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ORIGINAL PAPER

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

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Abstract Maturing adult sockeye salmon *Oncorhynchus nerka* were intercepted while migrating in the ocean and upstream in freshwater over a combined distance of more than 1,300 km to determine physiological and endocrine changes associated with ionoregulation. Sockeye migrating through seawater and freshwater showed consistent declines in gill Na^+/K^+-ATPase (NKA) activity, plasma osmolality and plasma chloride concentration. In contrast, plasma sodium concentration became elevated in seawater as fish approached the river mouth and was then restored after sockeye entered the river. Accompanying the movement from seawater to freshwater was a significant increase in mRNA for the NKA α1a subunit in the gill, with little change in the α1b subunit. Potential endocrine signals stimulating the physiological changes during migration were assessed by measuring plasma cortisol and prolactin (Prl) concentrations and 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). Plasma cortisol and prolactin concentrations were high in seawater suggesting a preparatory endocrine signal before freshwater entry. Generally, the mRNA expression for GR1, GR2 and MR declined during migration, most notably after fish entered freshwater. In contrast, PrlR mRNA increased throughout migration, particularly as sockeye approached the spawning grounds. A highly significant association existed between gill PrlR mRNA and gill NKA α1a mRNA. GH1R mRNA also increased significantly, but only after sockeye had migrated beyond tidal influence in the river and then again just before the fish reached the spawning grounds. These findings suggest that cortisol and prolactin stimulate ionoregulation in the gill as sockeye salmon adapt to freshwater.

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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, particularly for anadromous Pacific salmon (*Oncorhynchus* spp.) during their reproductive migration to natal streams (Hinch et al. 2006). The majority of Pacific salmon are semelparous and failure to migrate to the spawning grounds in freshwater to reproduce means an individual does not contribute genetically to future generations, resulting in no lifetime fitness (Dingle 1980). Maturing adults that are leaving the ocean for freshwater undergo physiological transformations to maintain osmotic and ionic balance (Shrimpton et al. 2005). This ability is dependent on the regulation of water and ions by several organs (gills, intestine, urinary bladder and kidney), with the gills playing the primary role in the maintenance of ion balance in fish acclimated to both freshwater and seawater (Marshall 2002). The principle enzyme involved in ion-absorption and ion-secretion in the gill is Na^+/K^+-ATPase (NKA) (McCormick 2001). Juvenile salmon in freshwater, preparing to migrate to the ocean, increase gill NKA activity (Shrimpton et al. 2001) and a comparable decrease in gill NKA activity occurs before maturing adult salmon in seawater return to freshwater to spawn (Shrimpton et al. 2005). The decline in NKA activity may promote a higher tolerance to the lower levels of salinity in freshwater, the converse of the parr-smolt transformation. Furthermore, two isoforms of the NKA α-subunit (α1a and α1b) have been identified to change in rainbow trout (*Oncorhynchus mykiss*) transferred among waters of different salinity (Richards et al. 2003). In freshwater, NKA α1a is the abundant isoform present in lamellar chloride cells in the gills, while NKA α1b becomes the dominant isoform after seawater acclimation and is found primarily in filamental chloride cells (McCormick et al. 2009).

The neuroendocrine system is the primary link between seasonal environment changes and physiological adaptations; therefore, the hormonal control of NKA is critical for maintaining homeostasis in both freshwater and seawater (McCormick 2001). The sensitivity of target cells to specific hormones 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) both increase when fish move from seawater to freshwater (Manzon 2002) raising the possibility that both hormones are involved in the acclimation to freshwater, yet increased cortisol has also been linked to seawater acclimation (McCormick 2001). Thus, 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 (MR), has been identified (Colombe et al. 2000). Potentially, cortisol may function through an MR in freshwater ionoregulation in concert with Prl.

In the present study, we investigated which hormonal signals have a role in stimulating physiological modifications in wild adult sockeye salmon that were intercepted in the ocean and in freshwater while migrating to their natal spawning grounds in the Fraser River watershed, British Columbia, Canada. Gill NKA activity, plasma osmolality, plasma chloride concentration, plasma sodium concentration and mRNA in the gill for NKA α1a and α1b were measured to assess physiological changes that occurred during migration in seawater, following freshwater entry and during upstream migration. To elucidate what endocrine signals may be associated with the physiological changes, plasma cortisol and Prl concentrations were measured and 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, Canada, and at several locations during their upstream migration through the Fraser River system in both 2003 and 2006 over a combined distance of greater than 1,300 km. Sampling was timed to intercept the peak of the spawning run for the Late Shuswap sockeye salmon population, although multiple spawning populations of sockeye salmon were caught at each sampling location. Population identification was determined a posteriori using tissue biopsy and variation in genetic markers, as outlined by Beacham et al. (2004). The data reported here are only for Late Shuswap sockeye, which resulted in sample size varying among locations and between the years. The mean length and weight of fish sampled in 2003 and 2006 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, and were not significantly different between 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) (Fig. 1). Salinities off the north coast of British Columbia are greater than 32‰. In the more coastal areas of Johnstone and Georgia Straits, salinity levels fluctuate seasonally from the river run-off in the spring, but levels usually exceed 27‰ in August and September when sockeye are migrating through the region. At the mouth of the Fraser River, however, surface water salinities fluctuate with the tide. In this area, sockeye prefer to be below the halocline. For their upstream migration, sockeye were caught in the lower Fraser River at Cottonwood (C, ~11 km), an estuarine site, at Whonnock (W, ~50 km), the first freshwater sampling site beyond the saltwater intrusion, and in 2006 at a tag-site just past Whonnock (~75 km). Sampling occurred as the fish moved upstream, at Yale (Y, ~170 km), Lytton (L, ~255 km), Savona (S, ~369 km), and at one of the main spawning areas for the Late Shuswap, the Adams River (AR, 484 km). Fish were sampled twice on the spawning areas; when they had just arrived as healthy gravid fish and when they had become sexually mature after being on the spawning grounds for approximately two weeks. (Sexually mature fish were 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 (2003) and in Georgia Strait; purse seine off the coast of the Queen Charlotte Islands (2006) and off the coast of Vancouver Island near Port Hardy and in Johnstone Strait; gill netting in the Fraser River at Cottonwood, Whonnock, and Yale; dip net at Lytton; and beach seining at Savona and on the spawning areas.

Tissue sampling

The fish sampled were part of normal test fisheries, First Nations Fisheries, or stock assessment operations and the type of treatment for each fish was dependent 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. Fish captured by troll line were landed and sampled within minutes. When using gill nets, the soak time was reduced to less than 15 min and only fish that were still moving vigorously at capture were sampled. Handling stress has been shown to result in a release of stress hormones, notably cortisol which shows a significant increase within approximately 10 min for rainbow trout at 13°C (Sumpter et al. 1986). To assess whether constraint in the seine differentially affected plasma cortisol concentration, we compared the sequence fish were sampled with plasma cortisol concentration for two marine sites where sockeye were captured, but found no relationship for either site ($F_{1,45} = 0.39$, $P = 0.53$; $F_{1,25} = 0.69$, $P = 0.41$). We cannot rule out that an increase in cortisol occurred before we sampled the first fish. With this exception, however, the effect of sampling protocol on endocrine, ionoregulatory and molecular vari-

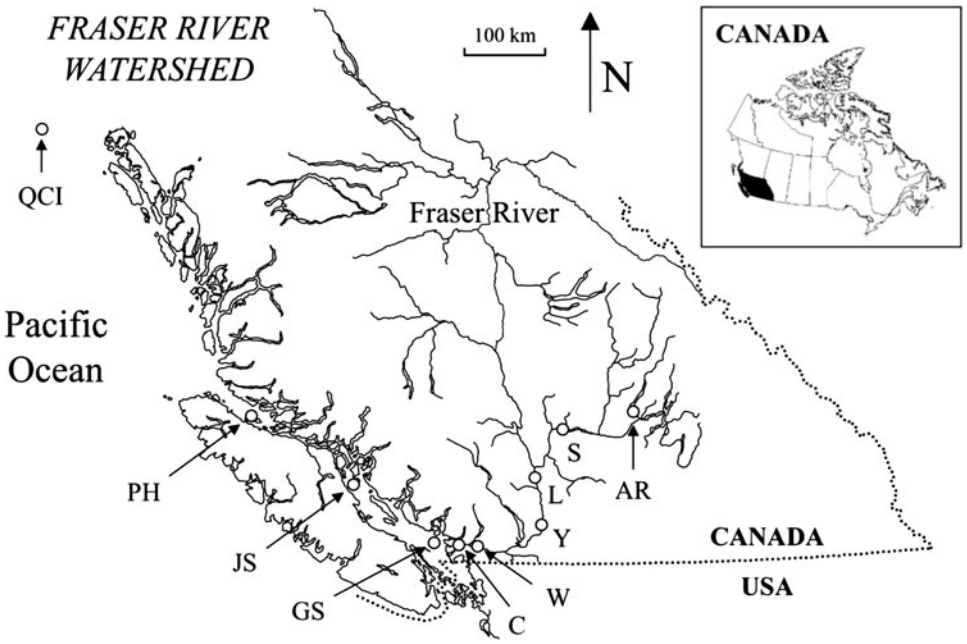

Fig. 1 Map of the Fraser River watershed and coastal British Columbia, Canada showing locations where Late Shuswap stock were intercepted during migration. Samples were collected off the Queen Charlotte Islands (*QCI*), Port Hardy (*PH*), Johnstone Strait (*JS*), and Georgia Strait (*GS*) in seawater. 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*)

ables measured in our study is expected to be minimal. Endocrine changes associated with stress lead to alterations in ionoregulation after 1 h (Shrimpton et al. 2001). In rainbow trout hepatocytes, changes in GR mRNA levels are seen approximately 8 h following cortisol treatment (Sathiyaa and Vijayan 2003) and changes in rainbow trout gill MR mRNA levels occur by 8 h after a 30 s handling stress (Yada et al. 2007). In response to changes in salinity, gill NKA α1 subunit mRNA change significantly by 24 h and reach maximal changes by 72 h (Richards et al. 2003). All fish were killed via cerebral percussion and both blood and gill tissue samples were taken immediately. Blood was collected from the caudal vasculature and a section of gill arch was taken to determine NKA activity and 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) and weight (g) were measured and an adipose fin clip removed for stock identification. All sampling protocols were approved by the University of British Columbia Animal Care Committee.

Gill Na^+/K^+-ATPase activity and plasma analysis

Gill NKA activity was measured using the microassay protocol of McCormick (1993). In brief, gill filaments were homogenized in SEI buffer (150 mM sucrose, 10 mM Na_2EDTA, 50 mM imidazole, pH 7.3) containing 0.1 % sodium deoxycholate (SEID). After centrifugation (5,000*g* for 30 s), the supernatant was used to determine the activity by relating ATP hydrolysis to the oxidation of NADH, measured at 340 nm for 10 min at 25°C in the presence and absence of 0.5 mmol L^{-1} ouabain on a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). Protein content was then measured using a bicinchoninic acid protein assay (Pierce, Rockford, IL, USA). Specific activities were expressed as μmol ADP mg^{-1} of protein h^{-1}.

Plasma samples were thawed, vortexed and centrifuged for 5 min before analysis. Osmolality was measured in duplicate using a model 5500 Wescor Vapour Pressure meter (Logan, UT, USA). Plasma sodium was measured using a model 410 Cole-Parmer single channel flame photometer (Montreal, PQ, Canada). 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, USA); values were checked against a chloride standard before and after approximately 10 duplicates. Plasma cortisol concentrations were quantified with an enzyme immunoassay (EIA) using methods outlined by Carey and McCormick (1998). Plasma Prl was measured according to the radioimmunoassay (RIA) protocol of Hirano et al. (1985).

Gill mRNA expression

Gill tissue (approximately 20–50 mg) was homogenized in a Geno/Grinder 2000 (BT&C, Inc., Burlington, ON, Canada). Samples were extracted for total RNA using *RNeasy Mini Kit* (Qiagen, Mississauga, ON, Canada). Isolated RNA was dissolved in 30 μL RNase-free deionized water and treated to eliminate genomic DNA. Concentration and purity of RNA 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, USA). 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 (qRT-PCR)].

Messenger RNA (mRNA) was measured for genes involved in ion regulation using qRT-PCR. We used primers designed for *O. mykiss* by Madsen et al. (2008) for the NKA α1a-isoform and by Richards et al. (2003) for the NKA α1b-isoform. To assess the role of hormones in response to migration and changes in salinity, receptor mRNA for cortisol, growth hormone and prolactin were measured. Primers designed for *O. mykiss* by Sathiyaa and Vijayan (2003) for GR1 and by McCormick et al. (2008) for GR2 and MR, by Very et al. (2005) for GH1R and by Kiilerich et al. (2007) for PrlR were used. *β*-actin primers designed by Sathiyaa and Vijayan (2003) for *O. mykiss* were used as a reference gene.

To verify that all primers 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, USA) using *Taq* DNA polymerase (Invitrogen, Burlington, ON, Canada). 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 s at 95°C (denaturation), 1 min at 58°C (annealing) and 5 s at 72°C (extension). In the last cycle, the extension time was increased to 5 min. 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.

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, USA) at the University of British Columbia—Okanagan (Kelowna, BC, Canada). 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). β-actin showed 98% similarity to the *O. mykiss* β-actin gene (GenBank Accession No. AF157514) and 100% similarity to the *O. nerka* β-actin gene (GenBank Accession No. AB481206.1).

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, Canada). The resulting PCR products were then cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). The purified plasmids (QIAprep, Miniprep Kit; Qiagen, Mississauga, ON, Canada) obtained were then sequenced using the Beckman-Coulter CEQ 8000 (Mississauga, ON, Canada) at the University of Northern British Columbia (Prince George, BC, Canada). All of the 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). PrlR showed 100% similarity to the *O. mykiss* PrlR (GenBank Accession No. EU084744.1). GH1R receptor sequence revealed a 95% similarity to the *O. mykiss* GH1R (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., Streetville, ON, Canada). All qRT-PCR reactions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min (ABI Prism 7300, Applied Biosystems Inc., Foster City, CA, USA). Melt curve analysis was performed after each reaction to confirm the presence of a single reaction product. 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 more than 1:1,000,000 starting copies for the NKA α1a isoform, 1:190,000 starting copies for NKA α1b isoform, 1:1700 starting copies for GR1, 1:350 starting copies for GR2, 1: 22,000 starting copies for MR, 1:3600 starting copies for PrlR, 1:1800 starting copies for GH1R, and 1:7800 starting copies for β-actin. Genomic DNA contamination, therefore, was considered to be negligible.

Randomly selected samples were serially diluted to develop a standard curve relating threshold cycle to concentration of cDNA for each primer set. Slopes were linear and similar for all genes, suggesting that the amplification efficiency in the qRT-PCR reactions did not differ among genes. The relative expression of the target genes, therefore, could be normalized to a reference gene by utilizing the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001). Gene expression levels were normalized relative to the reference gene, β-actin. Relative expression of mRNA was related to samples collected from the QCI location, the farthest seawater sampling location from freshwater entry. To verify normalization of qRT-PCR results, elongation factor 1α mRNA was also used and there were no appreciable differences between normalization methods; primers from Richards et al. (2003).

Statistical analyses

We analyzed the data using two approaches. First, sockeye salmon 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 on any of the variables measured. The analysis was repeated for freshwater locations to determine significant changes while fish migrated upstream. Sampling was more extensive in 2006 than 2003 and data were not directly compared between the 2 years. For the second approach, we used a one-way ANOVA to compare across all sampling locations in seawater and freshwater to determine where significant changes in the variables occurred during the migration. When significant differences were found, a Bonferroni test was conducted to identify differences between sample locations. Linear regression analysis was then used to test for relationships between relative mRNA levels for genes that showed similar temporal patterns of change. Statistical significance was evaluated at a level of $P < 0.05$. All values are expressed as means ± 1 sem.

Results

Adult Late Shuswap sockeye salmon exhibited significant physiological changes as they migrated from the ocean back to their natal spawning grounds. For fish captured in seawater, a number of variables changed significantly as fish migrated approximately 800 km from the Queen Charlotte Islands to the mouth of the Fraser River in the Georgia Strait. Variables that changed significantly in one or both years were gill NKA, plasma ion concentrations, plasma Prl concentration, gill NKA α1a mRNA, and gill PrlR mRNA (Table 1). For fish captured as they migrated upstream, all physiological variables measured differed significantly among sample sites in one or both years (Table 1).

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 both 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, and declined significantly before fish entered freshwater, remaining low while they migrated upstream (Fig. 2a).

Table 1 Results of one-way ANOVA for sockeye salmon captured migrating in seawater and captured migrating in freshwater

	Year	Seawater				Freshwater			
		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**
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**
Plasma cortisol concentration	2006	34	1.631	2	0.212	**26**	**44.53**	**2**	**<0.001**
Plasma prolactin concentration	2006	**24**	**5.419**	**2**	**0.013**	**47**	**8.122**	**5**	**<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 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
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**

Measurements of plasma variables also showed significant changes with location in migrating sockeye salmon. Plasma osmolality significantly declined throughout migration in both 2003 ($F_{6,48} = 17.9$; $P < 0.001$) and 2006 ($F_{10,75} = 13.5$; $P < 0.001$; Fig. 2b). In 2003, osmolality significantly declined prior to freshwater entry and continued declining during up river migration. 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 (Fig. 2c). Plasma chloride changes mirrored those of plasma osmolality with significant declines in both 2003 ($F_{6,48} = 8.09$; $P < 0.001$) and 2006 ($F_{10,76} = 12.2$; $P < 0.001$). Significant differences from the QCI chloride values were not observed until after freshwater entry, although plasma chloride at Cottonwood also differed significantly from Georgia Strait (Fig. 2d). Plasma sodium increased significantly in seawater as the fish migrated toward the mouth of the Fraser River, declined significantly as fish moved into freshwater, and rebounded as fish migrated up river.

Plasma cortisol differed significantly with location ($F_{5,54} = 11.5$; $P < 0.001$); values were high in seawater, dropped after freshwater entry and then increased significantly for fish on the spawning grounds (Fig. 3a). Plasma Prl also differed significantly with location ($F_{8,62} = 6.74$: $P < 0.001$); values were highest for fish captured at Johnstone Strait (~200 km from the river mouth), Whonnock (river 50 km) and Yale (river 170 km). The highest plasma Prl levels were for fish from Yale which differed significantly from fish sampled at Port Hardy (~375 km from the river mouth), Georgia Strait (~25 km from the river), Cottonwood (river 11 km), and all sites further upstream (Fig. 3b).

Gill NKA α1a mRNA levels significantly changed with location in both 2003 ($F_{6,41} = 5.91$; $P < 0.001$) and 2006 ($F_{10,69} = 7.70$; $P < 0.001$), with up to tenfold increases in mRNA copies throughout the migration, although only locations close to and on the spawning grounds were significantly different from QCI (Fig. 4a). In contrast, the change in gill NKA α1b subunit mRNA was smaller in migrating sockeye (Fig. 4b), although the effect of location was significant in 2006 ($F_{10,69} = 4.74$, $P < 0.001$), it was not in 2003 ($F_{6,41} = 1.81$; $P = 0.34$). The significant effect of location in 2006 was related to a more than twofold increase in mRNA copies for fish sampled at the Adams River site on arrival at the spawning area. Only significant changes for gill NKA α1b subunit mRNA were seen in freshwater (Table 1).

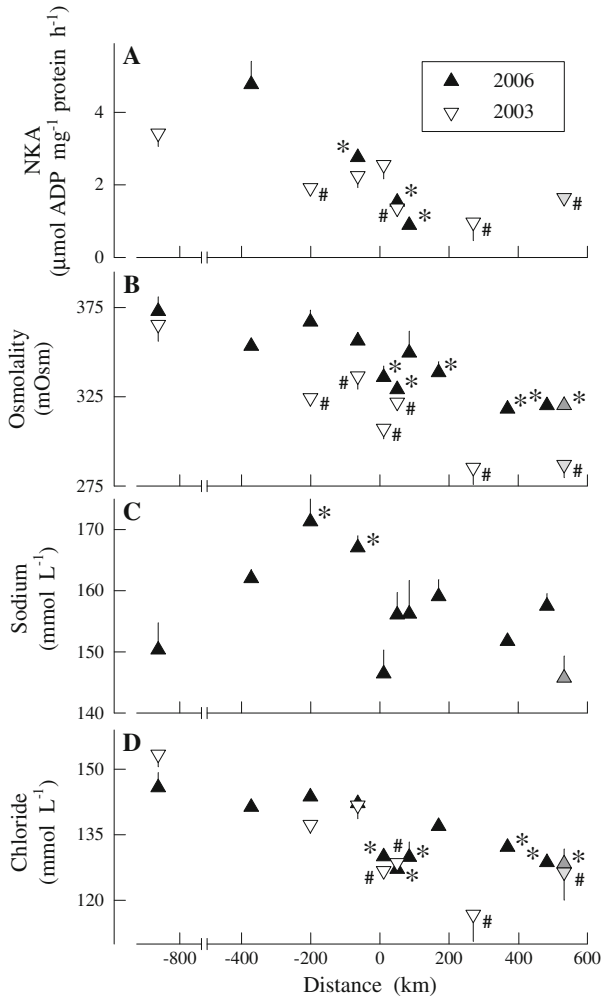

Fig. 2 **a** Gill Na^+/K^+-ATPase (NKA) activity (μmol ADP mg^{-1} of protein h^{-1}), **b** plasma osmolality (mOsm), **c** plasma sodium concentration (mmol L^{-1}), and **d** plasma chloride concentration (mmol L^{-1}) for maturing Late Shuswap sockeye salmon captured in seawater and freshwater during migration (distance, km). Negative and positive values on the distance axis represent capture locations in either seawater (−) or freshwater (+). The mouth of the Fraser River is defined as 0 km. Sampling locations are shown in Fig. 1. Spawners were captured in the Adams River, a major spawning tributary for the Late 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 sem for 2006 values and means − 1 sem for 2003 values. *Hashes* indicates value differs significantly from that of QCI, the sampling location farthest from the river mouth for 2003. *Asterisks* indicates value differs significantly from that of Port Hardy (NKA) or QCI for 2006, the farthest locations from the mouth of the Fraser River

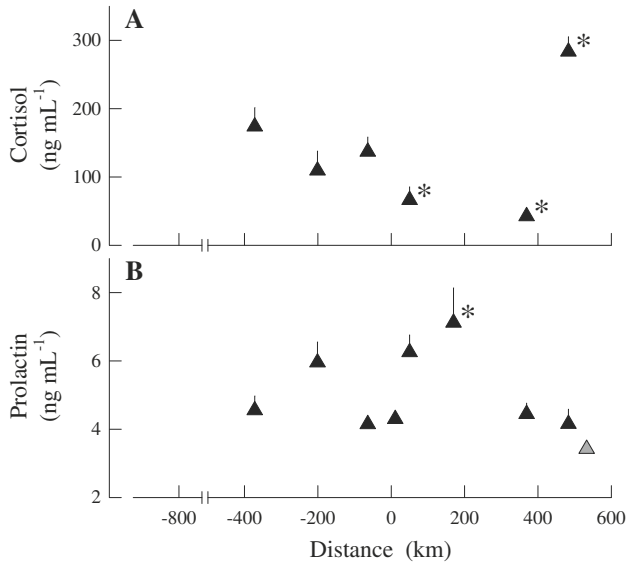

Fig. 3 **a** Plasma cortisol concentration (ng mL^{-1}) and **b** plasma prolactin concentration (ng mL^{-1}) for maturing Late Shuswap sockeye salmon captured in seawater and freshwater during migration (distance, km). See Fig. 1 for sample locations and Fig. 2 for description of figure. For cortisol sample size ranged from 6 (Savona) to 10 (Georgia Strait) and for prolactin sample size was 8 for all locations except 7 for Cottonwood. Values are means + 1 sem for fish caught in 2006. *Asterisks* indicates value differs significantly from that of Port Hardy

The steroid hormone receptor mRNA levels showed little change with location. They were generally lower in freshwater than seawater and significant effects of location were limited to freshwater sites (Table 1), being more pronounced in 2003 than in 2006. The decline in 2003 resulted in significant differences with location for GR1 mRNA ($F_{6,41} = 4.37$; $P < 0.005$), GR2 mRNA ($F_{6,41} = 4.11$; $P < 0.005$), and MR mRNA ($F_{6,41} = 13.49$; $P < 0.001$). Only the values for MR mRNA from the sample locations farthest up river differed significantly from QCI (Fig. 5). For 2006, none of the glucocorticoid receptor mRNA levels 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 higher MR mRNA values for fish captured in Johnstone Strait and at Savona differed significantly from fish holding on the spawning grounds (Fig. 5c).

Significant changes in PrlR mRNA levels were seen with location in both 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 freshwater, as well as during the upriver migration (Table 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 area. For 2006, QCI fish differed significantly from fish captured at Savona and on the spawning area in the Adams River (Fig. 6a). Gill PrlR mRNA expression was high at the same sample locations as gill NKA α1a mRNA and there was a highly significant linear relationship between these two genes ($F_{1,125} = 46.76$, $P < 0.001$; Fig. 7a). GH1R mRNA expression also showed significant changes in location in both 2003 ($F_{6,41} = 4.26$; $P < 0.005$)

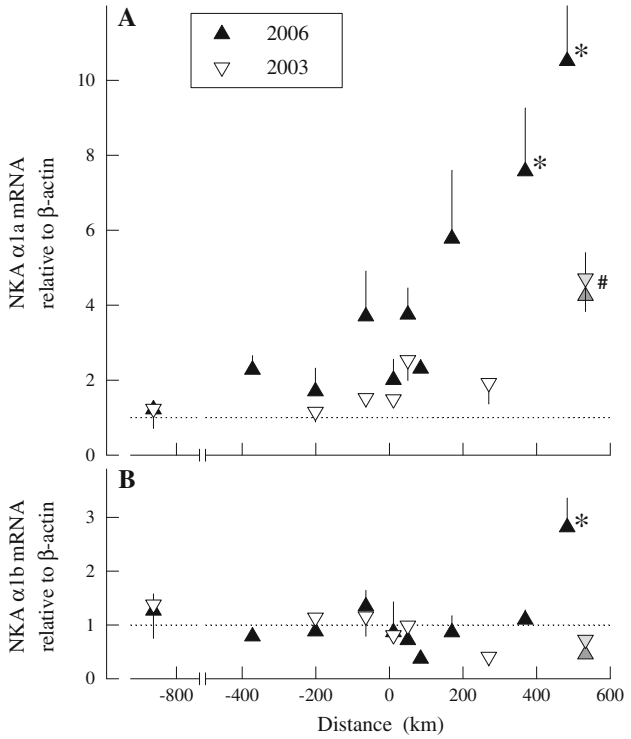

Fig. 4 **a** Gill Na^+/K^+-ATPase (NKA) isoform α1a mRNA (expressed relative to *β*-actin) and **b** gill NKA isoform α1b mRNA (expressed relative to *β*-actin) for Late Shuswap sockeye salmon captured in seawater and freshwater during migration to the spawning grounds (distance, km). Sample size ranged from 6 to 8 for either year. See Fig. 1 for sample locations and Fig. 2 for description of figure. *Hashes* and *asterisks* indicate significant differences between sampling locations relative to QCI, the farthest location from the river mouth, for samples caught in 2003 and 2006, respectively

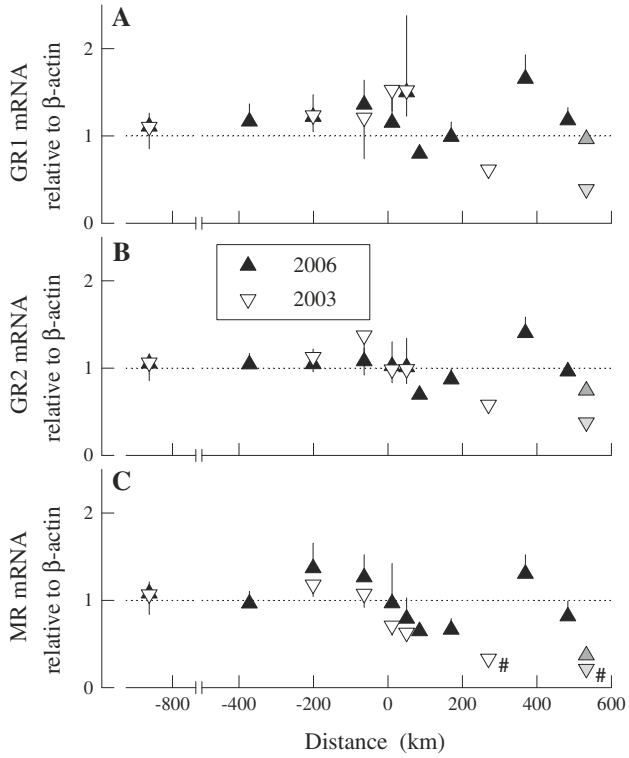

Fig. 5 **a** Gill glucocorticoid receptor 1 (GR1) mRNA (expressed relative to *β*-actin), **b** gill glucocorticoid receptor 2 (GR2) mRNA (expressed relative to *β*-actin) and **c** gill mineralocorticoid receptor (MR) mRNA (expressed relative to *β*-actin) for Late Shuswap sockeye salmon captured in seawater and freshwater during migration to the spawning grounds (distance, km). Sample size ranged from 6 to 8 for both years. See Fig. 1 for sample locations and Fig. 2 for description of figure. *Hashes* and *asterisks* indicate significant differences between sampling locations relative to QCI, the farthest location from the river mouth, for samples caught in 2003 and 2006, respectively

and 2006 ($F_{10,69} = 2.98$; $P < 0.005$). Little change was observed in GH1R mRNA in seawater, but values were higher in freshwater and differed significantly from QCI at Whonnock in 2003 and from QCI at the Adams River in 2006 (Fig. 6b). Gill GH1R mRNA was also high when gill NKA α1b mRNA was high and a significant linear relationship existed between these two genes ($F_{1,125} = 23.54$; $P < 0.001$; Fig. 7b).

Discussion

Physiological changes prior to freshwater entry

The results presented in this study provide evidence that sockeye salmon make ionoregulatory adjustments to prepare for freshwater well 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 had high salinity. Given that migrating adult Pacific salmon are thought to cover about 25 km per day as they approach the Fraser River (Hanson et al. 2008), this means that they are preparing for freshwater entry at least 1–2 weeks before they reach the Fraser River estuary waters. 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 with the decline in NKA activity prior to freshwater entry.

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 freshwater. There is not much evidence, however, for a link between high NKA

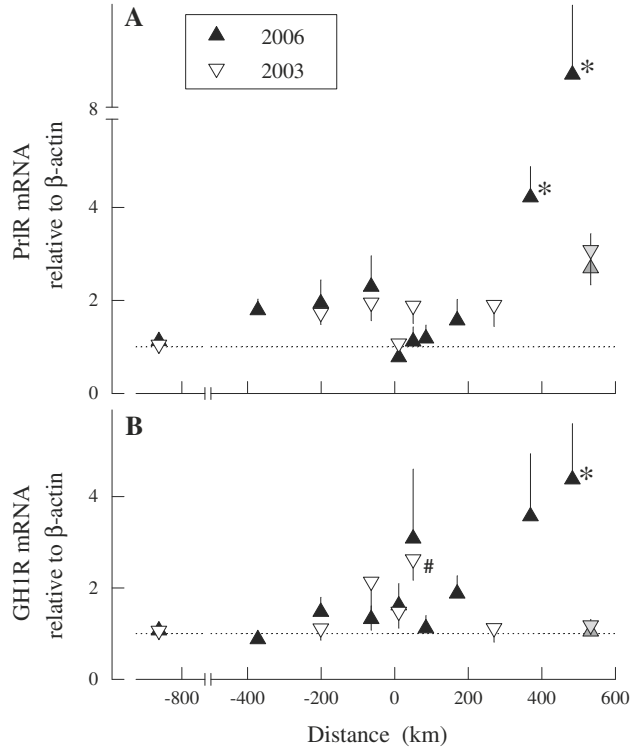

Fig. 6 **a** Gill prolactin receptor (PrlR) mRNA (expressed relative to *β*-actin) and **b** gill growth hormone 1 receptor (GH1R) mRNA (expressed relative to *β*-actin) for Late Shuswap sockeye salmon captured in seawater and freshwater during migration to the spawning grounds (distance, km). Sample size ranged from 6 to 8 for both years. See Fig. 1 for sample locations and Fig. 2 for description of figure. *Hashes* and *asterisks* indicate significant differences between sampling locations relative to QCI, the farthest location from the river mouth, for samples caught in 2003 and 2006, respectively

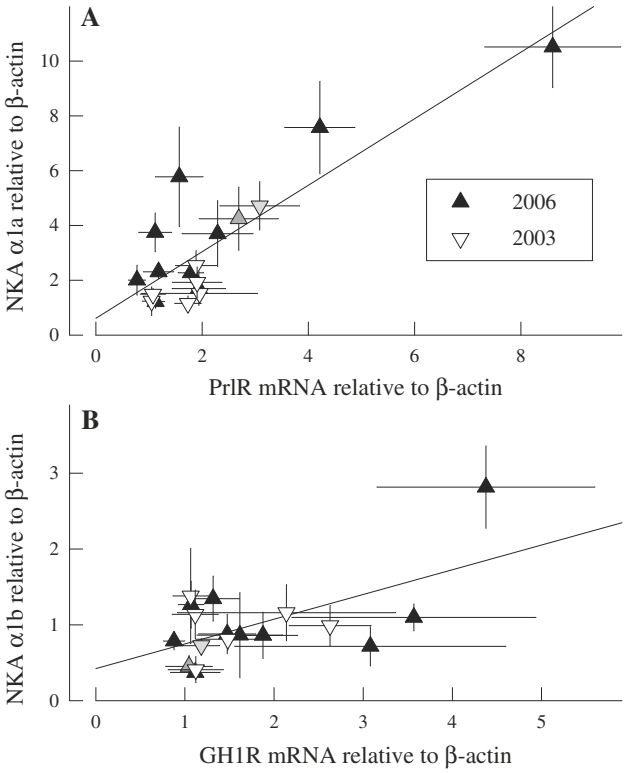

Fig. 7 **a** Relationship between gill prolactin receptor (PrlR) mRNA (expressed relative to *β*-actin) and gill Na^+/K^+-ATPase (NKA) α1a mRNA (expressed relative to *β*-actin). **b** Relationship between gill growth hormone 1 receptor (GH1R) mRNA (expressed relative to *β*-actin) and gill NKA α1b mRNA (expressed relative to *β*-actin). Values are means ± 1 sem

activity and subsequent in-river mortality. Cooke et al. (2006a, b) radio-tagged and biopsied migrating sockeye salmon in Johnstone Strait (~200 km away from the river mouth), and tracked them through the lower Fraser River and upstream to Mission, British Columbia (river85 km). Their study found no difference in NKA activity for sockeye salmon detected by the radio-telemetry receivers at Mission (successfully entered freshwater) and for fish not detected at Mission (failed river entry). In a similar radio-tagging study, however, Crossin et al. (2009) found gill NKA was significantly lower in sockeye that held in Georgia Strait before migrating upriver and spawned successfully compared to fish that did not hold before freshwater entry. Interestingly, 35% of the fish with high NKA and did not hold before river entry spawned successfully. It is possible that in our study, gill NKA activity may already be down-regulated by the time the fish are within 200 km of the river mouth as NKA activity for fish captured in Johnstone Strait was significantly lower than for fish sampled near QCI (2003 data; Fig. 2a). It is also possible, however, that the activity level of NKA measured does not accurately reflect ionoregulatory ability (and freshwater survival at this lifestage) perhaps because it is the change in α subunits of NKA that prepare fish for freshwater entry. The present results clearly show that it is up-regulation of gill NKA α1a mRNA, the freshwater isoform of NKA (Richards et al. 2003), prior to freshwater entry (2006 data; Table 1; Fig. 4a) that indicates the sockeye salmon gills are being remodeled for freshwater ionoregulation while the fish are still in seawater. Changes in gill NKA α1a mRNA, however, did not reach statistical significance among seawater sampling locations in 2003 probably as a result of our inability to sample sufficient numbers of Late Shuswap sockeye among the many thousands of sockeye salmon at these locations.

With a decline in gill NKA activity, it would be expected that adult sockeye progressively lose their ability to hypo-osmoregulate in seawater as they prepare for freshwater entry. The expectation, therefore, is that plasma ions increase while fish are still in the ocean. This was the case for plasma sodium, but not for plasma chloride or osmolality (Fig. 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 seawater (Shrimpton et al. 1994). Coho with lower NKA had a reduced ability to ionoregulate in seawater, resulting in greater mortality. Indeed, in the radiotelemetry studies of Cooke et al. (2006a, b) adult sockeye salmon that successfully passed Mission, British Columbia (river 85 km), 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 supporting the idea that some changes are indeed preparatory.

River entry produced significant drops in plasma sodium and chloride; a finding also seen by Onuma et al. (2010) for homing chum salmon (*O. keta*). The drop in plasma ions indicates that the transition to freshwater requires further physiological adjustments, possibly the reverse of those seen when juvenile smolts enter the ocean. When juvenile salmon smolts are transferred to seawater, there is an initial increase in plasma sodium concentration (Blackburn and Clarke 1987), which if unregulated is associated with higher levels of mortality (Shrimpton et al. 1994). Even so, perturbations in plasma ions following salinity transfer are often transitory while the animal fully adapts to the new environment over a period of a few days (Blackburn and Clarke 1987). In the present study, plasma sodium and chloride rebounded in the adult sockeye as the fish migrated upriver from Cottonwood (river 11 km) to Whonnock (river 50 km), a distance the fish would cover in approximately 2 days (Hanson et al. 2008). Ionic recovery is expected to enable the fish to swim more effectively because Brauner et al. (1992) demonstrated that deviations in plasma sodium concentration following salinity transfer reduced swimming performance.

There was a significant effect of location on plasma chloride and osmolality for fish in seawater in 2003, but not 2006. Thus, plasma chloride and osmolality decreased parallel to the change in gill NKA activity as fish migrated towards the river estuary (Fig. 2b, d). These 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 the case for plasma sodium in our study. Onuma et al. (2010) also found a significant increase in plasma sodium in maturing chum salmon as they approached coastal waters, but no significant change in plasma chloride. The drop in plasma osmolality, however, apparently benefits the fish as Cooke et al. (2006b) showed that tagged late-run sockeye salmon that successfully entered the river also had a 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 become 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 remodeling of the gill in seawater?

Considerable evidence exists for endocrine factors signaling the physiological changes that accompany migration, including those observed here. Cortisol has long been hypothesized to be the saltwater adapting hormone and there is considerable evidence that this hormone may also play a role in freshwater ionoregulation. For example, cortisol treatment was found to stimulate chloride cell proliferation and increase ion uptake in freshwater rainbow trout (Laurent and Perry 1990). Further, cortisol has been shown to increase both NKA α1a and NKA α1b mRNA in the gills of juvenile Atlantic salmon (*Salmo salar*) held in freshwater (McCormick et al. 2008; Tipsmark and Madsen 2009), but not for Atlantic salmon acclimated to seawater (Tipsmark and Madsen 2009). Levels of cortisol in our seawater fish were higher than for fish captured after freshwater entry, suggesting that cortisol stimulated physiological adjustments in seawater fish to enable successful freshwater entry. It is possible that the higher cortisol levels in the ocean fish represent the effect of stress from capture. Our values, however, were similar to (Donaldson and Fagerlund 1970) or lower than (Onuma et al. 2010) values previously reported for anadromous maturing Pacific salmon intercepted during migration and lower than reported for landlocked kokanee (*O. nerka*) during migration (Carruth et al. 2000).

The biological effect of cortisol depends not only on the plasma concentration of the hormone, but on the number and affinity of the intracellular receptors in the gills (Shrimpton and McCormick 1999). Experimental evidence suggests that GR is important for the transition from freshwater to seawater (Bury et al. 2003; Kiilerich et al. 2007). In contrast, the role and function of MR is poorly understood, but cortisol may act through an MR during freshwater acclimation (Colombe et al. 2000). Given the work of Sathiyaa and Vijayan (2003) on autoregulation of GR, the high plasma cortisol concentrations for marine fish would result in high mRNA levels expressed for at least one of the cortisol receptors. The limited change in mRNA levels for all three receptors for fish sampled at the different locations suggest that gene transcription also remains high throughout migration. The similar pattern and limited difference in mRNA expression for GR1, GR2 and MR, however, makes

it difficult to determine if function differs for the different cortisol receptor types. The significant decline in plasma cortisol concentration after freshwater entry was associated with drops in mRNA for all three receptors; but changes were only significant for MR in 2003 (Fig. 5c). Our results suggest that GR1, GR2 and MR, therefore, all function in freshwater ionoregulation.

Two closely related pituitary hormones, Prl and GH, are also involved ionoregulation. It is generally accepted that Prl is important for freshwater ionoregulation, while GH is important for seawater ionoregulation among 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). The two hormones, therefore, should differentially affect NKA α subunits expression. A recent study by Tipsmark and Madsen (2009) showed that Prl injection decreased gill NKA α1b mRNA, while GH injection increased NKA α1b mRNA. These authors, however, found no effect of Prl treatment on gill NKA α1a mRNA which contrasts with the correlation we found between PrlR and NKA α1a mRNA.

Changes in gill PrlR expression have been detected in response to changes in environmental salinity (Manzon 2002). Gill PrlR mRNA increased in the 2006 samples for adult sockeye salmon in seawater as they approached the river mouth (Table 1; Fig. 6a). Earlier studies have shown that circulating levels of Prl increase while adult Atlantic salmon are still in seawater (Andersen et al. 1991). We also saw significant changes in Prl in fish captured in the ocean and levels were high for marine fish. Previous studies have shown Prl to be less than 0.5 ng mL^{-1} for fish in seawater which increased to greater than 3 ng mL^{-1} after transfer to freshwater for adult coho salmon (Sakamoto et al. 1991) and tilapia, *Oreochromis mossambicus*, (Yada et al. 1994). Additionally levels of Prl 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 plasma Prl and gill PrlR mRNA increased while salmon were still in seawater suggest that Prl is stimulating physiological changes in the gill that are preparatory for freshwater entry.

Physiological changes during freshwater migration

Even though we present evidence that sockeye salmon prepare for freshwater in advance of entry, these maturing sockeye were not in an ionoregulatory steady state during their up river migration which lasted almost one month (Table 1). Gill NKA activity, plasma osmolality, sodium and chloride consistently decline with time and distance upriver. Generally, the lowest values for each measurement after the initial perturbation at Cottonwood (river 11 km) were observed in the present study as fish approached or first arrived at the spawning area. The physiological measures of freshwater migrating fish suggest continuous ionic and ionoregulatory changes throughout freshwater as well as coastal migration.

Modifications of gill NKA α1a and α1b subunits also indicated that physiological changes continued after sockeye salmon moved into freshwater (Fig. 4). Isoform switching in gills following salinity transfer has previously suggested that the switching between these two α-subunits contributes to the change in the salmonid gill from an ion-absorbing epithelium in freshwater to an ion-secreting epithelium in seawater (Richards et al. 2003). Our results are consistent with this model. The expression of NKA α1a significantly increased in wild sockeye salmon following movement into the river, and while migrating upstream and onto the spawning grounds. Similar findings for gill NKA α1a were reported for wild anadromous Arctic char (*Salvelinus alpinus*) migrating from seawater into freshwater (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 of these salmonid examples 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 freshwater. This lack of change in gill NKA α1b isoform expression was also found for saltwater acclimated rainbow trout when transferred to freshwater (Richards et al. 2003). There was a significant increase in α1b mRNA for sockeye salmon that were recent arrivals on the spawning grounds (Fig. 4b). 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 in seawater?

Cortisol appears to play less of a role in stimulating physiological changes that occurred during up river migration as circulating concentrations were significantly lower in freshwater. Lower cortisol receptor mRNA and lower plasma cortisol levels suggest that the stimulatory role of cortisol diminishes after adult sockeye have entered freshwater. Although, expression of mRNA for GR1, GR2 and MR did not significantly differ with location for fish caught in 2006, mRNA for MR did drop significantly for samples collected

in 2003. Additionally, mRNAs for all three receptors were generally lower in freshwater than seawater indicating little difference between GR and MR function during migration for sockeye. Conversely, there is evidence in juvenile Atlantic salmon that MR plays a role in ionoregulation in freshwater; MR transcript levels remain unchanged during smolting, but significantly increased at the onset of de-smoltification (Kiilerich et al. 2007). It is not surprising that changes in plasma cortisol do not correlate with changes in NKA α1a mRNA as sockeye migrate upstream as it requires at least 24 h to upregulate transcription of the NKA α1 subunits (Richards et al. 2003). It is interesting that changes in cortisol receptor mRNA do not correlate with NKA α1a mRNA. Our data, therefore, suggests that for adult salmon migrating in freshwater adult salmon, other endocrine factors also play a role in freshwater ionoregulation.

In contrast, the mRNA expression for PrlR and GH1R increased significantly in the gills of sockeye salmon migrating up river. The mRNA levels for both receptors were highest for fish arriving at the spawning grounds but declined with maturation to a spawning state while sockeye held 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 freshwater, but then increased up to fivefold after entry into freshwater.

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. This may be due to residence time on spawning areas that differ among stocks (D.A. Patterson, unpublished data). Shrimpton et al. (2005) examined Quesnel River sockeye and the present study examined Late Shuswap sockeye; however, in both studies gill α1a and α1b isoforms increased as sockeye migrated up river. The higher mRNA levels of PrlR and GH1R may have an ionoregulatory role and modify gill NKA to limit osmotic perturbations. The correlations between PrlR mRNA and NKA α1a mRNA suggest that prolactin is directly stimulating the gill to uptake ions in freshwater. 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) 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 do not support this suggestion, but rather that GH is directly driving the increase in 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. Semelparous salmon resort to protein degradation as fuel stores are depleted in the later stages of migration resulting in a marked increase in ammonia production (Mommsen et al. 1980). Exhaustive exercise also elevates white muscle and plasma ammonia levels (Tang et al. 1992). 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 (Wilkie 2002). The increase in NH_3 production may, therefore, make NH_4^+ transport via the basolateral NKA necessary.

We have shown that cortisol and prolactin stimulate ionoregulatory changes in adult sockeye salmon during their homing migration. Based on the plasma cortisol concentrations and mRNA levels for the cortisol receptors, our findings suggest that cortisol induces a physiological response before freshwater entry during the migration of adult sockeye salmon. 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.

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