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Expression of corticoid-regulatory genes in the gills of Atlantic salmon (*Salmo salar*) parr and smolt and during salinity acclimation

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Abstract

In teleost fishes, cortisol is the major corticoid and has both glucocorticoid and mineralocorticoid actions. However, how fish tissues discriminate between these distinct corticosteroid actions is unclear. In mammals, the major factors responsible for intracellular corticosteroid regulation are glucocorticoid receptors (*grs*) and the mineralocorticoid receptor (*mr*), but their role in osmoregulation of fish is unclear. 11β-hydroxysteroid dehydrogenases (*hsd11bs*) control the levels of intracellular corticosteroids by converting from bioactive forms to inert forms. To investigate how Atlantic salmon (*Salmo salar*) respond to cortisol in different physiological or environmental conditions, we performed comparisons of parr and smolt, and osmotic challenge experiments to examine the physiological responses and gill transcript levels of genes underlying cortisol-signalling, including *gr1*, *gr2*, *mr*, *hsd11b2* and *hsd11b3*. Because cortisol may interact with growth hormone and prolactin during salinity changes, transcript levels encoding growth hormone receptors (*ghr1*, *ghr2*) and the prolactin receptor (*prlr*) were also examined. *Hsd11b2* transcript levels in seawater-acclimated fish were consistently lower compared to fish acclimated to fresh water. After transfer to seawater, *prlr* transcript levels in fish significantly decreased and transcript levels of *ghr1*, *ghr2* and *hsd11b3* showed no change or were slightly higher than those of freshwater control groups. *Gr1*, *gr2* and *mr* transcript levels were slightly but consistently higher in fish acclimated to fresh water relative to seawater. Our results indicate that changes in corticosteroid receptor and *hsd11b2* transcript levels in the gills may be important mechanisms that regulate corticoid signals to achieve ion homeostasis in Atlantic salmon.

KEYWORDS
cortisol, *gr*, *hsd11b*, *mr*, osmoregulation

1 | INTRODUCTION

The endocrine system is a key controller of osmoregulatory processes in teleosts, and studies over the past few decades have demonstrated that a number of hormones are involved in regulating ion and water balance (Takei & McCormick, 2013). In many teleost species, the combination of cortisol and growth hormone (GH) promotes acclimation to seawater, whereas cortisol and prolactin (PRL), acting either separately or in combination, support freshwater acclimation (McCormick, 2001).

Gill ionocytes are important for ion uptake in fresh water and ion secretion in seawater (Evans, 2002; Hiroi & McCormick, 2012; Shaughnessy & Breves, 2021; Shih et al., 2023). The number and size of gill ionocytes are increased by GH and cortisol (Takei & McCormick, 2013). The expression of ion transporters and channels that are important for salt secretion in seawater, such as of Na^+/K^+-ATPase (NKAα1b), Na^+-K^+-Cl^- cotransporter 1 and the cystic fibrosis transmembrane regulator (CFTR, an apical chloride channel), in the gills is promoted by cortisol and GH. In salmonids, these hormones can act both independently and synergistically (Madsen, 1990; Pelis & McCormick, 2001). PRL is a hyper-osmoregulatory hormone in many teleosts (Bern, 1983; Breves et al., 2014), promoting acclimation to fresh water. In some teleost species cortisol also has a role in promoting ion uptake in fresh water (Cruz et al., 2013), stimulating freshwater-type ion transporters and channels, such as Na^+/K^+-ATPase (NKA α1a), Na^+/H^+ exchanger 3 (NHE3) and Na^+/Cl^- cotransporter (NCC) (Hiroi et al., 2008). The dual osmoregulatory role of cortisol likely depends on its interaction with other hormones such as GH and PRL (Takei & McCormick, 2013).

Cortisol has multiple roles in teleosts, regulating metabolism, growth, immune response, stress response and osmoregulation (Wendelaar Bonga, 1997). Although aldosterone is the main mineralocorticoid hormone in terrestrial vertebrates, it is generally accepted that teleosts do not produce substantial levels of circulating aldosterone (Bern, 1967; Kiilerich et al., 2011). This is consistent with the absence of the aldosterone synthase activity of *cyp11b* in Japanese eel (*Anguilla japonica*) (Jiang et al., 1998). For osmoregulation in fishes, cortisol appears to be a bifunctional hormone for both hypo- and hyper-saline acclimation in teleosts (Laurent & Perry, 1990; Madsen, 1990; Mancera et al., 2002; Redding et al., 1991). However, it is unclear how fish use cortisol differentially in diverse salinity environments for the development of seawater or freshwater ionocytes in the gill. Two possibilities can be considered which may determine what levels of cortisol are perceived by osmoregulatory organs such as the gill to promote an appropriate physiological response. First, both the abundance and affinity of corticosteroid receptors in ionocytes (or their precursor cells) will determine what levels of cortisol are perceived by the gill to promote an appropriate physiological response (Shrimpton & McCormick, 1999). Second, the presence of steroidogenic enzyme(s) in osmoregulatory tissues/cells can facilitate conversion of active forms (cortisol) to inactive forms (cortisone) to optimize or fine-tune the levels of cortisol needed for osmoregulation.

The first molecular cloning of both the teleost glucocorticoid receptor (*gr*) and mineralocorticoid receptor (*mr*) were reported in rainbow trout (*Oncorhynchus mykiss*) (Colombe et al., 2000; Ducouret et al., 1995; Takeo et al., 1996). Currently, it is widely believed that most teleost possess two isoforms of *gr*, which are now referred to as *gr1* and *gr2*, and a single *mr* (Bury et al., 2003; Greenwood et al., 2003). However, the roles of *gr1*, *gr2* or *mr* for osmoregulatory action of cortisol vary among fish species (Breves & Shaughnessy, 2024). In zebrafish (*Danio rerio*), cortisol action for ion uptake is mediated by *gr* rather than *mr* (Cruz et al., 2013). In medaka (*Oryzias latipes*) embryo, *gr2* has strong effects on regulating the number of epidermal ionocytes (Trayer et al., 2013). In freshwater-acclimated Mozambique tilapia (*Oreochromis mossambicus*), cortisol stimulates ionocyte differentiation through the *mr* (Wu et al. Wu et al., 2023). Since the two known *grs* in salmonids have different affinities for cortisol (Bury et al., 2003), understanding their relative abundance will be important to determining the potential of the gill to respond to changes in cortisol levels.

In mammals, the major factors responsible for intracellular corticosteroid regulation are two distinct forms of the steroidogenic enzyme 11β-hydroxysteroid dehydrogenase (*hsd11b*). *Hsd11b* type 1 (*hsd11b1*) is principally found in glucocorticoid target tissues, such as liver, gonad and kidney (Agarwal et al., 1989), and acts as an amplifier of glucocorticoid action (Kotelevtsev et al., 1997). *Hsd11b* type 2 (*hsd11b2*) is localized mainly in mineralocorticoid target tissues such as kidney and colon (Albiston et al., 1994) and acts as the key physiological factor for converting bioactive glucocorticoids (cortisol or corticosterone) to inert forms (cortisone or 11-dehydrocorticosterone) to protect the *mr* (Agarwal et al., 1989; Edward et al., 1988; Funder et al., 1988). *Hsd11b* in fish was first reported in rainbow trout (Kusakabe et al., 2003), Japanese eel (*Anguilla japonica*) and Nile tilapia (*Oreochromis niloticus*) (Jiang et al., 2003). These teleost *hsd11b* have higher homology to mammalian *hsd11b2* than *hsd11b1*. Analyses of enzymatic activity of *hsd11b*s in rainbow trout and Japanese eel demonstrate conversion of cortisol to cortisone (Jiang et al., 2003; Kusakabe et al., 2003), but no conversion of cortisone to cortisol (Kusakabe et al., 2003). *Hsd11b2* transcripts are ubiquitously found in rainbow trout and gill is one of the tissues that strongly expresses *hsd11b2* (Kusakabe et al., 2003). Thus, it is possible that *hsd11b2* can convert cortisol to cortisone to regulate intracellular cortisol levels in gills.

In mammals, another key glucocorticoid regulator is *hsd11b1*, which catalyses the conversion of cortisone to cortisol. However, the orthologue of mammalian *hsd11b1* is seemingly absent in teleosts (Baker, 2004). A search of genome databases revealed that *hsd11b* type 3 (*hsd11b3*) was found in sea urchin, amphioxus, sea squirt, shark, teleosts, amphibians and mammals, suggesting that *hsd11b3* is the ancestral form of mammalian *hsd11b1* (Baker, 2010). However, the function of *hsd11b3* is still unknown. Rainbow trout *hsd11b3* transcript levels are abundant in gill, brain and pituitary, and are found at lower levels in other tissues (Kusakabe et al., Shizuoka University, oral communication, 7th International Symposium on Fish Endocrinology, 1–6 September 2012). It is therefore possible that *hsd11b3* in teleost gills acts to increase intracellular cortisol (as *hsd11b1* does in mammals), although the enzymatic activity of *hsd11b3* has not yet been determined in teleosts.

Our overall goal of this study was to further examine the role of cortisol in smolt development (which includes large increases in salinity tolerance) and salinity acclimation of Atlantic salmon. Specifically, we examined gill transcript levels of corticosteroid-regulating factors *gr1*, *gr2*, *mr*, *hsd11b2* and *hsd11b3*, and plasma cortisol in Atlantic salmon (*Salmo salar*) parr and smolt. The transcript levels and plasma cortisol were also investigated during transfer from fresh water to seawater and vice versa. Because of the potential interaction of cortisol with the pituitary hormones, PRL and GH, we also examined changes in gene expression of gill GH receptors (*ghr1* and *ghr2*) and the PRL receptor (*prlr*).

2 | MATERIALS AND METHODS

2.1 | Experimental animals and sampling

Atlantic salmon parr from the Kensington State Fish Hatchery (CT, USA) were brought to the Conte Anadromous Fish Research Center in October 2010 (MA, USA) and raised in 1.5-m diameter tanks supplied with Connecticut River water (water hardness 170–250 mg L^{-1}) at 4 L/min with supplemental aeration. Fish were reared under a natural photoperiod and fed with automatic feeders during daylight hours.

Parr (10.6–12.8 cm) and smolt (15.7–17.5 cm) were sampled on 12 May 2011, which is the time of peak smolt development for this strain of Atlantic salmon (McCormick et al., 2019). Fish were the same age (16 months from hatching) and size differences were due to natural variation in growth among individuals. At the time of sampling, there were clear morphological differences in silvering, parr marks and black fin margins between parr and smolt.

A freshwater to seawater transfer experiment (FW → SW) was conducted beginning on 3 November 2010. Parr (11.6–13.3 cm, 10 months from hatching) were sampled from their stock tanks and then transferred to identical 1.5-m diameter tanks containing either fresh water or seawater (25 ppt). This salinity was chosen based on previous experiments indicating that direct transfer of parr to higher salinities results in substantial mortality. Each tank had recirculating flow-through water with biological and charcoal filtration and supplemental aeration. A 50% water change occurred every third day. Ten parr in fresh water (control) and seawater were sampled at 0 (fresh water only), 2, 5 and 14 days after seawater exposure as described below. Fish were not fed during the experiment to avoid the impact of differential feeding due to salinity change on the physiological responses.

A seawater to freshwater transfer experiment (SW → FW) was conducted beginning on 18 January 2006. Prior to the experiment 100 parr (10.8–13.4 cm, 12 months from hatching) were first gradually acclimated to 30 ppt over a period of 20 days in recirculating tanks as described above. They remained at this salinity for 20 days prior to the beginning of the study. A 50% water change occurred every third day. Ten parr in seawater (control) and fresh water were sampled at 0 (seawater only), 2, 5, 8 and 14 days after freshwater exposure as described below. Fish were not fed during the experiment to avoid the impact of differential feeding due to salinity on the physiological responses.

Fish were sampled between 10:00 and 12:00 Eastern Standard Time. At the time of sampling, fish were rapidly removed from their tanks and placed in a lethal dose of tricaine methanesulfonate (MS-222, 200 mg L^{-1} neutralized to pH 7.0). Fork length to the nearest millimetre and mass to the nearest 0.1 g were recorded, and blood was sampled from the caudal vessels using 1-mL ammonium heparinized syringes. Blood collection was completed within 5 min of the first disturbance of the tank. Blood was centrifuged at 3200*g* for 5 min at 4°C, and the plasma was aliquoted and stored at −80°C. The second gill arch was removed and the gill filaments trimmed from ceratobranchials and placed in an autoclaved 1.5-mL microcentrifuge tube and frozen immediately at −80°C for later extraction of total RNA.

All animal procedures were reviewed and approved by the US Geological Survey's Eastern Ecological Science Center and/or the Shizuoka University Animal Care and Use Committee.

2.2 | Plasma analyses

Plasma cortisol levels were measured by a validated direct competitive enzyme immunoassay described by Carey and McCormick (1998). Sensitivity as defined by the dose–response curve was 1–400 ng mL^{-1}. The lower detection limit was 0.3 ng mL^{-1}. Using a pooled plasma sample, the average intra-assay variation was 5.5% ($n = 10$) and the average inter-assay variation was 8.8% ($n = 10$). Plasma chloride was measured using a Buchler-Cotlove Chloridometer (ExpotechUSA) using external standards.

2.3 | Gill NKA activity assay

Gill NKA activity was determined using a temperature-regulated nicotinamide adenine dinucleotide-linked microplate method (McCormick, 1993). Gill samples were homogenized in 150 μL of 0.1% sodium deoxycholate in Sucrose-EDTA-Imidazole buffer, pH 7.3 and centrifuged at 3000*g* for 30 s. Ten microlitres of this homogenate was run in duplicate in the presence and absence of 0.5 mM ouabain. Protein concentrations were determined using a bicinchoninic acid protein assay (Pierce). Both assays were run on a BioTek Synergy 2 spectrophotometer using Gen5 software (BioTek).

2.4 | RNA extraction

Total RNA was extracted using Isogen (Nippon Gene). The RNA concentration was determined by measuring absorbance at 260 nm using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies).

RNA purity was assessed by the ratio of absorbance at 280/260 nm (1.8–2.0) and 260/230 nm (2.0–2.2). RNA quality was checked by agarose (1%) gel electrophoresis, confirming sharp bands of 28S/18S rRNA.

2.5 | Real-time quantitative PCR

One microgram of total RNA was incubated with two units of TURBO DNase I (Ambion; Life Technologies) at 37°C for 30 min to eliminate genomic DNA contamination. DNase I was inactivated by adding 0.1 volume of DNase inactivation reagent (Ambion, Life Technologies) for 5 min at room temperature. Subsequently, single-strand complementary DNA (cDNAs) were synthesized from the DNase I-treated RNA using a High-Capacity cDNA archive kit as described by the manufacturer (Applied Biosystems).

Real-time quantitative reverse transcription PCR (RT-PCR) was performed for the relative quantitation of transcript levels using an ABI PRISM 7900 sequence detection system (Applied Biosystems). *Hsd11b2*, *hsd11b3*, *gr1*, gr2 and *mr* expressions were determined

TABLE 1 Sequences of primers and probes used for real-time quantitative PCR.

Gene name	Primer/ probe	Sequence (5′ → 3′)	Description	Accession number
11β-hydroxysteroid dehydrogenase 2	*hsd11b2*_F	TGCGCCACGAAATGGAA	Forward primer	XP_014032144
	*hsd11b2*_P	CAAAGTGTCCACCATACTGCCATCCTCAT	Probe	
	*hsd11b2*_R	GGTTACTAGACTGACCTGTCTT	Reverse primer	
11β-hydroxysteroid dehydrogenase 3	*hsd11b3*_F	GCCCACCCTTGAGCAGAGTA	Forward primer	XM_014169312
	*hsd11b3*_P	CGGTTCCATTGTGGTCGTCTC	Probe	
	*hsd11b3*_R	GGGCTGCACATTTTTCCTAATAA	Reverse primer	
Glucocorticoid receptor 1	*gr1*_F	CGCAGCAGAACCAACAGCTG	Forward primer	XM_014198060
	*gr1*_P	TGAGAACTTTGCTCTGTTGGAGGCG AGTAT	Probe	
	*gr1*_R	ATGAGGGCGTTCAAGTACAGA	Reverse primer	
Glucocorticoid receptor 2	*gr2*_F	TCCAGCAGCTTTGCCAGTTCA	Forward primer	XM_014198678
	*gr2*_P	AGGCAGGGTGGTGGCGCTG	Probe	
	*gr2*_R	TTGCCCTGGGTTGTACACGA	Reverse primer	
Mineralocorticoid receptor	*mr*_F	AGCTGATTGAGCCAGAGGTGGTG	Forward primer	XM_014209388
	*mr*_P	TTGCGGGCTACGACAACACCC	Probe	
	*mr*_R	CAGGTGGTCGGTGGTGTCA	Reverse primer	
Growth hormone receptor 1	*ghr1*_F	CTGGGAAGTTGAGTGCCAGACT	Forward primer	AY462105
	*ghr1*_R	CACAAGACTACTGTCCTCCGTTGA	Reverse primer	
Growth hormone receptor 2	*ghr2*_F	AAGGCTGAGGGAAAGGAGAAAGAG	Forward primer	NM_001123594
	*ghr2*_R	ACCTCCCCCGTCAGCATTCAC	Reverse primer	
Prolactin receptor	*prlr*_F	CTGGAAGTGTACGTCAACGAGAA	Forward primer	XP_013994342
	*prlr*_R	TTGAGGCAGCCAACATCTTG	Reverse primer	
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*_F	GCAGCTACGCTGAGATCAAG	Forward primer	BT059114
	*gapdh*_R	AAGATGGAGGAGTGGGTGTC	Reverse primer	
Elongation factor 1 alpha	*ef1a*_F	ATCTCCAAGAATGGGCAAAC	Forward primer	NM_001173967
	*ef1a*_R	CCATCTTGTTGACCGATACG	Reverse primer	
Ribosomal protein L7	*rpl7*_F	GTCTGTGCGTGAGCTGATTT	Forward primer	NM_001140480
	*rpl7*_R	GTCCTCCACACAGATGATGC	Reverse primer	

using TaqMan assays (ABI Universal PCR Master Mix, Applied Biosystems). Sequences of primers and probes for *hsd11b2*, *gr1*, *gr2* and *mr* were designed based on the previous trout studies (Kusakabe et al., 2006; Yada et al., 2007). Primers and a TaqMan probe for *hsd11b3* were designed using Primer Express version 1.5 (Applied Biosystems). *Ghr1*, *ghr2* and *prlr* gene expressions were determined by SYBR green assay (KAPA SYBR FAST qPCR Master Mix, Kapa Biosystems). Primers for *ghr1*, *ghr2* and *prlr* were designed using a primer design online tool (GenScript Real-time PCR Primer Design, https://cellculturedish.com/genscript-real-time-pcr-taqman-primer-probes-design-tool/). PCR was performed in a 10-μL reaction volume using 1 μL of 10-fold diluted reverse-transcribed cDNAs. PCR conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A standard curve was generated by four-fold serial dilution of brain cDNA. Melting curve analysis was performed after PCR amplification for the SYBR Green assay. The PCR products were confirmed as a single peak. To confirm that the DNase-treated RNA samples were free of genomic DNA, 10 samples were randomly selected. Real-time quantitative RT-PCR was performed for all target genes without reverse transcription to confirm that the samples did not give any signal. Correlation coefficients of the standard curves ranged from 0.99 to 1.00 and the efficiency of the reaction ranged from 99% to 100% (slope = −3.3). The transcript levels are indicated as relative messenger RNA (mRNA) levels. The mean of the initial control group was assigned a value of 1.0. For normalization of data, 18S ribosomal RNA, glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), elongation factor-1 alpha (*ef1a*) and 60S ribosomal protein L7 (*rpl7*) transcript levels were tested. The 18S ribosomal gene was measured by TaqMan assay using commercially available primers and probes (Eukaryotic 18S rRNA Endogenous Control, Applied Biosystems). *Gapdh*, *ef1a* and *rpl7* gene expressions were determined by SYBR green assay (KAPA SYBR FAST qPCR Master Mix). The mean expression level of the four internal control genes (18S ribosomal, *gapdh*, *ef1a* and *rpl7*) was used for a value of internal control. The average transcript level obtained from an internal control gene was calculated, then the transcript levels of the internal control gene for each sample were divided by the average value. We repeated this calculation for all four internal control genes. By calculating the average transcript levels of four internal control genes, the variation in expression of the internal control genes can be eliminated. The nucleotide sequences for primers and probes are listed in Table 1.

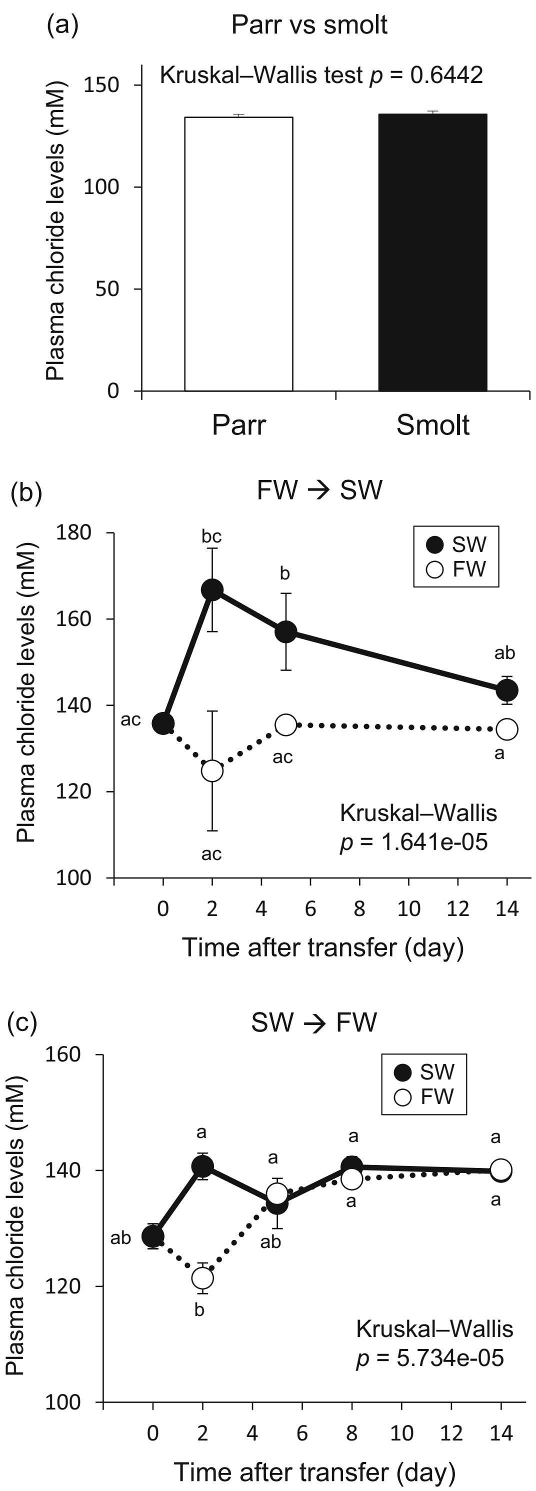
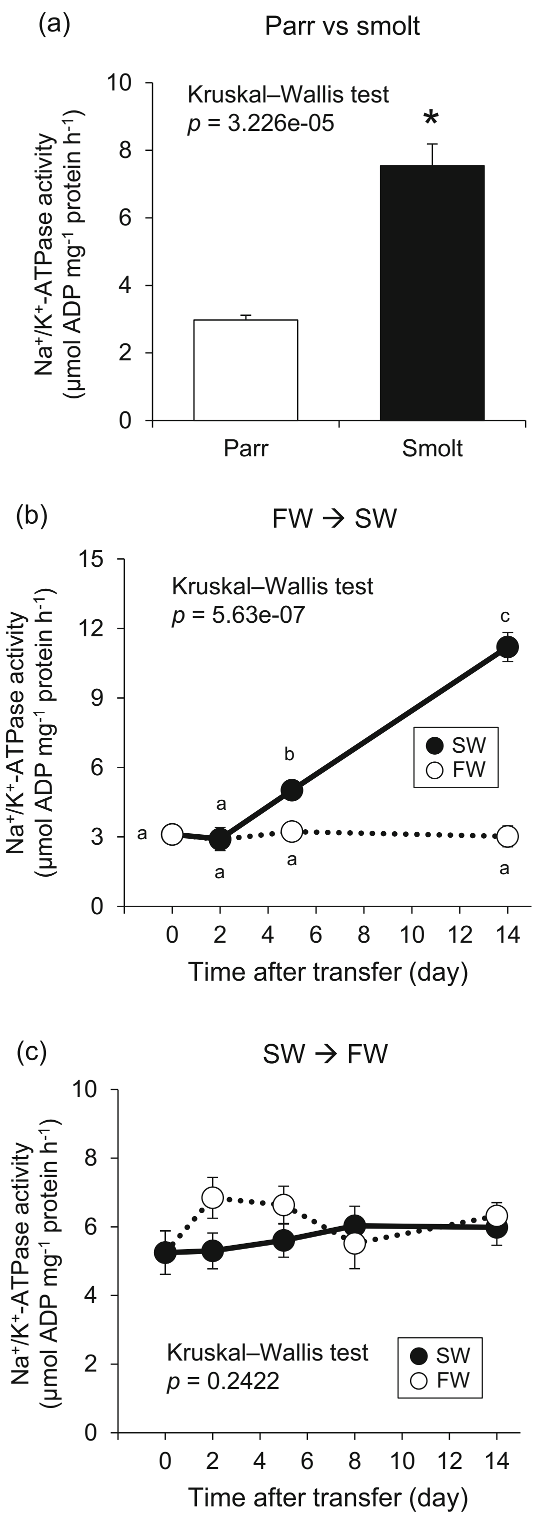


FIGURE 1 Plasma chloride levels in Atlantic salmon. (a) Comparison of parr and smolt (n = 12 for parr and n = 12 for smolt). Values are the means ± standard error (SE). Differences in chloride levels between parr and smolt were analysed using the Kruskal–Wallis test. (b) Seawater (SW) acclimation experiment. Plasma samples were collected at 0, 2, 5 and 14 days after seawater transfer. (c) Freshwater (FW) acclimation experiment. Plasma samples were collected at 0, 2, 5, 8 and 14 days after freshwater transfer. For (b) and (c), values are indicated as the means ± SE. Differences in chloride levels between sampling points were analysed using the Kruskal–Wallis test. Values not sharing a letter are significantly different by the Steel–Dwass test (p < 0.05).

2.6 | Statistical analyses

Differences in plasma chloride, cortisol concentrations and transcript levels between parr and smolt were analysed using the Kruskal–Wallis test. Differences in plasma chloride, cortisol and transcript levels in the seawater or freshwater transfer and the fasting experiments were analysed using the Kruskal–Wallis test, followed by the Steel–Dwass test. A p value of 0.05 or lower was considered to be statistically significant. All statistical analyses were performed using R statistical software (version 4.4.2, R Core Team, 2024).

At the time of publication, data were not publicly available from the Japan Society for the Promotion of Science.



3 | RESULTS

3.1 | Plasma chloride levels

In May 2011, there was no significant difference in the plasma chloride levels between parr and smolt in fresh water (Figure 1a). Plasma chloride levels of parr transferred from fresh water to seawater in December were significantly higher after 4 days in seawater compared to controls in fresh water (Figure 1b). The levels of plasma chloride of fish in seawater were within the physiological range (150–170 mM). Plasma chloride levels of fish transferred from seawater to fresh water in January were significantly lower after 2 days of freshwater transfer compared to seawater controls (Figure 1c).

3.2 | Gill Na+/K + -ATPase activity

Gill Na^+/K^+-ATPase activity was significantly higher in smolt than in parr (Figure 2a). Gill Na^+/K^+-ATPase activity in fish transferred from fresh water to seawater was significantly higher than those in freshwater controls after 5 days (Figure 2b) and continued to increase through day 14. Gill Na^+/K^+-ATPase activity of fish transferred from seawater to fresh water showed no significant differences throughout the 14-day study (Figure 2c).

3.3 | Plasma cortisol levels

Plasma cortisol levels were significantly higher in smolt than in parr (Figure 3a). Freshwater to seawater transfer experiments showed trends of increases in plasma cortisol levels after 2 days of transfer (fresh water to seawater) or 5 days of transfer (fresh water to fresh water, control fish) (Figure 3b). Plasma cortisol levels 2 days after transfer of fish from seawater to fresh water were significantly higher than those of control fish (Figure 3b).

3.4 | Gill transcript levels in parr and smolt

Transcript levels of *hsd11b2*, *hsd11b3*, *gr1*, *gr2*, *mr*, *prlr* and *ghr1* in the gills showed no difference between parr and smolt (Figure 4a–g).

FIGURE 2 Na^+/K^+-ATPase activity in the gills of Atlantic salmon. (a) Comparison of parr and smolt (n = 12 for parr and n = 12 for smolt). Values are the means ± standard error (SE). Differences in chloride levels between parr and smolt were analysed using the Kruskal–Wallis test. The asterisk indicates a significantly higher value (*p < 0.05). (b) Seawater (SW) acclimation experiment. Gill samples were collected at 0, 2, 5 and 14 days after seawater transfer. (c) Freshwater (FW) acclimation experiment. Gill samples were collected at 0, 2, 5, 8 and 14 days after freshwater transfer. For (b) and (c), values are indicated as the means ± SE. Differences in chloride levels between sampling points were analysed using the Kruskal–Wallis test. Values not sharing a letter are significantly different by the Steel–Dwass test (p < 0.05).

Transcript levels of *ghr2* (Figure 4h) were significantly higher in the gills of smolt compared to parr.

3.5 | Changes in transcript levels during seawater- and freshwater-acclimation in gills

Hsd11b2 transcript levels in fish transferred from fresh water to seawater remained constant throughout the experiment, whereas the *hsd11b2* transcript levels in freshwater-transferred fish (controls) significantly increased after 5 days of transfer (Figure 5a). *Hsd11b2* transcript levels in fish transferred from seawater to fresh water were significantly higher than seawater controls after 8 days of the transfer (Figure 5b). The freshwater to seawater transfer experiment showed no significant difference of gill *hsd11b3* transcript levels between groups (Figure 5c). *Hsd11b3* transcript levels in fish transferred from seawater to fresh water did not change over time, whereas the seawater controls significantly increased between 8 and 14 days (Figure 5d).

Gr1 transcript levels in fish transferred from fresh water to seawater tended to be lower than those in freshwater controls (Figure 6a). *Gr1* transcript levels in fish transferred from seawater to fresh water tended to be higher than seawater controls (Figure 6b). *Gr1* transcript levels significantly increased over time in freshwater groups during the seawater to freshwater transfer experiment. The freshwater to seawater transfer experiment showed no significant difference of gill *gr2* transcript levels between groups (Figure 6c). *Gr2* transcript levels in fish transferred from seawater to fresh water were significantly higher 8 days after transfer compared to 2-day freshwater controls (Figure 6d). *Mr* transcript levels after 2 and 14 days of transfer from fresh water to seawater were significantly lower than those of freshwater (control) fish (Figure 6e). *Mr* transcript levels in fish transferred from seawater to fresh water showed no significant difference in gill *mr* transcript levels between seawater and freshwater groups by the Steel–Dwass test (Figure 6f).

Prlr transcript levels in fish transferred from fresh water to seawater significantly decreased after 5 and 14 days of transfer, whereas the *prlr* transcript levels of freshwater fish (control) significantly increased after 2 and 5 days (Figure 7a). *Prlr* transcript levels in fish transferred from seawater to fresh water significantly increased after 2 days from the transfer and further increased after 5, 8 and 14 days of transfer (Figure 7b). *Prlr* transcript levels of control fish remained the same throughout the experiment (Figure 7b).

In the freshwater to seawater experiment, *ghr1* transcript levels were not impacted by salinity (Figure 7c). *Ghr1* transcript levels in

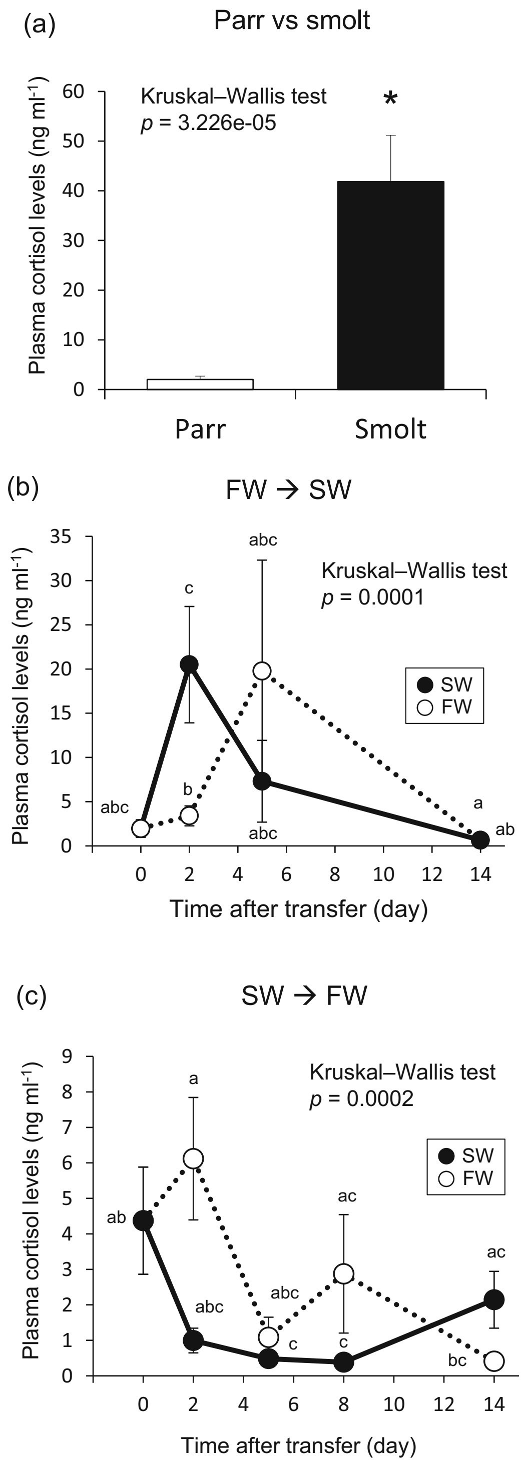


FIGURE 3 Plasma cortisol concentrations in Atlantic salmon. (a) Comparison of parr and smolt ($n = 12$ for parr and $n = 12$ for smolt). Values are the means ± standard error (SE). Differences in cortisol levels between parr and smolt were analysed using the Kruskal–Wallis test. The asterisk indicates a significantly higher value (*$p < 0.05$). (b) Seawater (SW) acclimation experiment. Plasma samples were collected at 0, 2, 5 and 14 days after seawater transfer. (c) Freshwater (FW) acclimation experiment. Plasma samples were collected at 0, 2, 5, 8 and 14 days after freshwater transfer. Plasma samples were collected at 0, 7 and 14 days after fasting. For (b) and (c), values are indicated as the means ± SE. Differences in chloride levels between sampling points were analysed using the Kruskal–Wallis test. Values not sharing a letter are significantly different by the Steel–Dwass test ($p < 0.05$).

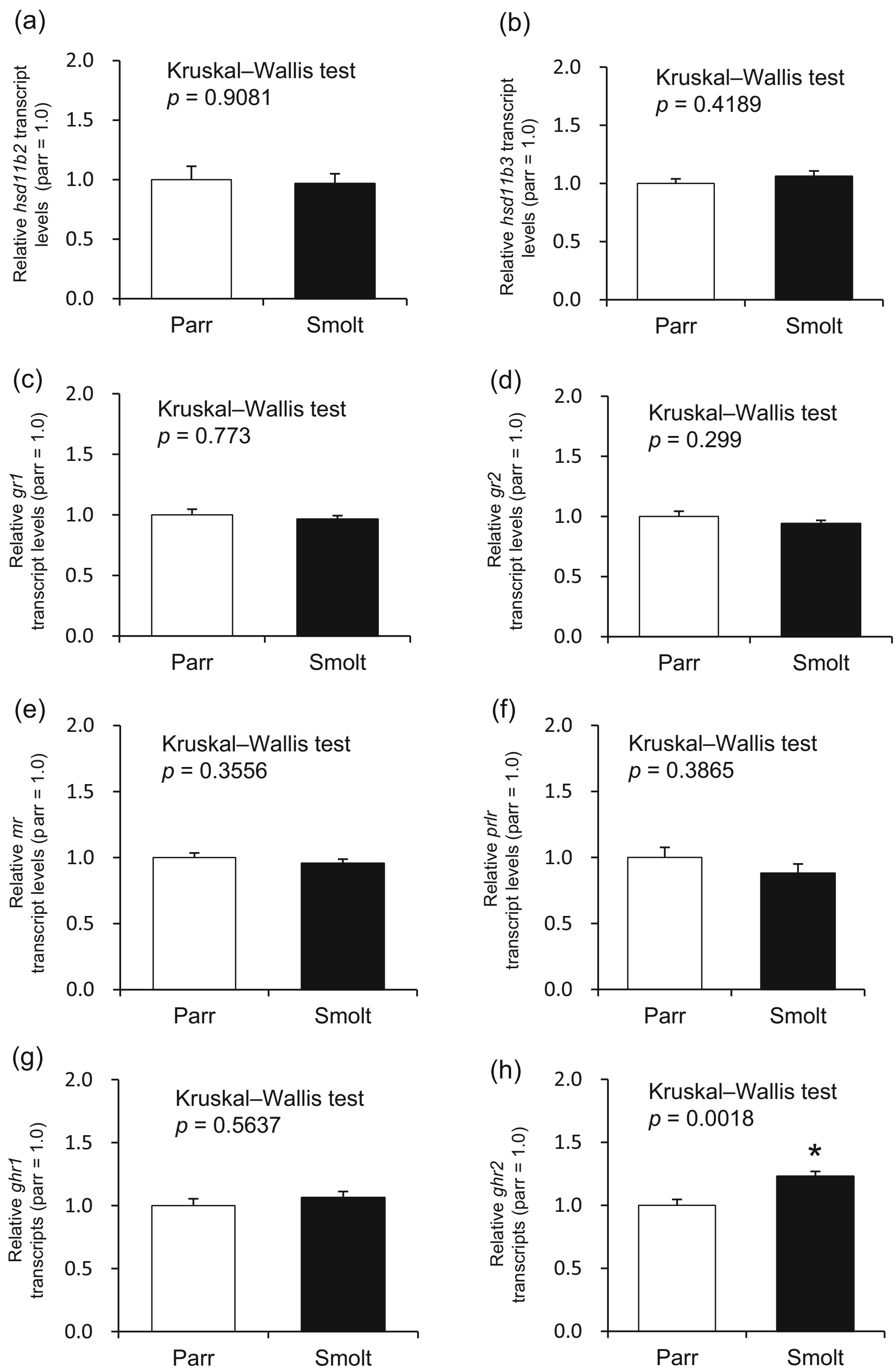


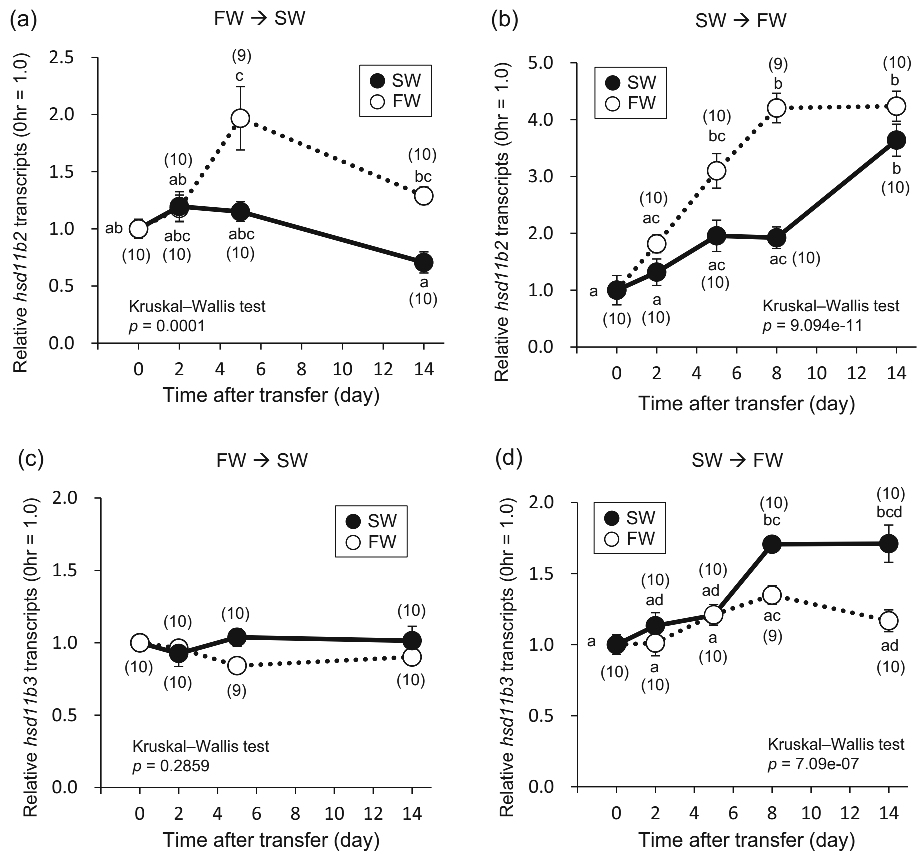
FIGURE 4 Comparison of transcript levels between Atlantic salmon parr and smolt ($n = 12$ for parr and $n = 12$ for smolt). *Hsd11b2* (a), *hsd11b3* (b), *gr1* (c), *gr2* (d), *mr* (e), *prlr* (f), *ghr1* (g) and *ghr2* (h). Values are the means ± standard error. The relative expression values of parr fish were set to 1.0. Differences in transcript levels between parr and smolt were analysed using the Kruskal–Wallis test. The asterisk indicates a significantly higher value (*$p < 0.05$).

fish transferred from seawater to fresh water significantly increased after 2 days of transfer, whereas they remained constant in fish remaining in seawater (control fish) (Figure 7c). *Ghr2* transcript levels were not significantly influenced by salinity in the freshwater to seawater or seawater to freshwater experiments (Figure 7e,f).

4 | DISCUSSION

When salmon migrate to seawater, substantial physiological changes occur that facilitate adaptation to high salinity environments (McCormick, 2001). The endocrine system controls expression of transporters and channels responsible for salt and water transport. To

FIGURE 5 Gill *hsd11b2* transcript levels of the Atlantic salmon during seawater (SW) acclimation (a) and freshwater (FW) acclimation (b) experiments. Atlantic salmon gill *hsd11b3* transcript levels during seawater acclimation (c) and freshwater acclimation (d) experiments. The values were corrected with internal controls and the relative expression values at day 0 were set to 1.0 (refer to Materials and Methods). Differences in transcript levels between sampling points were analysed using the Kruskal–Wallis test. Values not sharing a letter are significantly different by the Steel–Dwass test (p < 0.05). For the seawater acclimation experiment, gill samples were collected at 0, 2, 5 and 14 days after seawater transfer. For the freshwater acclimation experiment, gill samples were collected at 0, 2, 5, 8 and 14 days after freshwater transfer. The numbers in parentheses in each graph indicate the number of samples at each sampling point.



study the coordination of osmoregulatory systems in gills of Atlantic salmon during parr–smolt transformation and during seawater- and freshwater acclimation, we examined changes in transcript levels of key osmoregulatory genes with three experiments: comparison between parr and smolt, and time courses of fresh water to seawater and seawater to freshwater exposures. To evaluate the effects of feeding activity, we also conducted a 2-week fasting experiment and examined the same factors examined in the salinity acclimation experiments.

Comparison of parr and smolt showed no significant differences of plasma chloride levels in fresh water. One important alteration that occurs before downstream migration of salmonids into seawater is the development of seawater-type ionocytes in the gills (McCormick et al., 2013). GH and cortisol are known to be two important endocrine factors that promote this development (McCormick, 2001). We hypothesized that smolt prepared for downstream migration would have higher expression levels of key osmoregulatory genes than parr. However, only *cftr1* transcript levels were different between parr and smolt (Figure S1C). Nonetheless, gill NKA activity was higher in smolt, and because sampling occurred at the peak of smolt development, transcriptional changes in *atp1a1a* and *atp1a1b* likely occurred earlier, as shown in previous studies (Christensen et al., 2018; Nilsen et al., 2007). Gill *ghr2* transcript levels were slightly but significantly higher in smolt than in parr. Plasma cortisol levels were significantly higher in smolt than in parr, which is consistent with previous studies (Specker & Schreck, 1982; Langhorne & Simpson, 1986). Cortisol implantation induced *cftr1* expression, but not *cftr2* expression in Atlantic salmon (Singer et al., 2003). In vitro incubation with cortisol stimulated transcript levels of *cftr1* in gills from both freshwater- and seawater-acclimated Atlantic salmon (Kiilerich et al., 2007a). The results of the present study support the idea that *cftr1* expression is under the control of circulating cortisol during smolt development.

Regarding the seawater and freshwater acclimation, physiological data revealed that the fish used in this study successfully acclimated to the changes in environmental salinity in each experimental condition. One of the largest transcriptional changes observed in the present study was the response of gill *prlr* to salinity, which significantly decreased after transfer from fresh water to seawater and increased after transfer from seawater to fresh water. Kiilerich et al. (2007b) reported that gill *prlr* transcript levels decreased during smolt development but increased after seawater transfer, whereas Nilsen et al. (2007) found that *prlr* levels decreased after a month in seawater. The latter is consistent with our results and indicates that gill *prlr* transcription is elevated in fresh water relative to seawater. Decreases in gill *prlr* transcript abundance following seawater transfer have also been shown in Mozambique tilapia (Breves et al., 2010; Sandra et al., 1995; Shiraishi et al., 1999). Two prolactin receptor paralogs have been found in Mozambique tilapia (Fiol et al., 2009) and black sea bream (*Spondyliosoma cantharus*; Huang et al., 2007), and gill *prlr1/a* is elevated in fresh water and *prlr1/b* elevated in seawater in the black sea

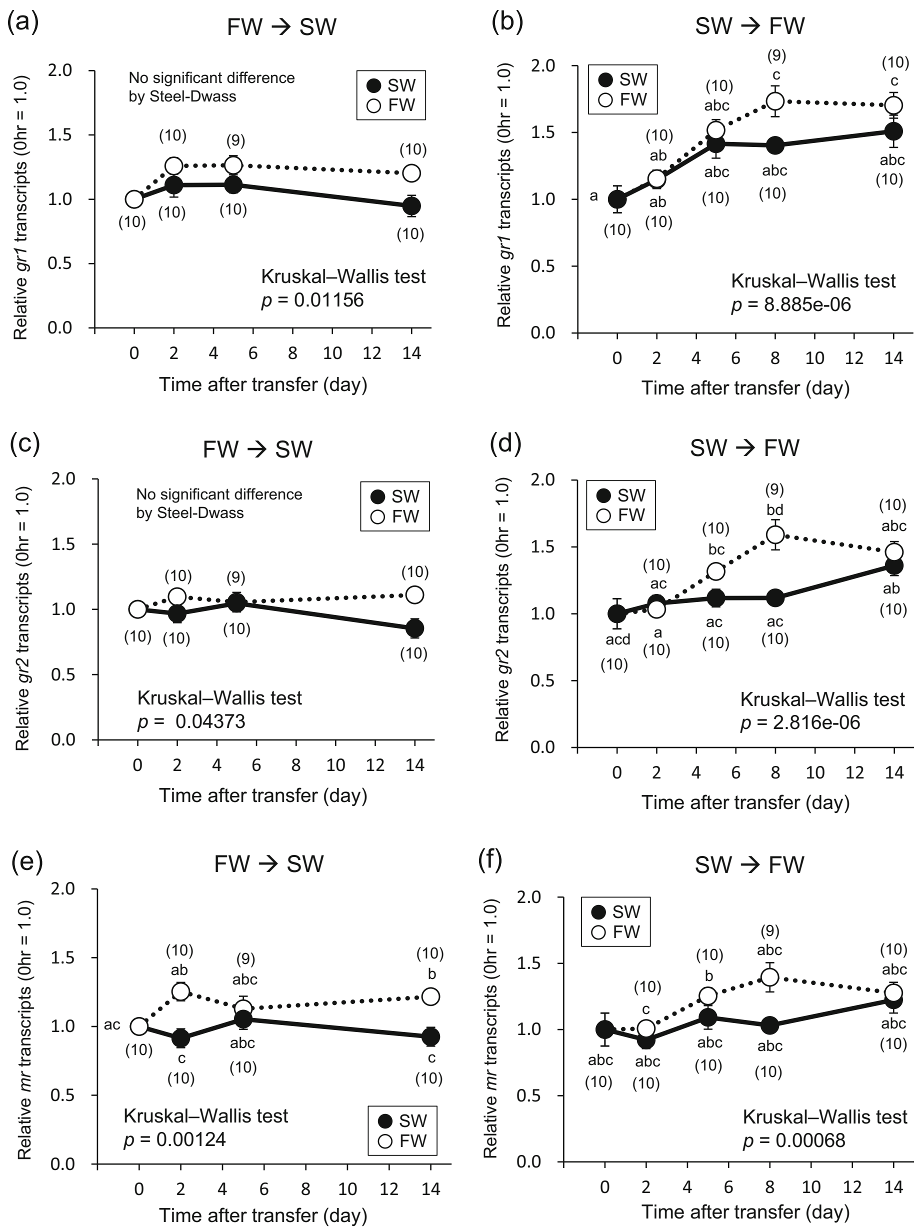


FIGURE 6 Atlantic salmon gill *gr1* transcript levels during seawater (SW) acclimation (a) and freshwater (FW) acclimation (b) experiments. Atlantic salmon gill *gr2* transcript levels during the seawater acclimation (c) and freshwater acclimation (d) experiments. Atlantic salmon gill *mr* transcript levels during the seawater acclimation (e) and freshwater acclimation (f) experiments. The values were corrected with internal controls and the relative expression values at day 0 were set to 1.0 (see Materials and Methods). Differences in transcript levels between sampling points were analysed using the Kruskal–Wallis test. Values not sharing a letter are significantly different by the Steel–Dwass test ($p < 0.05$). For the seawater acclimation experiment, gill samples were collected at 0, 2, 5 and 14 days after seawater transfer. For the freshwater acclimation experiment, gill samples were collected at 0, 2, 5, 8 and 14 days after freshwater transfer. The numbers in parentheses in each graph indicate the number of samples at each sampling point.

bream. The pattern of *prlr* transcript levels measured in the present study were most similar to those of *prlr1/a* observed in tilapia, where transcript levels were upregulated after freshwater exposure. Further examination of Atlantic salmon, their tissue distribution and response to salinity could enhance our understanding of the existence of two *prlr* isoforms. Prunet et al. (1985) demonstrated that plasma PRL levels decreased after seawater transfer and increased after freshwater transfer in rainbow trout but did not change consistently in response to changes in salinity in Atlantic salmon (Prunet et al., 1989). In contrast, Young et al. (1989) found that plasma PRL of coho salmon (*Oncorhynchus kisutch*) decreased within 24 h of exposure to seawater and remained low for 14 days. The strong effect of salinity on circulating PRL and PRL receptor highlights the key role for PRL signalling in regulating osmoregulatory physiology of Atlantic salmon, particularly during acclimation to fresh water.

Previous studies have demonstrated an important role for GH in osmoregulation of many euryhaline teleost fish (Takei & McCormick, 2013). Kiilerich et al. (2007b) and Nilsen et al. (2007) both

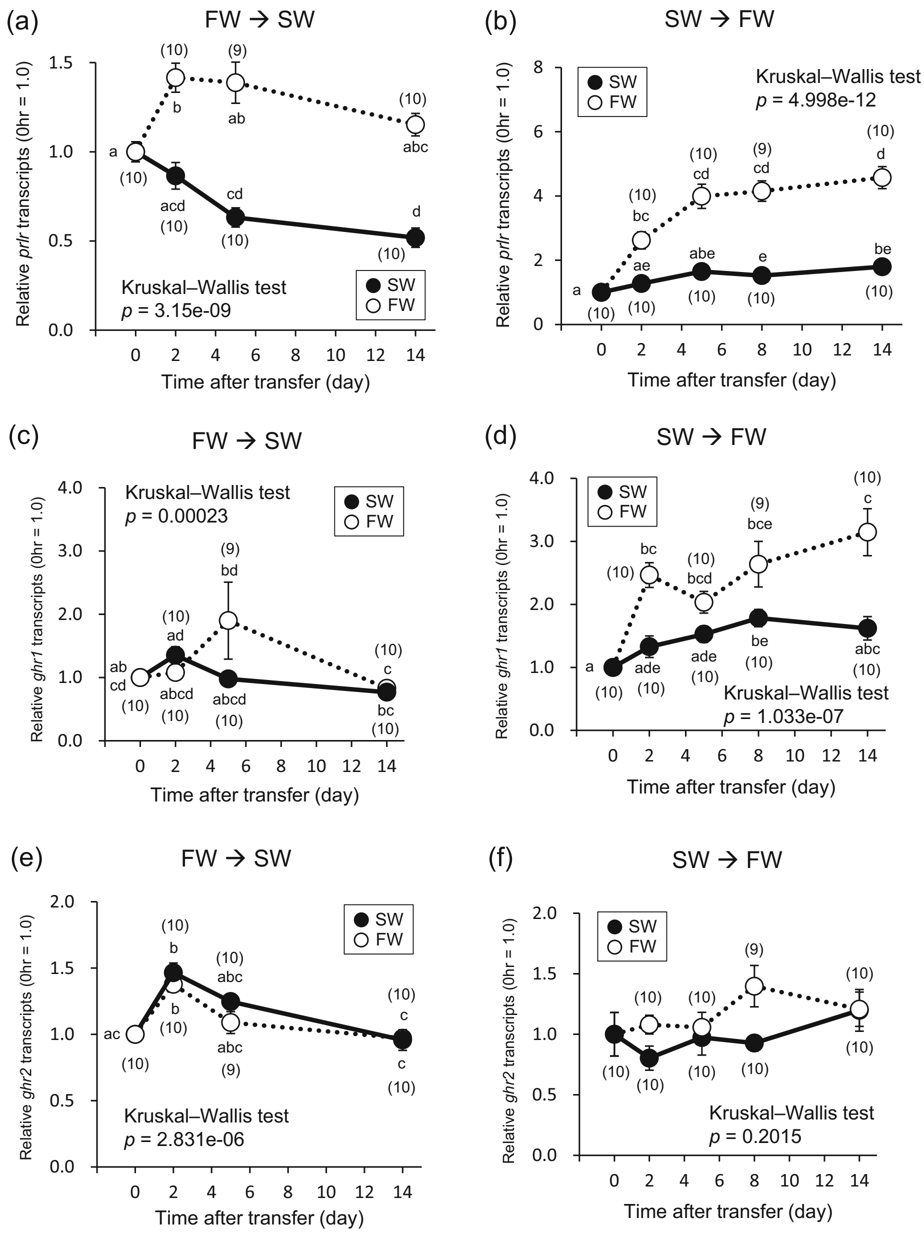


FIGURE 7 Atlantic salmon gill *prlr* transcript levels during seawater (SW) acclimation (a) and freshwater (FW) acclimation (b) experiments. Atlantic salmon gill *ghr1* transcript levels during seawater acclimation (c) and freshwater acclimation (d) experiments. Atlantic salmon gill *ghr2* transcript levels during seawater acclimation (e) and freshwater acclimation (f) experiments. The values were corrected with internal controls and the relative expression values at day 0 were set to 1.0 (refer to Materials and Methods). Differences in transcript levels between sampling points were analysed using the Kruskal–Wallis test. Values not sharing a letter are significantly different by the Steel–Dwass test ($p < 0.05$). For the seawater acclimation experiment, gill samples were collected at 0, 2, 5 and 14 days after seawater transfer. For the freshwater acclimation experiment, gill samples were collected at 0, 2, 5, 8 and 14 days after freshwater transfer. The numbers in parentheses in each graph indicate the number of samples at each sampling point.

found increased gill *ghr* transcription during smolt development, but conflicting results regarding changes following seawater exposure. Breves et al. (2017) found that gill *ghr* transcription did not change during smolt development, but was elevated following exposure of smolt to seawater. The results of the present study revealed that changes in gill *ghr* transcription in parr were not strongly influenced by environmental salinities, and thus may not be critical to salinity acclimation for this developmental stage.

Cortisol is known to be one of the major osmoregulatory hormones in bony fishes. Plasma cortisol levels stayed high for several days after seawater transfer in American eel (*Anguilla rostrata*; Forrest Jr. et al., 1973) and Atlantic sturgeon (*Acipenser oxyrinchus*; McCormick et al., 2020). Transient increases of plasma cortisol within 6–24 h after seawater transfer have been observed in tilapia (Assem & Hanke, 1981), juvenile chinook salmon (*Oncorhynchus tshawytscha*; Strange & Schreck, 1980), parr, smolt and adult Atlantic salmon (McCormick et al., 2013, 2019; Nichols & Weisbart, 1985), and striped bass (*Morone saxatilis*; Madsen et al., 1994). In some cases, however, no meaningful change of plasma cortisol was observed during seawater exposure in tilapia (Dean et al., 2003), although Assem and Hanke (1981) reported the increase of plasma cortisol at the early phase of seawater exposure in the same species. Thus, plasma cortisol levels following seawater exposure are variable depending on species, developmental stage and/or physiological status. Although plasma cortisol levels increase slightly, the metabolic clearance rate of cortisol increases to a much greater extent following seawater exposure of Atlantic salmon, indicating that both synthesis and catabolism are increased (Nichols & Weisbart, 1985). A number of previous studies using exogenous cortisol and corticosteroid receptor blockers have shown that cortisol is a major endocrine factor for osmoregulatory homeostasis (Takei & McCormick, 2013). Thus, it is possible that teleosts, particularly euryhaline fishes, have tissue-specific mechanisms that can promote a response to cortisol in osmoregulatory tissues. Flores et al. (2012) showed that *gr1*, *gr2* and *mr* in the gills of rainbow trout did not change after transfer to 70% seawater for up to 2 weeks. Yada et al. (2008) reported that gill *gr1*, *gr2* and *mr* in steelhead (*Oncorhynchus mykiss*) significantly increased after acclimation to 100% seawater for a month. Kiilerich et al. (2007b) showed that gill *gr* transcript levels were significantly increased and *mr* were significantly decreased after 24 h of seawater exposure in Atlantic salmon. Thus, the responses of *gr1*, *gr2* and *mr* expression in response to salinity change in salmonids appear to be inconsistent. In the present study, changes in *gr1*, *gr2* and *mr* expression were relatively small, at least in comparison with changes in *atp1a1a*, *atp1a1b* and *cftr1* transcript levels following seawater transfers (Figures S2 and S3). Nonetheless, the fact that transcript abundance of all three corticosteroid receptors was decreased in seawater and increased in fresh water indicates that regulation of these receptors may be important in the Atlantic salmon's response to salinity change, especially during freshwater acclimation.

Another possible cortisol-regulating factor that may be involved in salinity acclimation is the tissue level of *hsd11b*. Kiilerich et al. (2007b) found that transcript levels of gill *hsd11b2* after 24 h in seawater were significantly increased early in smolt development but decreased late in smolt development, indicating a developmental difference in response to salinity. In the present study, 2 weeks of fasting induced increases of *hsd11b2* transcript levels (as well as *gr1*, *gr2* and *mr* transcripts), although these were small compared to the changes observed due to salinity (Figure S5). Our results indicate that for Atlantic salmon parr, exposure to seawater decreases and exposure to fresh water increases the abundance of *hsd11b2* transcript levels. The lower levels of gill *hsd11b2* in seawater conditions would reduce the conversion of cortisol to cortisone, resulting in higher cortisol within gill cells. This would, in turn, increase the binding of cortisol to its receptors and promote the transcription of ion transporters and channels in gill ionocytes that are critical for seawater acclimation. Localizing *hsd11b2* in the gill could support determination of whether it is more abundant in gill ionocytes and whether it co-localizes with corticosteroid receptors.

Hsd11b3 transcripts were detectable in Atlantic salmon gill. The seawater to freshwater experiment showed that gill *hsd11b3* transcript levels were significantly higher in seawater fish. However, freshwater to seawater and fasting experiments (Figure S2D) showed no significant change of gill *hsd11b3* transcript levels. These results do not support the hypothesis that multiple types of *hsd11b*s are differentially expressed in the gills and optimize levels of corticosteroids involved in osmoregulation. Determining the enzymatic activity of *hsd11b3* through a transient transfection assay or other approaches could help characterize the enzyme's function.

In conclusion, salinity change had slight but consistent impacts that resulted in higher levels of gill *gr1*, *gr2* and *mr* transcripts in fresh water. Even stronger upregulation by freshwater exposure was seen for gill *prlr* expression, indicating that both PRL and cortisol signalling are important for freshwater acclimation. The coincident increases in corticosteroid and prolactin levels indicate a possible interaction between prolactin and cortisol, which could be further examined. Decreased transcription of gill *hsd11b2* during seawater acclimation likely promotes elevated intracellular cortisol that will promote the development of seawater ionocytes and associated ion transporters. Innovation in methods for measurement of intracellular cortisol in specific cell types (such as seawater ionocytes in the gill) would help test the role of *hsd11b2* in smolt development and seawater acclimation.

AUTHOR CONTRIBUTIONS

M.K., A.M.R. and S.D.M. performed the experiments and analyzed the data. T.Y. and G.Y. helped supervise the project. M.K. and S.D.M. wrote the manuscript in consultation with A.M.R., T.Y. and G.Y.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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