium] increased significantly in seawater as the fish migrated toward the mouth of the Fraser River, then declined significantly as fish moved into fresh water, but rebounded as fish migrated up river. Plasma [chloride] changes mirrored those of plasma osmolality (Figure 2.2d) with significant declines in 2003 ($F_{6,48} = 8.09$; $p < 0.001$) and 2006 ($F_{10,76} = 12.2$; $p < 0.001$), except significant differences from the QCI values were not observed until after freshwater entry.

Gill NKA α1a subunit showed significant changes with location in 2003 ($F_{6,41} = 5.91$; $p < 0.001$) and 2006 ($F_{10,69} = 7.70$; $p < 0.001$) with mRNA copies increasing by up to 10-fold throughout the migration, although only locations close to and on the spawning grounds were significantly different from QCI (Figure 2.3a). In contrast, gill NKA α1b subunit mRNA showed less change in migrating sockeye (Figure 2.3b), although the effect of location was significant in 2006 ($F_{10,69} = 4.74$, $p < 0.001$), but not in 2003 ($F_{6,41} = 1.81$; $p = 0.34$). The significant effect of location in 2006 is related to the over two-fold increase in mRNA copies for fish sampled at the Adams River site when the fish arrived on the spawning grounds and was just seen in fresh water (Table 2.1).

The steroid hormone receptors showed little change with location, although generally there tended to be a decrease in mRNA for each receptor as the fish got closer to the spawning grounds and significant effects of location were limited to freshwater sites (Table 2.1). This trend was more pronounced in 2003 than in 2006. The decline in 2003 resulted in significant differences with location for GR1 ($F_{6,41} = 4.37$; $p < 0.005$), GR2 ($F_{6,41} = 4.11$; $p < 0.005$), and MR ($F_{6,41} = 13.49$; $p<0.001$). Only the values for MR from the sample locations furthest up river differed significantly from QCI (Figure 2.4). For 2006, none of the glucocorticoid receptors differed with location; GR1 ($F_{10,69} = 0.74$; $p = 0.68$) and GR2 ($F_{10,69}$

= 1.51; p = 0.15). MR mRNA was affected by location for the 2006 samples ($F_{10,69}$ = 2.45; p < 0.05); however, the value for QCI did not differ from the other sampling locations. The slightly higher MR mRNA values for fish captured in Johnstone Strait and at Savona differed significantly from the fish sampled on the spawning grounds (Figure 2.4c).

Significant changes in PrlR expression were seen with location for 2003 ($F_{6,41}$ = 3.11; p < 0.05) and for 2006 ($F_{10,69}$ = 15.36; p < 0.001). Generally PrlR mRNA increased as the fish were approaching fresh water and during the upriver migration (Table 2.1). The Bonferroni test did not reveal that any values differed significantly from QCI in 2003, although the value for Cottonwood near the mouth of the Fraser River differed significantly from the fish on the spawning grounds. For 2006, QCI fish differed significantly from fish captured at Savona and on the spawning grounds in the Lower Shuswap (Figure 2.5a). GH1R mRNA expression also showed significant changes in location for 2003 ($F_{6,41}$ = 4.26; p < 0.005) and for 2006 ($F_{10,69}$ = 2.98; p < 0.005). Little change was observed in GH1R mRNA in seawater, but values were higher in fresh water and differed significantly from QCI at Whonnock for 2003 and from QCI at Lower Shuswap for 2006 (Figure 2.5b). A significant relationship existed between the gill PrlR mRNA and gill NKA α1a mRNA ($F_{1,125}$ = 46.76, p < 0.001; Figure 2.6a). A significant relationship also existed between the gill GH1R mRNA and gill NKA α1b mRNA ($F_{1,125}$ = 23.54; p < 0.001; Figure 2.6b).

The length and weight of fish sampled in 2003 and 2006 for each location did not differ significantly. The mean length and weight for the 2003 and 2006 Late Shuswap stock were 60.3 ± 2.6 cm and 2.6 ± 0.4 kg, and 58.0 ± 3.1 cm and 2.4 ± 0.3 kg, respectively.

Table 2.1: Results of one-way ANOVA for sockeye salmon captured migrating in seawater and captured migrating in fresh water.

		Seawater				Fresh water			
	year	n	F	df	p	n	F	df	p
Gill NKA activity	2006	**17**	**9.210**	**1**	**0.008**	**8**	**15.116**	**1**	**0.008**
	2003	**23**	**7.088**	**2**	**0.005**	**32**	**6.084**	**3**	**0.003**
Plasma Sodium concentration	2006	**37**	**7.549**	**3**	**0.001**	**50**	**3.680**	**6**	**0.005**
	2003	-	-	-	-	-	-	-	-
Plasma Chloride concentration	2006	37	0.836	3	0.484	**50**	**3.388**	**6**	**0.008**
	2003	**23**	**9.109**	**2**	**0.002**	32	1.009	3	0.103
Plasma Osmolarity	2006	37	2.374	3	0.088	**49**	**4.996**	**6**	**0.001**
	2003	**23**	**8.866**	**2**	**0.002**	**32**	**8.613**	**3**	**<0.001**
Gill NKA α1a mRNA	**2006**	**35**	**3.132**	**3**	**0.040**	**45**	**4.849**	**6**	**0.001**
	2003	17	0.352	2	0.710	**31**	**5.556**	**3**	**0.004**
Gill NKA α1b mRNA	2006	35	1.204	3	0.325	**45**	**6.448**	**6**	**<0.001**
	2003	17	0.111	2	0.896	31	1.351	3	0.279
Gill Glucocorticoid Receptor 1 mRNA	2006	35	0.290	3	0.832	44	0.912	6	0.497
	2003	17	0.066	2	0.937	**31**	**9.269**	**3**	**<0.001**
Gill Glucocorticoid Receptor 2 mRNA	2006	35	0.016	3	0.997	45	2.239	6	0.060
	2003	17	0.388	2	0.685	**31**	**7.079**	**3**	**0.001**
Gill Mineralocorticoid Receptor mRNA	2006	35	0.913	3	0.446	45	2.319	6	0.053
	2003	17	0.181	2	0.837	**31**	**11.249**	**3**	**<0.001**
Gill Growth Hormone 1 Receptor mRNA	2006	34	1.519	3	0.230	46	1.892	6	0.107
	2003	17	1.271	2	0.311	**31**	**4.504**	**3**	**0.011**
Gill Prolactin Receptor mRNA	**2006**	**35**	**2.957**	**3**	**0.048**	**46**	**15.595**	**6**	**<0.001**
	2003	17	3.065	2	0.079	31	2.925	3	0.052

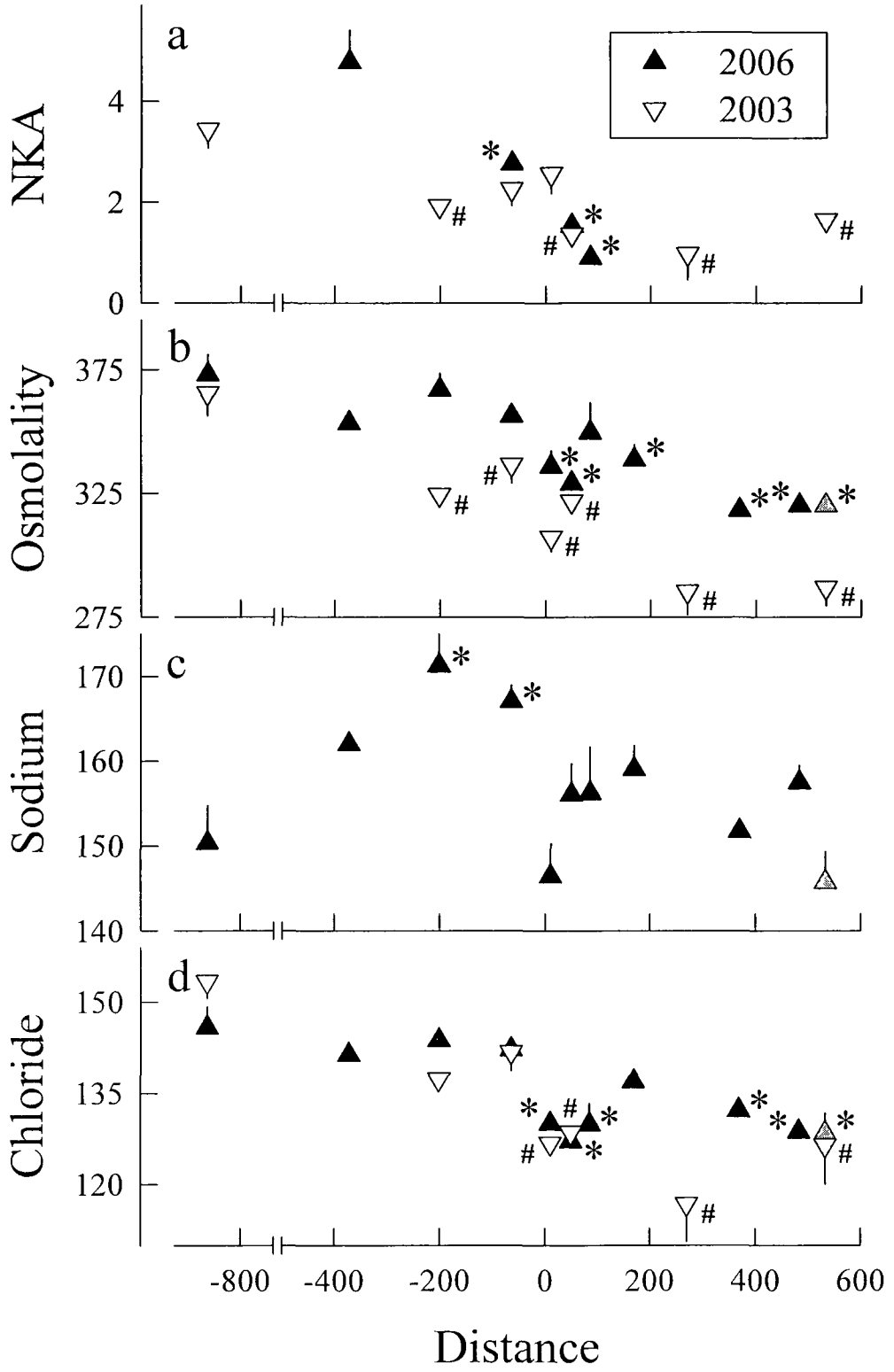

Figure 2.2: (a) Gill Na^+,K^+-ATPase (NKA) activity (μmol ADP • mg^{-1} of protein • h^{-1}), (b) plasma osmolality (mOsm), (c) plasma [sodium] (mmol • L^{-1}), and (d) plasma [chloride] (mmol • L^{-1}) for maturing Late Shuswap sockeye salmon captured in seawater and fresh

water during migration (distance, km). Negative and positive values on the X-axis represent capture locations in either seawater (-) or fresh water (+). The mouth of the Fraser River is defined as 0 km. Sampling locations are shown in Figure 2.1. Spawners were captured in the Adams River, a major spawning tributary for the Lower Shuswap stock. Shaded symbols are offset from sampling location and were fish holding on the spawning grounds for approximately 2 weeks. Sample size ranged from 4 (Lytton, 2006) to 10 (Adams River, 2003). Values are means + 1 se for 2006 values and means – 1 se for 2003 values. # indicates value differs significantly from that of QCI, the sampling location farthest from the river mouth for 2003. * indicates value differs significantly from that of Pt. Hardy (NKA) or QCI for 2006, farthest values from the mouth of the Fraser River.

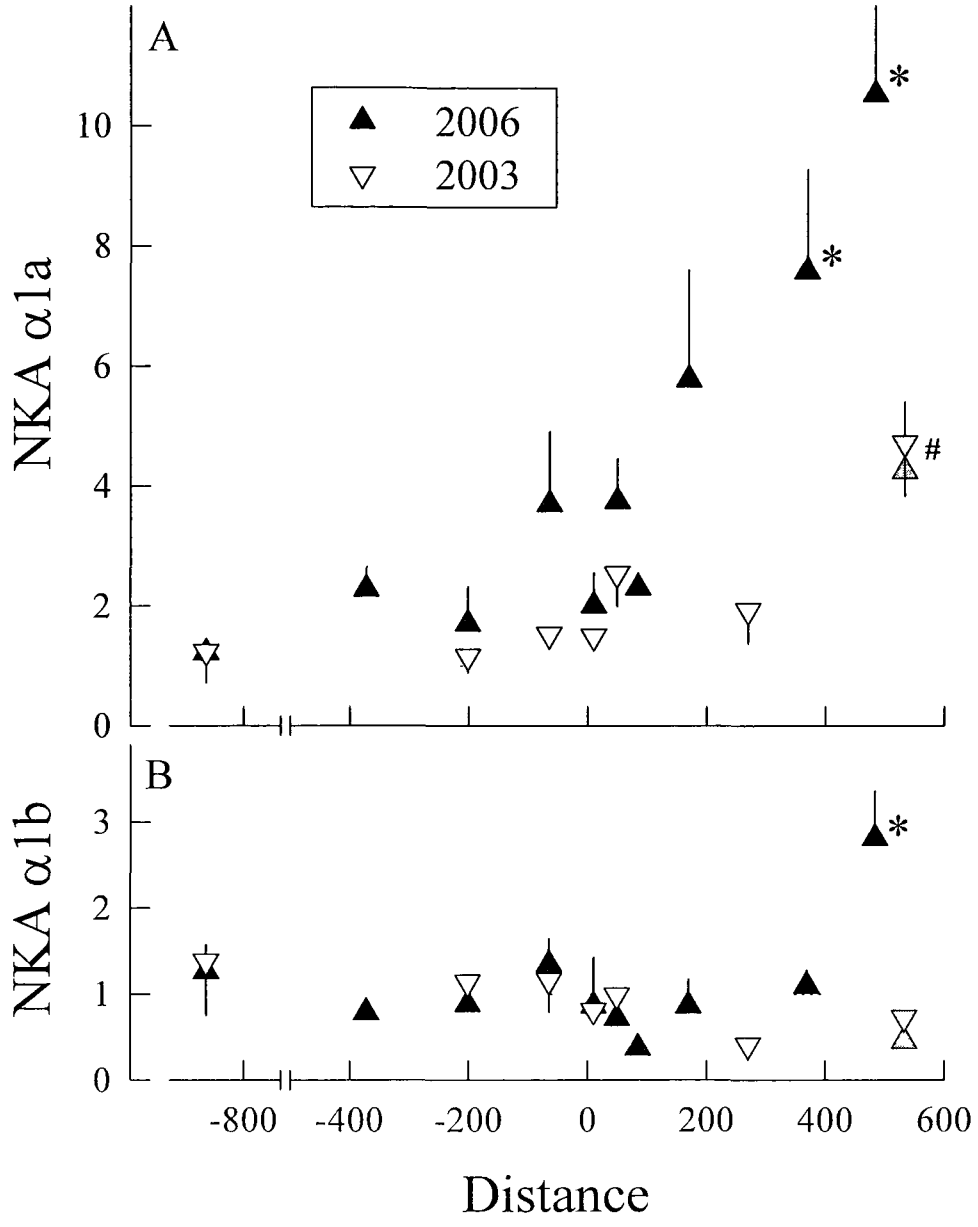

Figure 2.3: (a) Gill Na^+,K^+-ATPase (NKA) isoform α1a mRNA (expressed relative to β-actin) and (b) gill NKA isoform α1b mRNA (expressed relative to β-actin) for Late Shuswap sockeye salmon captured in seawater and fresh water during migration to the spawning grounds (distance, km). Sample size ranged from 6-8 for either year. Negative and positive values represent capture locations in either seawater (-) or fresh water (+). Values are means + 1 se for 2006 values and means – 1 se for 2003 values. The mouth of the Fraser River is defined as 0 km. Sampling locations are shown in Figure 2.1. Shaded symbols are offset from sampling location and were fish holding on the spawning grounds for approximately 2 weeks. # and * indicate significant differences between sampling locations relative to QCI, the farthest location from the river mouth, for samples caught in 2003 and 2006, respectively.

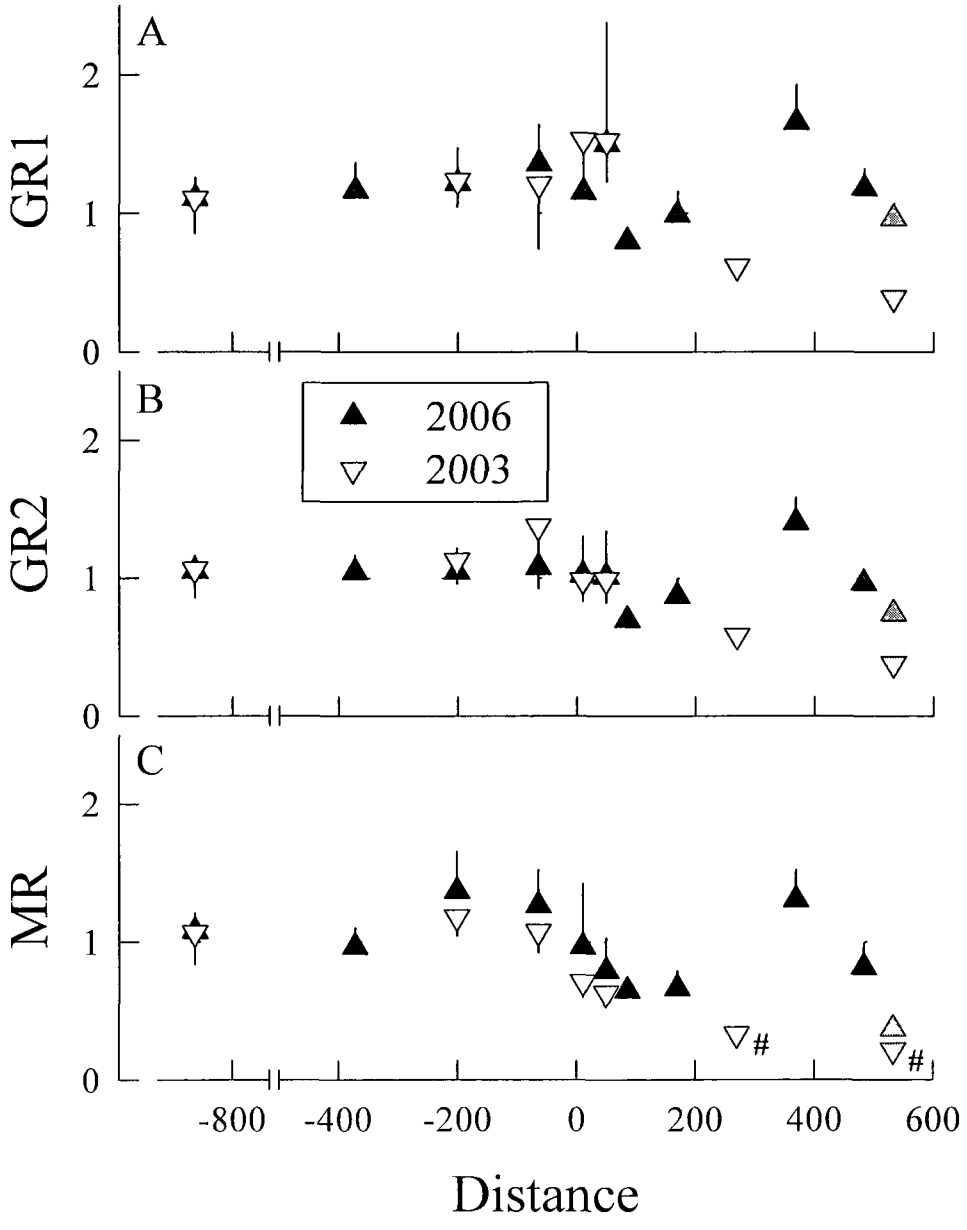

Figure 2.4: (a) Gill Glucocorticoid Receptor 1 (GR1) mRNA (expressed relative to β-actin), (b) gill Glucocorticoid Receptor 2 (GR2) mRNA (expressed relative to β-actin) and (c) gill Mineralocorticoid Receptor (MR) mRNA (expressed relative to β-actin) for Late Shuswap sockeye salmon captured in seawater and fresh water during migration to the spawning grounds (distance, km). Sample size ranged from 6-8 for both years. Values are means + 1 se for 2006 values and means – 1 se for 2003 values. Negative and positive values represent capture locations in either seawater (-) or fresh water (+). The mouth of the Fraser River is defined as 0 km. Sampling locations are shown in Figure 2.1. Shaded symbols are offset from sampling location and were fish holding on the spawning grounds for approximately 2 weeks. # and * indicate significant differences between sampling locations relative to QCI, the farthest location from the river mouth, for samples caught in 2003 and 2006, respectively.

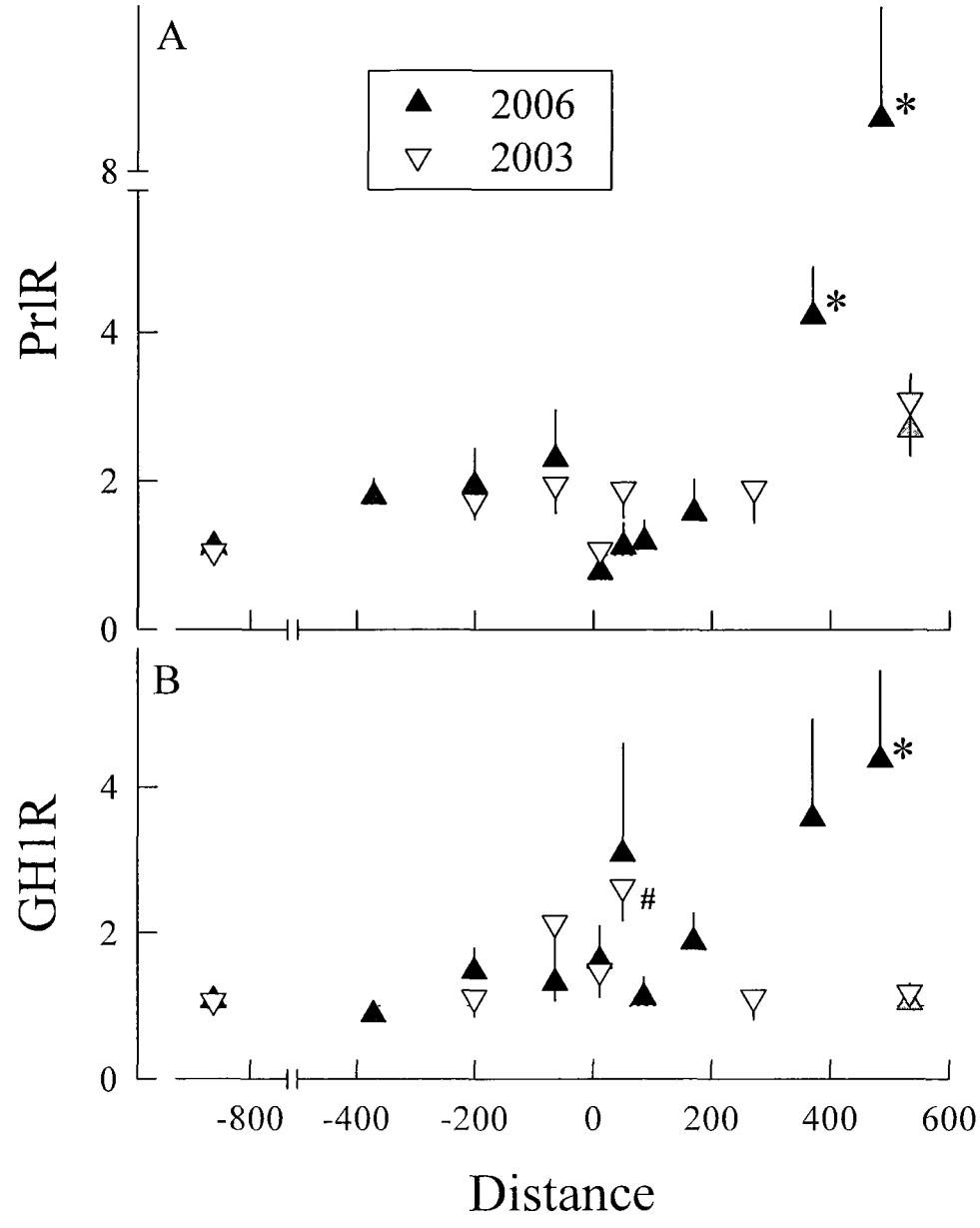

Figure 2 5: (a) Gill Prolactin Receptor (PrlR) mRNA (expressed relative to β-actin) and (b) gill Growth Hormone 1 Receptor (GH1R) mRNA (expressed relative to β-actin) for Late Shuswap sockeye salmon captured in seawater and fresh water during migration to the spawning grounds (distance, km). Sample size ranged from 6-8 for both years. Negative and positive values represent capture locations in either seawater (-) or fresh water (+). The mouth of the Fraser River is defined as 0 km. Values are means + 1 se for 2006 values and means – 1 se for 2003 values. Sampling locations are shown in Figure 2.1. Shaded symbols are offset from sampling location and were fish holding on the spawning grounds for approximately 2 weeks. # and * indicate significant differences between sampling locations relative to QCI, the farthest location from the river mouth, for samples caught in 2003 and 2006, respectively.

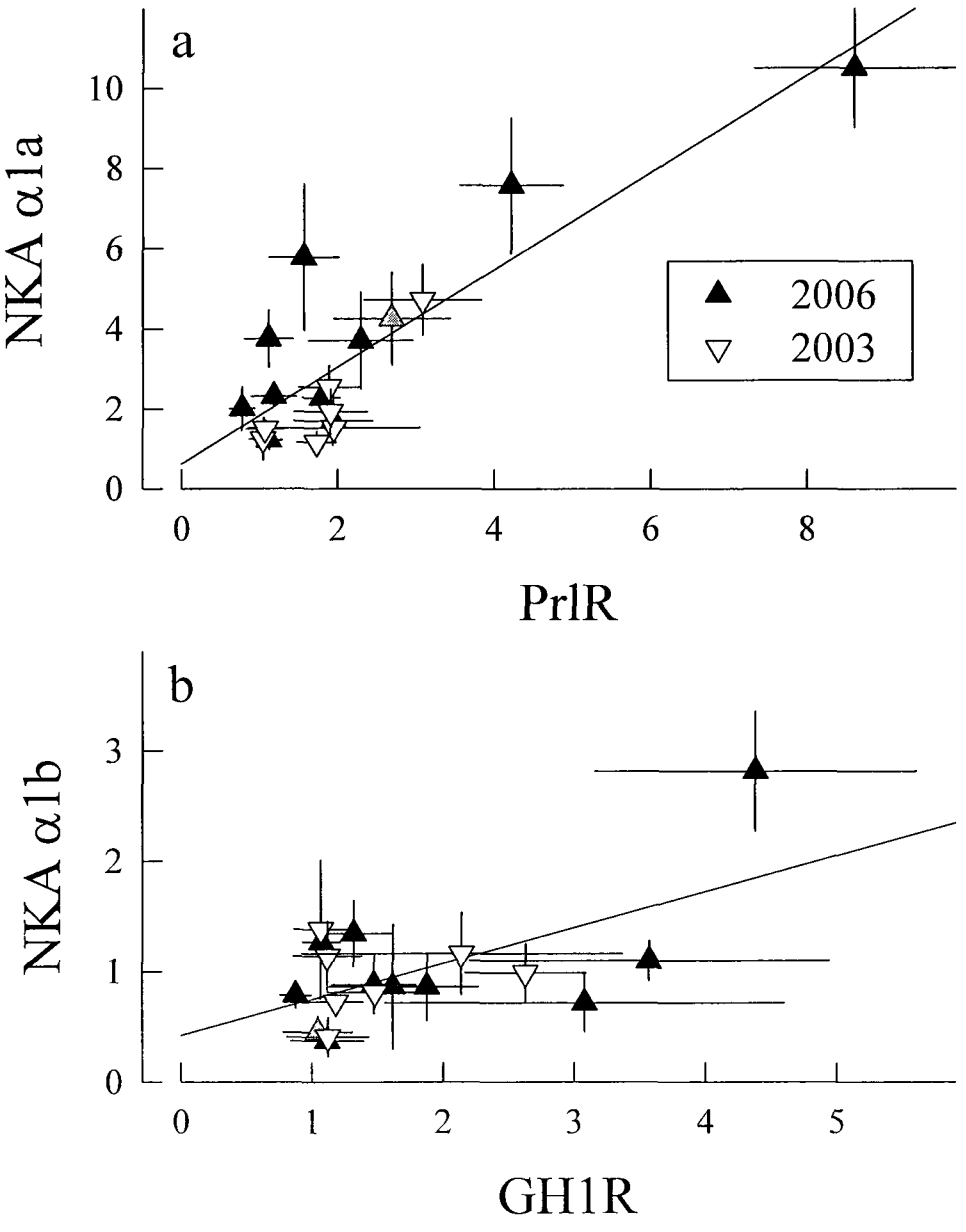

Figure 2.6: (a) Relationship between gill Prolactin Receptor (PrlR) mRNA (expressed relative to β-actin) and gill Na^+,K^+-ATPase (NKA) α1a mRNA (expressed relative to β-actin). (b) Relationship between gill Growth Hormone 1 Receptor (GH1R) mRNA (expressed relative to β-actin) and gill NKA α1b mRNA (expressed relative to β-actin). Values are means ± 1 se.

DISCUSSION

Physiological changes preparatory to freshwater entry

The results presented in this study provide evidence that sockeye salmon make ionoregulatory adjustments to prepare for fresh water in advance of river entry. Gill NKA activity declined significantly while fish were still in the ocean; lower in fish at ~200 km (Johnstone Strait) compared to fish ~800 km (QCI) away (Fig. 2A), even though both locations were in full strength seawater. Uchida *et al.* (1997) and Shrimpton *et al.* (2005) showed earlier that a decrease in gill NKA activity occurs prior to freshwater entry in maturing chum salmon *O. keta* and sockeye salmon and the ability to ionoregulate in seawater decreases before fish enter fresh water. Changes in gill NKA activity are also observed during the parr-smolt transformation; NKA activity increases several fold higher in smolts compared to freshwater parr activities (Shrimpton *et al.*, 2000). The increase in NKA activity, which occurs long before seawater exposure, allows the gill to secrete excess monovalent ions and is linked to an increased development in saltwater tolerance (Marshall, 2002). Migratory adult sockeye showed similar preparatory changes in NKA activity prior to freshwater entry. This suggests that the decrease in NKA activity prepared the fish for freshwater entry while they were still in seawater.

It would be expected that fish with low NKA activity, therefore, are better prepared for freshwater entry; higher NKA activity would be indicative of a seawater fish, not one that is preparing to migrate into fresh water. There is little evidence, however, for a link to in-river mortality for high NKA activity. Cooke *et al.* (2006a,b) found that there was no difference in NKA activity for sockeye salmon that successfully entered fresh water and for fish that failed river entry. Fish for these studies were tagged in Johnstone Strait (~200 km

away from the river mouth), tracked through the lower Fraser River and upstream to Mission, British Columbia (river km 85 km). Gill NKA activity for fish in Johnstone Strait captured in 2003 was significantly lower than for fish sampled near QCI. Gill NKA activity may already be down-regulated by the time the fish are within 200 km of the river mouth. It is also possible, however, that the activity level of NKA measured does not reflect all the changes in α subunits that prepare fish for freshwater entry. The up-regulation of gill NKA α1a mRNA in 2006, the freshwater isoform (Richards *et al.*, 2003) prior to freshwater entry (Table 1; Fig. 3) indicates that remodeling for freshwater ionoregulation occurs in the gill while the fish are still in seawater. Gill NKA α1a mRNA, however, did not differ significantly among seawater sampling locations for 2003, likely due to the less extensive sampling than in 2006. Little change occurred in the gill NKA α1b mRNA during seawater migration. It is interesting to note that the overall effects of gill NKA activity reflect the mRNA expression of NKA α1b more than α1a. The microassay method of McCormick (1993) to measure gill NKA activity does not differentiate among the different α-isoforms and is unable to distinguish between the α1a and α1b subunits. The activity per molecule of NKA may be greater in seawater than fresh water or the method may favour measurement of α1b activity over that of α1a activity (McCormick *et al.*, 2009).

With a decline in gill NKA activity, it would be expected that adult sockeye lose their ability to hypo-osmoregulate in seawater as they get ready to move into fresh water. The expectation, therefore, is that plasma ions increase while fish are still in the ocean. This is the case for plasma sodium (Table 2.1); but not plasma chloride or osmolality (Figure 2.2). In juvenile coho salmon (*O. kisutch*), there is an inverse relationship between gill NKA activity and magnitude of perturbation in plasma sodium concentration following transfer to salt

water (Shrimpton *et al.*, 1994b). Fish with lower NKA had a reduced ability to ionoregulate in salt water resulting in greater mortality. Adult sockeye, however, are migrating toward fresh water and the loss of hypo-osmoregulatory ability appears to be preparative. Indeed, in radiotelemetry studies, Cooke *et al.* (2006a,b) found that adult sockeye salmon that successfully passed Mission, British Columbia (river km 85), had significantly higher plasma sodium levels than fish that failed to enter the river. Thus, the successful fish appeared to have greater perturbations in their plasma sodium while they were still in seawater.

River entry results in a significant drop in plasma sodium indicating that the transition to fresh water is not without further physiological adjustments. The physiological changes observed are comparable to those seen in juvenile smolts as they enter the ocean. Previous work has shown that when transferred to seawater, there is an initial increase in plasma sodium concentrations (Blackburn and Clarke, 1987) and greater perturbations are associated with higher levels of mortality (Shrimpton *et al.*, 1994b). The perturbations in plasma ions following salinity transfer are usually transitory as the animal fully adapts to the new environment within a few days (Blackburn and Clarke 1987). Plasma sodium concentrations rebounded in the adult sockeye as the fish migrated upriver from Cottonwood (river km 11) to Whonnock (river km 50) a distance the fish would migrate in approximately 2 days (Hanson *et al.*, 2008). The recovery from the ionic perturbation would be expected to enable the fish to swim more effectively as Brauner *et al.* (1992) demonstrated that deviations in plasma sodium concentrations following salinity transfer reduced swimming performance.

Parallel to the change in gill NKA activity, plasma [chloride] and osmolality decreased as fish migrated towards the river entrance (Figure 2.2b and 2.2d); there was a significant effect of location on plasma [chloride] and osmolality for fish in seawater in 2003,

but not 2006. The findings are surprising as lower NKA activity would be expected to correspond with an increase in plasma [chloride] and osmolality for fish maintained in seawater as was seen for plasma [sodium]. The drop in plasma osmolality, however, apparently benefits the fish as Cooke *et al.* (2006b) showed that tagged late-run sockeye salmon which successfully entered the river had lower plasma osmolality. The decline in [chloride] and osmolality is difficult to interpret, however, but it may be due to changes in gill morphology. Active chloride secretion in the seawater gill involves the basolateral NKA and sodium, potassium, two chloride (NKCC) cotransporter, apical membrane CFTR anion channels, and paracellular sodium-selective conductance (Marshall, 2002). It is likely that as the gill remodels for freshwater entry, the junctions between the mitochondrial rich cells and the accessory cells becomes less conductive for sodium and lead to the increased plasma [sodium] observed. The lack of a change in NKA α1b mRNA may indicate that chloride secretion is less affected.

What signals the remodelling of the gill in seawater?

There is considerable evidence that endocrine factors signal the physiological changes that accompany migration. Cortisol has long been hypothesized to be the saltwater adapting hormone, but recent evidence also suggests that this hormone plays a role in freshwater ionoregulation. Cortisol treatment was found to stimulate chloride cell proliferation (Laurent and Perry, 1990; Laurent *et al.*, 1994; Sloman *et al.*, 2001) and increase ion uptake in freshwater rainbow trout (Laurent and Perry, 1990). The biological effects of cortisol will not only depend on plasma concentration of the hormone, but number and affinity of the intracellular receptors in the gills (Shrimpton and McCormick 1999; Marsigliante *et al.*,

2000). In the present study, we measured the receptors for cortisol, GR, subtypes GR1 and GR2, and MR. There is experimental evidence that suggests that GR is important for the transition from fresh water to seawater. For example, changes in GR expression have been detected during seawater adaption for the European eel, *Anguilla anguilla* (Marsigliante *et al.*, 2000), rainbow trout (Bury *et al.*, 2003) and Atlantic salmon, *Salmo salar* (Kiilerich *et al.*, 2007b). Alternatively, the role and function of MR is not as well understood, but recent studies suggest that cortisol may act through an MR in freshwater acclimation (Colombe *et al.*, 2000; Sloman *et al.*, 2001). Our results for the cortisol receptors mRNA show little change while fish are migrating in seawater (Figure 2.4) – suggesting that cortisol does not have a preparatory role for freshwater entry. If cortisol played a stimulatory role for physiological changes that prepare adult sockeye for freshwater entry we would have expected to see an increase in one of the three receptors while fish were in seawater that paralleled our physiological data. The lack of any change in steroid receptor mRNA indicates that other endocrine factors are involved.

There is evidence that the two closely related pituitary hormones, Prl and GH, are involved in the control of ionoregulation (Hoar, 1988). It is generally accepted that Prl is important for freshwater ionoregulation, while GH is important for seawater ionoregulation in a large and phylogenetically diverse number of teleosts (McCormick, 2001). During freshwater acclimation, pituitary and plasma Prl levels increase to regulate hydromineral balance by decreasing water uptake and increasing ion retention (Manzon, 2002). Earlier studies examined the effects of Prl injection on the expression of NKA α – subunit (Seidelin and Madsen, 1999; Deane *et al.*, 1999). Unfortunately, the protocol used did not distinguish

between the α – isoforms (i.e. α1a, α1b, α 1c) and more recent work has shown that they are differentially expressed during freshwater and seawater acclimation (Richards et al. 2003).

Prolactin receptors (PrlR) are abundant within the gill (Killerich *et al.*, 2007a) and changes in PrlR expression have been detected in response to changes in environmental salinity (Manzon, 2002). Although PrlR mRNA increased as adult sockeye approached the river mouth, the effect of location on PrlR mRNA for fish in seawater was only significant in 2006, but not 2003 (Table 2.1; Figure 2.5). Earlier studies have shown that circulating levels of Prl increased while adult Atlantic salmon were still in seawater (Andersen *et al.* 1991). Additionally levels of Prl and GH mRNAs from pituitary of adult migratory chum salmon were significantly higher in fish sampled in the ocean than fish captured close to the river mouth (Onuma *et al.* 2003). Thus our results that demonstrate gill PrlR are active while fish are still in seawater are supported by these earlier studies. The lack of an increase in gill GH1R mRNA in the present study for seawater fish is consistent with GH functioning to stimulate salt extrusion in seawater (Björnsson 1997), but not the work of Onuma *et al.* (2003).

Physiological changes during freshwater migration

Even though we present evidence that sockeye salmon prepare for fresh water in advance of entry, maturing sockeye are not in an ionoregulatory steady state during up river migration (Table 2.1), a finding consistent with earlier work by Shrimpton *et al.* (2005). Gill NKA activity, plasma osmolality, [sodium] and [chloride] declined with distance fish migrate upriver. Generally, the lowest values for each measurement after the initial perturbation at Cottonwood (river km 11) were observed for fish approaching or first arriving on the

spawning grounds. The physiological measures of freshwater migrating fish suggest continued physiological changes throughout migration.

Modifications of gill NKA α1a and α1b subunits also indicated that physiological changes continued after sockeye salmon moved into fresh water (Figure 2.3). Isoform switching in gills following salinity transfer suggest that the NKA α1a- and α1b-isoforms play different roles in acclimation and the switching between these two α-subunits changes the salmonid gill from an ion-absorbing epithelium in fresh water to an ion-secreting epithelium in seawater (Richards *et al.*, 2003). Consistent with this model, the expression of NKA α1a significantly increased for wild sockeye salmon following movement from Cottonwood to Savona and onto the spawning grounds. Similar findings for gill NKA α1a were reported for wild anadromous Arctic char migrating from seawater into fresh water (Bystriansky *et al.*, 2007). These authors found that the relative mRNA expression of gill NKA isoform α1a was more than threefold higher in Arctic char migrating upstream than in fish collected in different sites in seawater. Both cases suggest that gill ionoregulatory changes necessary for freshwater acclimation continue following freshwater entry.

The NKA α1b isoform remained fairly constant and no significant changes were seen in response to movement from seawater to fresh water. This lack of change in gill NKA α1b isoform expression was also found for saltwater acclimated rainbow trout when transferred to fresh water (Richards *et al.*, 2003). However, there was a significant change in α1b mRNA for sockeye salmon that just arrived on the spawning grounds. Shrimpton *et al.* (2005) characterized spawning sockeye salmon by this significant increase in gill NKA α1b mRNA and speculated that the increased expression of NKA α1b might be stimulated by cortisol and the gene might be responding to higher cortisol levels found in spawning Pacific salmon.

Are the hormonal signals after freshwater entry the same as before?

The physiological changes that occurred during up river migration do not appear to be regulated by cortisol. Copies of mRNA for GR1, GR2 and MR did not significantly differ with location for fish caught in 2006, but did differ in 2003; although mRNA for all three receptors declined as fish approached the spawning grounds. There is evidence in juvenile Atlantic salmon that MR plays a role in ionoregulation in fresh water; MR transcript levels remain unchanged during smolting, but significantly increased at the onset of de-smoltification (Kiilerich *et al.*, 2007b). If cortisol was stimulating ionoregulation and the changes in NKA α1a, however, we would expect to see a greater magnitude of change for all three receptors at different stages in the migration that parallel our physiological data. Our data suggests that for migrating adult salmon MR does not play a role in ionoregulation in fresh water.

The mRNA for PrlR and GH1R showed significant increases in the gills of sockeye migrating up river. The mRNA levels for both receptors were highest for fish arriving at the spawning grounds and then declined for mature spawners that were holding in the Adams River. The high levels of PrlR mRNA in the gills suggest that Prl is playing an important role in ionoregulation throughout the freshwater migration. Such findings are in agreement with Onuma *et al.* (2003) working on chum salmon. These authors found that pituitary Prl mRNA decreased significantly before fish entered fresh water, but then increased up to five-fold after entry into fresh water.

Shrimpton *et al.* (2005) showed that gill NKA activities in spawners were generally higher than for pre-spawners holding on the spawning grounds and suggested that higher gill

NKA activities were an attempt to compensate for osmotic perturbation. We found less evidence of an osmotic perturbation during freshwater migration in our study; however, both gill α1a and α1b isoforms increased as sockeye migrated up river. The higher mRNA levels of PrlR and GHR may have an ionoregulatory role and modify gill NKA to limit osmotic perturbations. The correlations between PrlR and NKA α1a mRNAs suggest that prolactin is directly stimulating the gill to uptake ions in fresh water. We also found a correlation between GH1R and NKA α1b mRNAs. Given the prominent role that GH plays in smolting (Björnsson, 1997) and NKA α1b in seawater adaptation (Richards *et al.*, 2003; Chapter 1), the increases in mRNA for these two genes are less clear. An increase in NKA α1b in response to high levels of cortisol commonly observed in spawning Pacific salmon has been suggested (Shrimpton *et al.*, 2005). Our mRNA data for the cortisol receptors does not support this suggestion, but rather that GH is directly driving the increase in and NKA α1b due to the significant relationship between gill GH1R mRNA and gill NKA α1b mRNA. The reason for the increased NKA α1b mRNA may be linked to ammonia excretion. A possible mode of ammonia transport out of the plasma has been proposed that NH_4^+ will displace K^+ on the basolateral NKA. As branchial NKA activity is relatively low in freshwater versus seawater fishes, appreciable NH_4^+ transport via this route seems unlikely in freshwater fish (Wilke, 2002). In semelparous salmon during the spawning migration, however, fish rely on muscle protein as an energy source, resulting in a marked increase in ammonia production. Exhaustive exercise also elevates white muscle ammonia levels (Randall and Wright, 1995). The increase in NH_3 production may, therefore, make NH_4^+ transport via the basolateral NKA necessary.

Based on the mRNA levels for the cortisol receptors, our findings suggest that cortisol is not the primary hormone that induces a physiological response during freshwater migration of adult sockeye salmon. Instead, this study is the first to demonstrate an increase in gill PrlR mRNA that parallels the changes in gill NKA α1a. These changes are preparatory to freshwater entry and continue throughout the upriver migration. The molecular endocrine and physiological changes are likely necessary for the fish to limit ionic perturbations caused by changes in salinity and the physical exertion of migration.

EPILOGUE

The objective of my research was to determine whether the mineralocorticoid receptor (MR) for cortisol had a role in stimulating an ionoregulatory response when fish move between waters of different salinities. In my first study, physiological changes were assessed when rainbow trout were transferred from freshwater to an ion-poor treatment or a 75% saltwater treatment. Although plasma [cortisol] increased in both experimental groups, there was no change in mRNA levels for both receptors of cortisol. Given the work of Sathiyaa and Vijayan (2003) on autoregulation of GR, I would have expected a change in mRNA expression for GR1, GR2 or MR if cortisol had activated any of these receptors. My results, therefore, suggest that cortisol was not the primary hormone inducing a physiological response following the experimental challenges in rainbow trout. In my second study, gill tissues from migratory adult sockeye were analyzed to determine if physiological changes preparatory for freshwater entry or during freshwater migration were linked to cortisol and a mineralocorticoid class of receptor. I found no evidence that cortisol was binding to either GR or the MR in sockeye gill tissue, as mRNA for these hormone receptors did not show a relationship to the physiological changes measured. My findings suggest that cortisol does not stimulate a physiological response through MR during migration of adult sockeye.

The findings from both experiments are puzzling, given the extensive literature that proposes a role for cortisol as an important endocrine factor involved in ionoregulation. Previous studies have shown that plasma cortisol and gill CR concentration changes seasonally in conjunction with smolting in several salmonids, including coho salmon (Shrimpton, 1996) and Atlantic salmon (Shrimpton and McCormick, 1998a). The increase in circulating plasma cortisol was coincident with higher gill NKA activity (Shrimpton and

McCormick, 1998a; Shrimpton *et al.*, 2000). The rise in cortisol, therefore, has long been associated with enhanced saltwater tolerance in salmonids (Hoar, 1988).

There is also evidence that experimentally administered cortisol has an ionoregulatory effect. Madsen (1990a) showed that cortisol treatment directly improved the development of hypo-osmoregulatory ability in rainbow trout and that there was a synergistic interaction with GH in promoting chloride cell proliferation (Madsen, 1990b). The results from experimentally administered cortisol treatment, however, are not consistent. Cortisol treatment had no effect on gill NKA activity in an experiment on Atlantic salmon (Langdon *et al.,* 1984) and also in an experiment on coho salmon (Richman *et al.,* 1987). The contradictory findings may be due to different doses, routes of administration and hormone derivatives that influence how cortisol functions (Madsen, 1990c).

The equivocal support for an ionoregulatory role for cortisol does not necessarily explain why cortisol does not appear to be important in my studies; however, cortisol is also a primary stress hormone. Stress increases plasma [cortisol], as well as causes a decrease in the abundance of corticosteroid receptors in the gills (Maule and Schreck 1991; Shrimpton and Randall, 1994). The downregulation of CR results in a lower tissue response to cortisol (Shrimpton and McCormick, 1999), but the effect on mRNA for cortisol receptors has not been studied. Nonetheless, the physiological distinction between the functions of cortisol in stress and as a hypo-osmoregulatory promoter may be linked to the contradictory findings of this hormone among studies. It is possible that the rainbow trout used in the salinity transfer study were stressed, but it seems unlikely that this could solely account for the findings in my study. As I mentioned previously, rainbow trout are not typically anadromous and may not undergo these seasonal changes in sensitivity to cortisol associated with smolting. Plasma

[cortisol] did not change seasonally in steelhead-rainbow trout hybrids, but there was a significant rise in steelheads from March to May (McLeese *et al.*, 1994). Furthermore, there are differences observed in the endocrine response for rainbow trout transferred to abrupt salinity challenges (Sakamoto and Hirano, 1991) and ionoregulation may be dependent on size, strain of fish and state of maturity (Bern and Madsen, 1992).

The above discussion provides a potential explanation for why cortisol did not seem to function in ionoregulation in the experiment with rainbow trout, but does not explain why cortisol did not seem to be involved when sockeye were migrating to fresh water. The majority of studies have focused on the physiological changes that occur during the downstream migration of juvenile salmon and comparatively less is known about the migration of adults from the ocean back to the freshwater spawning grounds. Additionally, there has been little work on the physiological changes that occur in adult salmon transferred from salt water to fresh water. Rather, the focus of some research has been to determine how fish acclimate to ion-deficient water (for example Perry and Laurent, 1989; Sloman *et al.*, 2003). Sloman *et al.* (2003) found that spironolactone, an MR antagonist, inhibited the proliferation of chloride cells normally associated with acclimation to ion-deficient water. Spironolactone, however, was not developed specifically for fish as a receptor blocker and this antagonist may be influencing other endocrine pathways that prevented the changes in gill morphology.

It is possible that cortisol may function through intracellular receptors without an effect on mRNA levels for that receptor. Nongenomic pathways exist that control the rate of protein synthesis and degradation by altering gene expression and half-life of mRNA. For example, microRNAs (miRNAs) affect gene expression post-transcriptionally by repressing

translation and/or by promoting mRNA degradation (Bushati and Cohen, 2007). In zebrafish embryos, miRNA induce significant mRNA decay using general mRNA degradation machinery (Giraldez *et al.*, 2006). The Argonaute family of proteins regulate miRNA by the induction of silencing complexes. Within these complexes, miRNAs guide the Argonaute proteins to fully or partially complementary mRNA targets, which are then silenced post-transcriptionally (Bushati and Cohen, 2007). Suppression of miRNAs would therefore be expected to increase the half-life of mRNA and lead to a greater production of the protein; the methods that I used in my studies would not detect such a change. I found no evidence in the literature, however, that cortisol or other steroids influence miRNA function.

Alternatively, steroid hormones have been shown to rapidly influence a variety of functions through nongenomic mechanisms (Steinman *et al.*, 2010). The nongenomic model assumes that steroid hormones bind directly to receptors in the cell membrane to activate cellular signalling pathways. For example, Orchinik *et al.* (1991) found that injecting corticosterone rapidly suppressed the sexual behaviour of male salamanders *Taricha granulosa*; a behavioural response linked to corticosterone receptors on synaptic membranes. Rapid changes stimulated by steroids (seconds or minutes compared to hours or days) have been associated with behaviour, rather than genomic activation and the synthesis of new proteins (Steinman *et al.*, 2010). The literature, however, does not support an ionoregulatory response without modifying gene expression in teleosts.

Although I have been looking for a role for a single hormone believed to be responsible for ionoregulation, multiple hormones are often involved and work together to elicit a physiological response. Problems arise since it is not easy to differentiate and extract the hormones that respond solely to changes in salinity (Takei, 2008). Disentangling the

potential role that each hormone has during ionoregulation requires a better understanding of the linkages among the different hormone systems (Sloman *et al.*, 2003). Among the seawater adapting hormones, growth hormone, cortisol and insulin-like growth factor-I are long-acting hormones that promote saltwater acclimation (Takei, 2008). These hormones are called slow-acting hormones because the plasma levels tend to increase slowly after fish are transferred from freshwater to saltwater (Sakamoto *et al.*, 1993). Saltwater acclimation also involves oligopeptide hormones such as angiotensisn II, urotensins, vasoactive intestinal polypeptide, guanylins and natriuretic peptides, known as fast and short-acting hormones. These fast-acting hormones are secreted immediately after changes in environmental salinity and have been speculated to govern the whole process of saltwater acclimation (Takei, 2008). Therefore, if some of these hormones are lacking or are not present, the physiological response due to salinity change may be affected, stimulating an alternate or different signal to cortisol. For example, prolactin is an important hormone for freshwater acclimation for most teleosts, but the importance of prolactin in freshwater acclimation varies both between and within species (Manzon, 2002). The majority of research has focused on prolactin antagonizing effect on seawater or its role in ion-poor water. Further studies are required to examine the interaction between prolactin and cortisol on hypo-osmoregulation and whether some of the fast-acting hormones are present and needed for freshwater acclimation. My data, however, indicates that cortisol is not the primary hormone stimulating physiological responses during changes in salinity. The higher levels of plasma cortisol found in rainbow trout (Figure 1.1) and migrating adult sockeye salmon (data not shown) may be linked to stress and other processes that are required to maintain homeostasis.

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