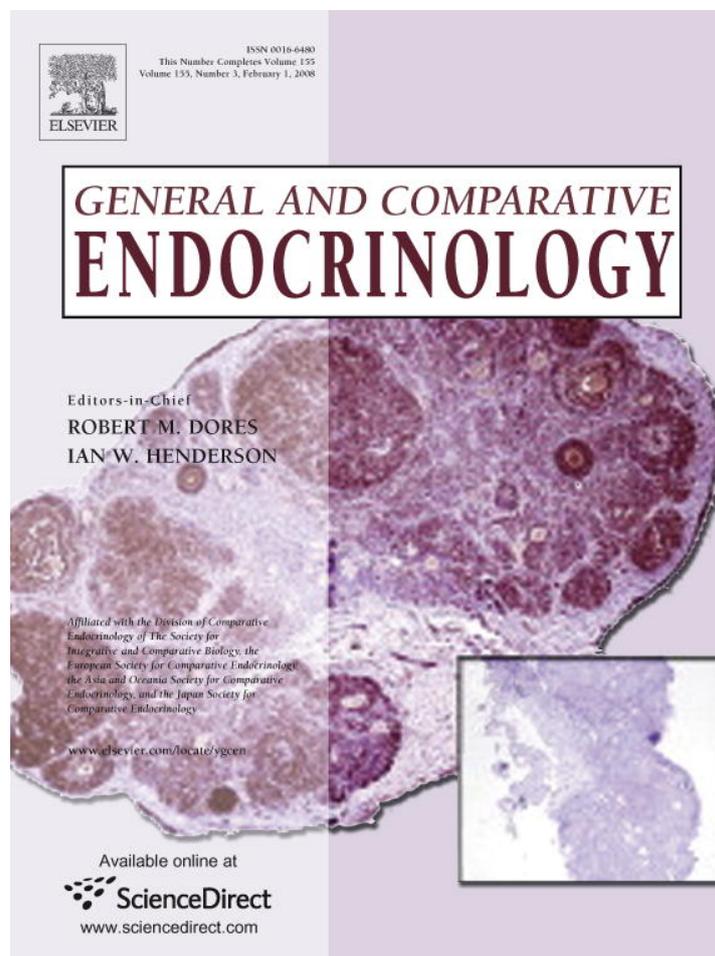


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Endocrine systems in juvenile anadromous and landlocked Atlantic salmon (*Salmo salar*): Seasonal development and seawater acclimation

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Abstract

The present study compares developmental changes in plasma levels of growth hormone (GH), insulin-like growth factor I (IGF-I) and cortisol, and mRNA levels of their receptors and the prolactin receptor (PRLR) in the gill of anadromous and landlocked Atlantic salmon during the spring parr-smolt transformation (smoltification) period and following four days and one month seawater (SW) acclimation. Plasma GH and gill GH receptor (GHR) mRNA levels increased continuously during the spring smoltification period in the anadromous, but not in landlocked salmon. There were no differences in plasma IGF-I levels between strains, or any increase during smoltification. Gill IGF-I and IGF-I receptor (IGF-IR) mRNA levels increased in anadromous salmon during smoltification, with no changes observed in landlocked fish. Gill PRLR mRNA levels remained stable in both strains during spring. Plasma cortisol levels in anadromous salmon increased 5-fold in May and June, but not in landlocked salmon. Gill glucocorticoid receptor (GR) mRNA levels were elevated in both strains at the time of peak smoltification in anadromous salmon, while mineralocorticoid receptor (MR) mRNA levels remained stable. Only anadromous salmon showed an increase of gill 11 β -hydroxysteroid dehydrogenase type-2 (11 β -HSD2) mRNA levels in May. GH and gill GHR mRNA levels increased in both strains following four days of SW exposure in mid-May, whereas only the anadromous salmon displayed elevated plasma GH and GHR mRNA after one month in SW. Plasma IGF-I increased after four days in SW in both strains, decreasing in both strains after one month in SW. Gill IGF-I mRNA levels were only increased in landlocked salmon after 4 days in SW. Gill IGF-IR mRNA levels in SW did not differ from FW levels in either strain. Gill PRLR mRNA did not change after four days of SW exposure, and decreased in both strains after one month in SW. Plasma cortisol levels did not change following SW exposure in either strain. Gill GR, 11 β -HSD2 and MR mRNA levels increased after four days in SW in both strains, whereas only the anadromous strain maintained elevated gill GR and 11 β -HSD2 mRNA levels after one month in SW. The results indicate that hormones and receptors of the GH and cortisol axes are present at significantly lower levels during spring development and SW acclimation in landlocked relative to anadromous salmon. These findings suggest that attenuation of GH and cortisol axes may, at least partially, result in reduced preparatory upregulation of key gill ion-secretory proteins, possibly a result of reduced selection pressure for marine adaptations in landlocked salmon.

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Keywords: Growth hormone receptor; Insulin-like growth factor I receptor; Cortisol; Glucocorticoid receptor; Mineralocorticoid receptor; 11 β -Hydroxysteroid Dehydrogenase Type 2; Parr-smolt transformation

1. Introduction

During parr-smolt transformation (smoltification) of Atlantic salmon (*Salmo salar*), several endocrine systems play important roles in regulating the timing and intensity

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of morphological, physiological and behavioral changes preparing the juvenile for a marine life. These preparatory changes are completed before the juveniles abandon their freshwater (FW) habitat and migrate to marine feeding grounds (McCormick et al., 1998). Among the many hormones involved in the regulation of smoltification, changes in circulating levels of growth hormone (GH), insulin-like growth factor I (IGF-I; Ágústsson et al., 2001; McCormick et al., 2000, 2002) and cortisol (McCormick et al., 2002; Shrimpton and McCormick, 1998b; Sundell et al., 2003) in concert with their receptors (Kiilerich et al., 2007; Shrimpton, 1996; Shrimpton and McCormick, 1998b) stimulate the preparatory development of seawater (SW) tolerance in osmoregulatory tissues. These hormones are involved in the proliferation and differentiation of SW-type chloride cells and increase gill Na^+ , K^+ -ATPase activity (NKA; McCormick, 2001; Sakamoto et al., 2001), allowing smolts to move rapidly from FW to full-strength SW with minimum osmotic disturbance (Hoar, 1988). Conversely, circulating prolactin (PRL; Prunet and Boeuf, 1989; Prunet et al., 1989) and PRL receptor (PRLR) mRNA (Kiilerich et al., 2007) levels decrease during smoltification. PRL is considered a FW-adaptive hormone, probably mediating its effect through impeding the osmotic permeability of gill epithelia (Manzon, 2002) and antagonizing the SW-adaptive effect of GH (Madsen and Bern, 1992).

In teleosts, cortisol actions were previously thought to be mediated primarily through glucocorticoid receptors (Mommsen et al., 1999). However, a mineralocorticoid-like receptor (MR) has been identified in fish (Colombe et al., 2000; Greenwood et al., 2003), showing equal or higher affinity to cortisol in salmonids (Sturm et al., 2005). In mammals, MRs are associated with 11β -hydroxysteroid Dehydrogenase type 2 (11β -HSD2), also found in teleosts (Jiang et al., 2003; Kusakabe et al., 2003), an enzyme that metabolises/inactivates cortisol, protecting the receptor from high glucocorticoid levels as its affinity is higher to cortisol than that of the GRs (Bury and Sturm, 2007). Hence, the actions of cortisol can be mediated through two receptors and regulated independently in different tissues and cells. GRs are known to regulate ion balance in salmonids (Prunet et al., 2006) and corticoidsteroid receptor (CR) abundance increase during smoltification in salmon (Shrimpton, 1996; Shrimpton and McCormick, 1998b). Although little is known about the role of MRs in teleosts, in mammals MRs stimulate ion retention (Rashid and Lewis, 2005). A similar role has been suggested in rainbow trout where proliferation of chloride cells in the gill is blocked with MR antagonist but not with GR antagonist when transferring fish to ion poor water (Sloman et al., 2001). Moreover, it has been proposed that cortisol has a dual action mediating both FW and SW acclimation, promoting both ion uptake and excretion, respectively, and that this is dependent on the presence of PRL and GH (McCormick, 2001; Sakamoto and McCormick, 2006). In teleosts, the dual roles of cortisol on these two different

osmoregulatory processes may be mediated through the differential binding to its two receptors, GRs and MRs.

In contrast to the normal anadromous lifecycle of Atlantic salmon, several landlocked Atlantic salmon populations complete their lifecycle in FW (McDowall, 1988). The smoltification-related developmental changes of these populations differ from their anadromous counterparts in morphology, immunology, and the degree of preparatory changes in hypo-osmoregulatory capacity (Johnston et al., 2005; Nilsen et al., 2003, 2007; Rønneseth et al., 2005). Landlocked salmon display a reduced development of the osmoregulatory machinery in the gill, including the synthesis and activity of NKA, levels of Na^+ , K^+ , 2Cl^- cotransporter (NKCC) and CFTR anion channel mRNA levels (Nilsen et al., 2003, 2007). Consequently, one may hypothesize that developmental traits associated with marine life in ancestral anadromous populations have been lost or suppressed in landlocked salmon due to reduced selection pressures for marine adaptations. No studies have yet addressed the endocrine basis for these differences between landlocked and anadromous Atlantic salmon during this critical period of development. The present study compares circulating hormone and receptor mRNA levels in the gills of anadromous and landlocked salmon during the spring smoltification period in FW and following SW acclimation in search of an endocrine basis for the previously observed differences in developmental changes in osmoregulatory proteins (Nilsen et al., 2007).

2. Materials and methods

2.1. Fish stocks, rearing conditions and sampling procedures

Juvenile Atlantic salmon from a non-anadromous, landlocked population (lake Byglandsfjord, South-central Norway) that migrate up tributaries to spawn (Dahl, 1928), and an anadromous population (River Vosso, South-western Norway) were brought to the aquatic laboratory of the Bergen high technology center in October and kept separately in 1 m^2 indoor tanks supplied with flow-through pH adjusted (6.9–7.1) FW with a constant rearing temperature of 8°C (± 0.2). The fish were exposed to simulated natural photoperiod (SNP; $60^\circ 25' \text{N}$) and fed a commercial dry diet (T. Skretting A/S, Stavanger, Norway) according to Austreng et al. (1987) for 8–12 h during the photo-phase. On March 5, a total of 150 anadromous (mean weight 29.4 ± 2.2 g) and landlocked (mean weight 21.7 ± 1.1 g) salmon were transferred into duplicate 1 m^2 indoor tanks (rearing volume 400 L, $n = 75$ in each tank) and reared as described above. The variance in mean weight is given as \pm standard error of the mean. In mid-May, sub-groups of both anadromous (mean weight 44.6 ± 2.4 g, $n = 10$) and landlocked (mean weight 39.1 ± 2.2 g, $n = 10$) salmon were transferred into full-strength SW (natural SW 34‰, 8°C) for 96 h SW challenge and one month for assessment of long-term post-smolt performance, while sub-groups of each strain remained in FW. All fish groups were kept in 1 m^2 indoor tanks with a rearing volume of 400 L.

Fish ($n = 10$) from both strains in FW were quickly dip netted out of the tanks and anaesthetized directly in 100 mg/L tricaine methanesulphonate (MS222; Sigma, St. Louis, MO, USA) on February 26, April 15, May 15 and June 18. Weight (g) and fork length (nearest mm) were measured before blood was drawn from the caudal vessels with heparinized syringes. Blood was stored on ice less than 30 min before centrifugation (1500g, 10 min, 4°C). Gill tissue for determination of mRNA levels was quickly dissected and frozen directly on dry ice. All samples were stored at

–80 °C until assayed. Samples were also obtained from juveniles after 96 h SW exposure ($n = 8$) on May 20 and from fish ($n = 10$) of both strains transferred to SW in mid-May and sampled on June 22.

2.2. Plasma hormones

Plasma cortisol levels were determined by a direct immunoassay according to Carey and McCormick (1998), with a few modifications as described by Stefansson et al. (in press). Plasma GH and IGF-I levels were assessed by radioassays validated for Atlantic salmon (Björnsson et al., 1994) and IGF-I (Moriyama et al., 1994).

2.3. Real-time quantitative PCR

Total RNA for quantification of mRNA levels was extracted from ~50 mg gill tissue using TRI Reagent® (Sigma, St. Louis, MO, USA) as outlined by Chomczynski (1993). Total RNA was quantified spectrophotometrically, purity assessed ($260/280 \geq 1.8$) and integrity checked by 1% agarose/formaldehyde gel electrophoresis. RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA) and cDNA reversely transcribed using 0.5 µg total RNA and random nonamers in conjunction with the Reverse Transcription Core kit (EUROGENTEC RT-RTCK-05, Liege, Belgium) following the manufactures instructions.

Gill GR, MR, 11β-HSD2 and PRLR mRNA levels were measured by Q-PCR SYBR Green assays according to Kiilerich et al. (2007) using a Mx3000p instrument (Stratagene, La Jolla, CA, USA) with standard software settings including adaptive baseline for background detection, moving average and amplification based threshold settings. Gill GHR, IGF-IR and IGF-I mRNA levels were measured by Q-PCR TaqMan assays according to Stefansson et al. (in press) using the ABI prism 7000 detection system platform (Applied Biosystems, Foster City, CA, USA). Primers and probes are shown in Table 1. Results are presented as relative expression (Livak and Schmittgen, 2001) using elongation factor 1A (Olsvik et al., 2005) as an internal control and anadromous parr (February 26) as calibrator. Elongation factor 1A did not change during development, SW acclimation or differ between strains.

2.4. Statistics

All statistical analyses were performed with Statistica 6.0. (StatSoft, Inc., Tulsa, OK, USA). The homogeneity of variance was tested using the Levene's *F*-test. When necessary, data were log transformed to meet the parametric assumptions of ANOVA (Zar, 1996). No significant differences were observed between replicate tanks, except for plasma cortisol which were consistently higher in fish of both strains sampled in the second replicate tank. This is probably due to stress induced elevation of cortisol levels (Carey and McCormick, 1998). Hence, only cortisol data from fish of both strains sampled in the first replicate tank was used. With the exception of cortisol, comparisons of all parameters in FW were analyzed using a nested ANOVA with tanks nested within time and strain, whereas a two-way ANOVA was used to test for overall differences in plasma cortisol levels. A two-way ANOVA was used to test for overall differences within strains between SW and FW, and between strains in SW. Significant ANOVAs were followed by Newman–Keuls post hoc tests. Data are presented as means ± standard error of the mean (SEM) and considered significant at the level of $P < 0.05$.

3. Results

3.1. Plasma GH and gill GH mRNA levels

Plasma GH levels in anadromous salmon increased from February through June, reaching peak levels in June, 7-fold higher than in February (Fig. 1A). In contrast, no increase in plasma GH levels occurred in landlocked sal-

Table 1
Primer and probe sequences used in real-time PCR assays

Gene	GenBank Accession No.	Forward primers	Reverse primers	Probes	Reference
GHR	AY462105	TGGGAAGTTGAGTGCCAGACT	CACAAGACTACTGTCTCCGTTGA	TGGGAGAGCCAGCCAGCCTGC	Stefansson et al. (2007)
IGF-IR	AY049954	TGAAGAGCCACCTGAGGTCACT	TCAGAGGTGGAGGTTGAGACT	CGGGCTAAAGACCCGTCCCAGTCC	
IGF-I	M81904	GTGTGCGGAGAGAGAGGCTTT	TGTGACCCGCTGAACCTG	TTTCAGTAAACCAACGGGCTATGG	
EF1A	AF321836	CCCCTCCAGGACGTTTACAAA	CACACGGCCACAGGTACA	ATCGGTGGTATTGGAAC	
GR	AF209873	ACGACGATGGAGCCGAAAC	ATGGCTTTGAGCAGGGATAG	Sybr Green	Kiilerich et al. (2007)
MR	AF209873	AGACTCGACCCCAACCAAG	CGTTAGTGGGACTGGTGCTC	Sybr Green	
11β-HSD2	BG934620	GCTGCCTATACTCTGCCA	GCCTGTGATGAAGACAGC	Sybr Green	
EF1A	AF321836	GAGAACCATTTGAGAAAGTTCGAGAAG	GCACCCAGGCATACCTTGAAG	Sybr Green	

References for a more detailed description of the assays are also listed.

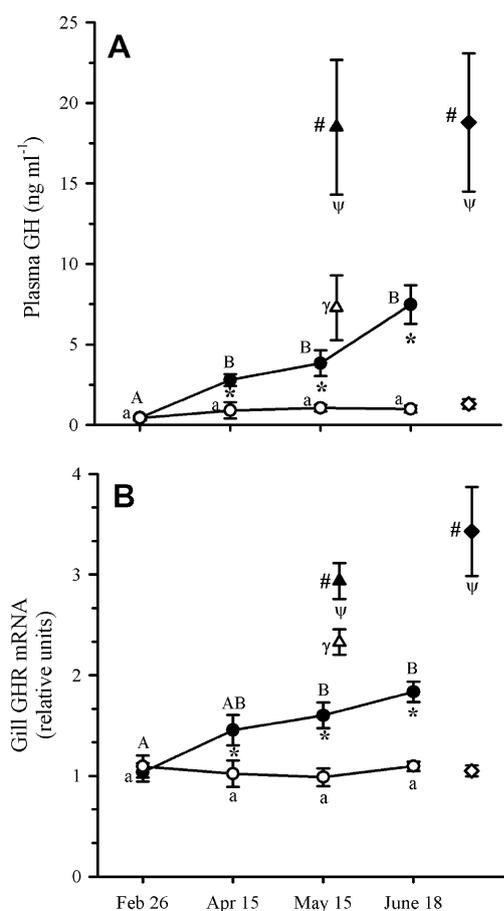


Fig. 1. Circulating GH (A) and gill GHR mRNA levels (B) in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 4 days in SW (34‰) exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after one month in SW are offset for clarity. mRNA levels are normalized to the internal reference gene, elongation factor 1A, and data expressed relative to parr on February 26 (relative units). Different capital and small letters denote differences ($p < 0.05$) between timepoints within anadromous and landlocked salmon in FW, respectively. When significant, differences between strains in FW (*) and SW (Ψ) are shown. Significant differences between FW and SW are indicated by # and γ in anadromous and landlocked salmon, respectively. Data are means ± SEM ($n = 8-10$).

mon during spring, resulting in lower GH levels than anadromous salmon from April onwards (Fig 1A). Gill GHR mRNA levels in anadromous salmon were significantly elevated in May and remained high in June, whereas gill GHR mRNA remained stable in landlocked salmon, resulting in lower GHR mRNA levels in landlocked salmon in April, May and June (Fig. 1B).

After 4 days of SW exposure in mid-May, circulating GH and gill GHR mRNA levels increased significantly in both strains, whereas after one month in SW elevated plasma GH and gill GHR mRNA levels were only observed in the anadromous strain (Fig. 1A and 1B). Plasma GH and gill GHR mRNA levels were significantly higher in the anadromous strain after SW transfer.

3.2. Plasma IGF-I, gill IGF-I and IGF-IR mRNA levels

Plasma IGF-I levels decreased significantly in both strains from February to April, remaining stable in May and June, with higher levels in the landlocked strain in June (Fig. 2A). Gill IGF-I mRNA levels in anadromous salmon increased from February to June, whereas no significant changes in IGF-I mRNA levels occurred during spring in landlocked salmon (Fig. 2B). Anadromous salmon exhibited a transient increase of gill IGF-IR mRNA levels in May, resulting in significant differences between strains in May (Fig. 2C).

After 4 days of SW exposure in mid-May, circulating IGF-I levels increased significantly in both strains (Fig. 2A), while gill IGF-I mRNA levels only increased in landlocked salmon (Fig. 2B). After one month in SW, plasma IGF-I levels were higher in landlocked than anadromous salmon (Fig. 2A), whereas no differences were observed in gill IGF-I mRNA levels (Fig. 2B). Gill IGF-IR mRNA levels did not change significantly following 4 days or one month of SW exposure in either strain (Fig. 2C), but gill IGF-IR mRNA was higher in anadromous than landlocked salmon after one month in SW.

3.3. Gill PRLR mRNA levels

Gill PRLR mRNA levels did not change significantly in either anadromous or landlocked salmon from February through June (Fig. 3), nor did levels differ between strains. Gill PRLR levels did not change significantly in either strain after 4 days of SW exposure in mid-May, but were reduced after one month in SW (Fig. 3).

3.4. Plasma cortisol, gill GR, MR and 11β-HSD2 mRNA levels

Plasma cortisol levels in anadromous salmon increased significantly from April to May, reaching peak levels in June, 5-fold higher than levels observed in February (Fig. 4A). No increase in plasma cortisol levels occurred in landlocked salmon during spring, resulting in lower cortisol levels in landlocked than anadromous salmon in May (Fig. 4A). Gill GR mRNA levels increased significantly in May in both strains, and did not differ between strains (Fig. 4B). Gill MR mRNA levels did not change significantly in anadromous nor landlocked salmon, and no differences were observed between strains (Fig. 4D). Anadromous salmon exhibited a transient increase of gill 11β-HSD2 mRNA levels in May, while no increase of gill 11β-HSD2 mRNA levels occurred in landlocked salmon, resulting in higher 11β-HSD2 mRNA levels in anadromous salmon in May (Fig. 4C).

Circulating cortisol levels did not change following SW exposure in either strain (Fig. 4A), yet plasma cortisol was significantly higher in anadromous than landlocked salmon after 4 days in SW in mid-May. Following short-term SW exposure in mid-May, gill GR (Fig. 4B), 11β-HSD2

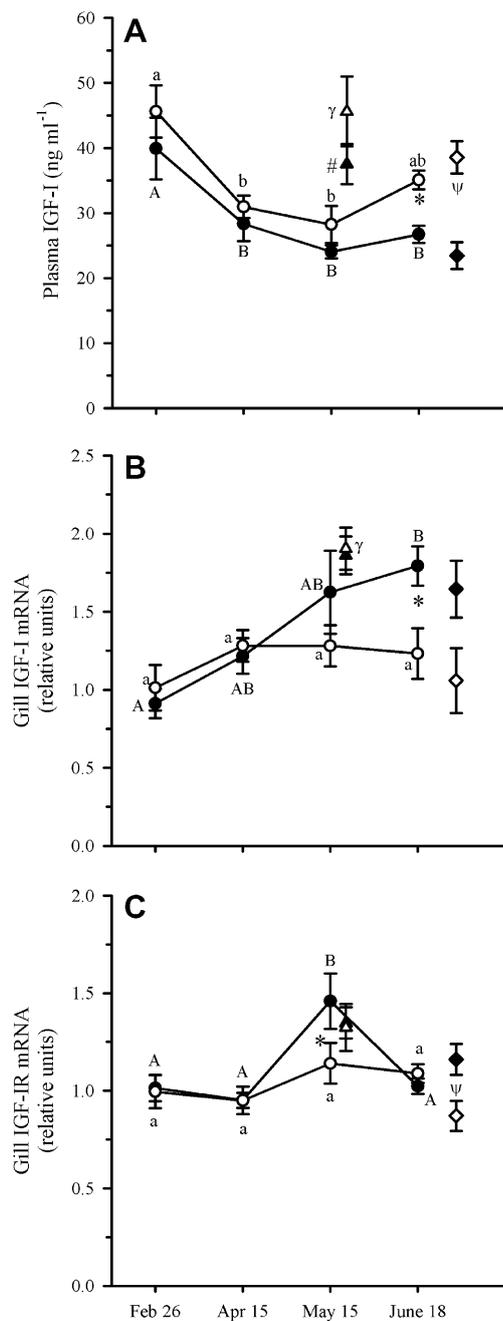


Fig. 2. Circulating IGF-I (A), gill IGF-I (B) and IGF-IR mRNA (C) levels in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 4 days in SW (34‰) exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after one month in SW are offset for clarity. mRNA levels are normalized to the internal reference gene, elongation factor 1A, and data expressed relative to parr on February 26 (relative units). Different capital and small letters denote differences ($p < 0.05$) between timepoints within anadromous and landlocked salmon in FW, respectively. When significant, differences between strains in FW (*) and SW (Ψ) are shown. Significant differences between FW and SW are indicated by # and γ in anadromous and landlocked salmon, respectively. Data are means ± SEM ($n = 8-10$).

(Fig. 4C) and MR (Fig. 4D) mRNA levels increased in both strains. After one month in SW, gill GR (Fig. 4B)

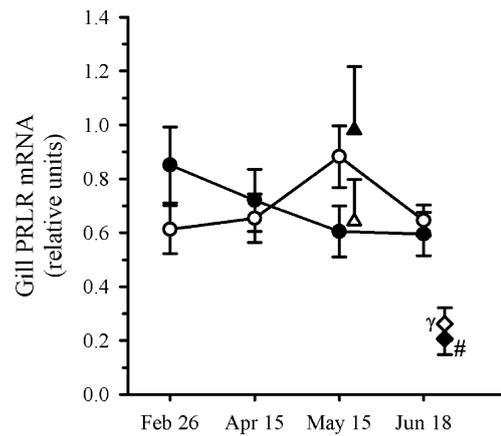


Fig. 3. Gill PRLR mRNA levels in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 4 days in SW exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after one month in SW are offset for clarity. mRNA levels are normalized to the internal reference gene, elongation factor 1A, and data expressed relative to parr on February 26 (relative units). No significant differences were observed. Data are means ± SEM ($n = 8-10$).

and 11β-HSD2 (Fig. 4C) mRNAs were significantly higher in anadromous than landlocked salmon.

4. Discussion

Developmental changes associated with smoltification in anadromous salmonids are mediated by temporal increases in endocrine systems, with GH, IGF-I and cortisol playing important roles (McCormick, 2001). Here we provide evidence that these endocrine systems, as judged by plasma hormone levels and their gill receptor mRNA levels, are attenuated during the spring development in landlocked compared with anadromous salmon, which corresponds well with recent molecular and physiological data showing that the landlocked strain display reduced preparatory synthesis and upregulation of key gill ion-secretory proteins during spring (Nilsen et al., 2007).

4.1. Freshwater (FW) development

4.1.1. Circulating GH and gill GHR mRNA

The present changes in circulating GH levels during smoltification in the anadromous strain is in good agreement with previous data (Björnsson, 1997), and supports the role for GH as a key regulator of growth (Björnsson et al., 2002) and development of hypo-osmoregulatory mechanisms (McCormick, 2001; Sakamoto and McCormick, 2006). Circulating GH levels were significantly lower in the landlocked strain, indicating reduced neuroendocrine GH stimulation (Ebbesson et al., 2003; unpublished results), synthesis (Ágústsson et al., 2003), secretion (Ágústsson et al., 2001) and/or clearance rates during the spring smoltification period in FW in the landlocked strain.

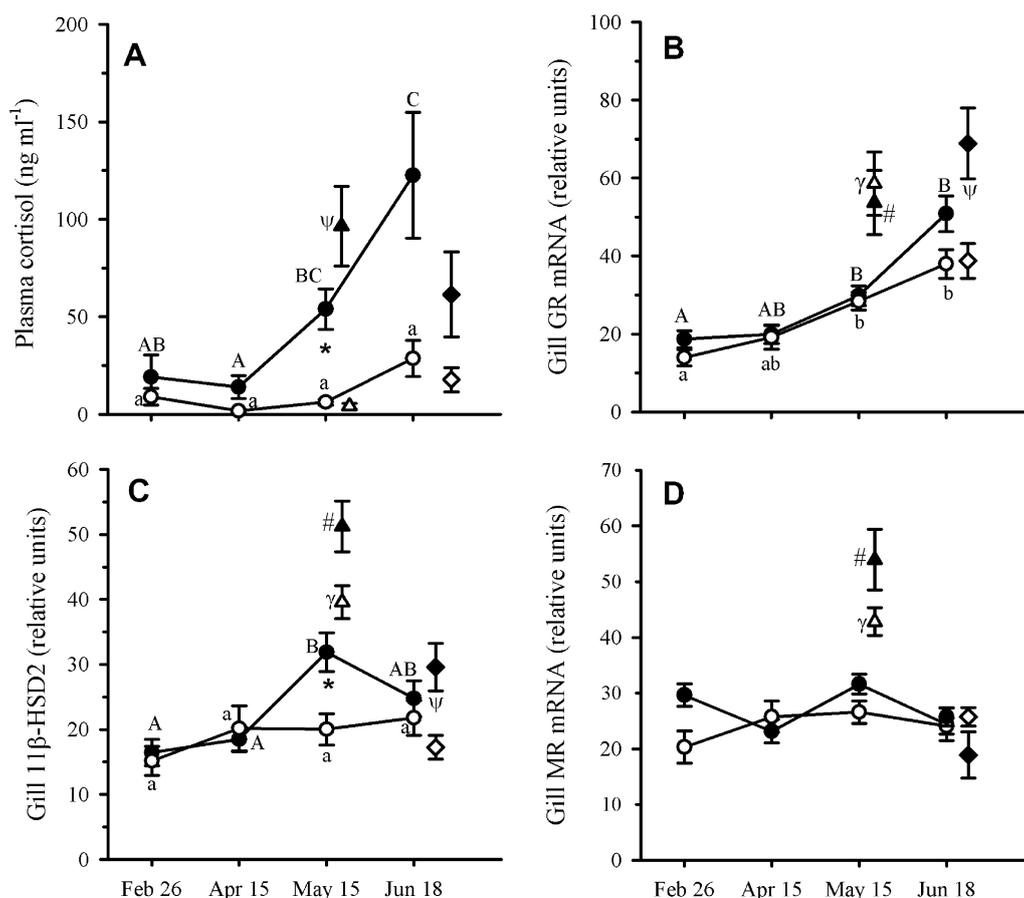


Fig. 4. Circulating cortisol (A), gill GR (B), 11 β -HSD2 (C) and MR (D) mRNA levels in anadromous (closed circles) and landlocked (open circles) Atlantic salmon in FW from February 26 through June 18. Symbols for anadromous (closed triangle) and landlocked salmon (open triangle) after 4 days in SW (34‰) exposure, and anadromous (closed diamond) and landlocked salmon (open diamond) in mid-June after one month in SW are offset for clarity. mRNA levels are normalized to the internal reference gene, elongation factor 1A, and data expressed relative to parr on February 26 (relative units). Different capital and small letters denote differences ($p < 0.05$) between timepoints within anadromous and landlocked salmon in FW, respectively. Asterisks (*) indicate significant differences between strains in FW. Significant differences between strains in SW are denoted with (Ψ). Data are means \pm SEM (cortisol, $n = 5-6$; gill GR, 11 β -HSD2 and MR mRNA, $n = 8-10$).

The present increase in gill GHR mRNA levels in the anadromous strain is similar to recent results in salmon during smoltification (Küllerich et al., 2007). Further, lower levels of plasma GH and gill GHR mRNA in landlocked salmon support the hypothesis that the GH system is essential for the development of hypo-osmoregulatory capacity, as they correlate with lower levels NKA activity and NKCC abundance during the spring smoltification period (Nilsen et al., 2003, 2007). This is similar to low plasma GH levels reported in non-smolting Atlantic salmon parr during the spring (Shrimpton et al., 2000; McCormick et al., in press). Together with preliminary *in situ* hybridization results showing localization of GHR mRNA in putative chloride cells (CC) of smolt gills (Ebbesson et al., unpublished results), the present findings suggest that increased GH signaling to the CCs may be an important mechanism underlying the differences in the preparatory increase in NKA synthesis and function between strains (Nilsen et al., 2007). Further support for the role of GH in hypo-osmoregulatory development is demonstrated in a recent study showing that circulating GH and gill GHR

mRNA levels do not change in anadromous salmon deprived of seasonal cues, which corresponds with reduced hypo-osmoregulatory (Stefansson et al., in press) and neuroendocrine (Ebbesson et al., in press) development.

4.1.2. Circulating IGF-I and gill IGF-I and IGF-IR mRNA

To our knowledge, key elements of the IGF-I system in landlocked Atlantic salmon have not previously been studied. In the present study, circulating IGF-I levels were similar between anadromous and landlocked salmon over the smoltification period, which contrasts the increase of plasma IGF-I previously observed during Atlantic salmon smoltification (Ágústsson et al., 2001; McCormick et al., 2000, 2002). On the other hand, gill IGF-I mRNA levels increased from February to June in anadromous but not in the landlocked salmon, suggesting that local IGF-I production is an important factor in stimulating the preparatory changes in synthesis, abundance and activity of ion-secretory proteins during smolting in the anadromous strain (Nilsen et al., 2007). Also, IGF-IR mRNA levels, which have not previously been described in salmon tissues

during smoltification, increased transiently in the gills of anadromous salmon in May, suggesting an increased sensitivity to IGF-I at peak smoltification. Together, the present data suggest that local IGF-I production can promote the preparatory FW development for SW in anadromous salmon, whereas in the landlocked strain, the lack of increased local IGF-I is reflected in the reduced preparatory development of ion transporters (Nilsen et al., 2007). It is interesting to note that plasma IGF-I did not increase during smolting of the anadromous strain in spite of large increases in plasma GH. In mammals, plasma IGF-I levels largely reflect production and release from the liver, but in fish, the origin of plasma IGF-I and its precise regulation are not known (Björnsson et al., 2002; Reinecke et al., 2005).

4.1.3. Circulating cortisol and gill GR, MR and 11 β -HSD2 mRNA

The increase in plasma cortisol levels in the anadromous strain is consistent with the characteristic rise of this hormone during smoltification in anadromous salmonids (McCormick et al., 2002; Shrimpton and McCormick, 1998b; Sundell et al., 2003). Cortisol is involved in several smoltification related processes and is acknowledged as one of the main regulators of hypo-osmoregulatory mechanisms. Numerous *in vivo* and *in vitro* studies in gill and intestine have demonstrated cortisol to increase NKA activity, NKA synthesis, NKCC and the permeability of membrane epithelium (McCormick, 2001). Circulating cortisol levels did not increase in the landlocked salmon during the spring smoltification period, which agrees with the lack of increase in cortisol levels in parr (non-smolting) anadromous salmon compared to upper mode (smolting) siblings (Shrimpton and McCormick, 1998b; McCormick et al., *in press*) and corresponds with a reduced preparatory induction of ion-secretory proteins in the gill (Nilsen et al., 2003, 2007; Shrimpton and McCormick, 1998b). While these data emphasize the importance of cortisol in the development of SW tolerance, low cortisol levels in landlocked salmon may also reflect metabolic differences between strains (Mommensen et al., 1999). The lower plasma GH levels in landlocked salmon compared with the anadromous may have led to lower sensitivity of the interrenal tissue to ACTH (Young, 1988), resulting in the observed lower levels of cortisol.

Gill GR mRNA levels increased with equal intensity in both anadromous and landlocked salmon during the spring smoltification period similar to that previously reported in salmonids (Mazurais et al., 1998; Mizuno et al., 2001; Kiilerich et al., 2007). The fact that there were no difference between strains further points toward the similarity between landlocked and lower mode Atlantic salmon, where gill CR concentration (B_{\max}) and affinity increased seasonally in both upper and lower mode, whereas cortisol levels were significantly higher in the smolting (upper mode) salmon (Shrimpton and McCormick, 1998b). *In vivo* treatment with GH increases gill CR abundance in salmo-

nids (Shrimpton et al., 1995; Shrimpton and McCormick, 1998a), but differences in GH levels between strains during the spring smoltification period appear to not have elicited differences in GR mRNA in the present study. It should be noted, however, that changes in receptor mRNA levels is not necessarily paralleled by changes in functional receptor abundance (rainbow trout: Singer et al., 2007). The similarities in GR mRNA levels between strains here may reflect independent seasonal/diel changes associated with an unknown factor or a residual parameter not abandoned by the landlocked salmon. However, the presence of two distinct GRs (GR1 and GR2) in teleosts (Bury et al., 2003; Bury and Sturm, 2007) leaves a fair chance that differential expression of these two GRs may occur during smoltification and/or differ between strains.

In agreement with Kiilerich et al. (2007), there were no significant changes in gill MR mRNA levels at peak smoltification in anadromous salmon, or any temporal changes in the landlocked salmon. To date we have only limited information on the physiological functions of MR-like protein in fish. In mammals MR is involved in ion retention (Rashid and Lewis, 2005), which would indeed suggest that the MR does not change during smoltification as long as the fish remain in fresh water. The increase in 11 β -HSD2 mRNA and cortisol in anadromous but not landlocked salmon in May, suggests that cortisol signaling via GRs is less active once the development of SW tolerance is achieved in anadromous smolts at peak smoltification (Nilsen et al., 2007).

4.1.4. Gill PRLR mRNA

Gill PRLR mRNA levels did not change during the spring smoltification period in either strain. However, in agreement with Kiilerich et al. (2007), there was a decreasing trend in the anadromous strain, while the landlocked fish showed a slight transient increase in May. In teleosts, PRL is generally thought to be a FW adapting hormone, reducing ion and water permeability across gill epithelium (Manzon, 2002). In salmonids, PRL has been shown to either decrease (Shrimpton and McCormick, 1998a) or have no effect (Madsen et al., 1995; Seidelin and Madsen, 1997) on gill NKA activity in FW.

4.2. Seawater (SW) acclimation

Salmonids, as most anadromous fish, display a remarkable plasticity in adjusting ion homeostasis in response to changes in environmental salinity (McDowall, 1997; Hiroi and McCormick, 2007). This plasticity may arise as part of the smolting process, or an endocrine regulatory response to salinity exposure (Evans et al., 2005; McCormick, 2001). As discussed above, landlocked salmon have a reduced stimulation of both the endocrine system and the preparatory increase in ion-secretory proteins during the spring smoltification period in FW. Here, we also discuss these endocrine systems during two periods of the SW life-stage, an early SW acclimation period where in both

strains the osmoregulatory system appears quite plastic (Nilsen et al., 2007) and is accompanied by increases in specific endocrine systems. The second period, one month in SW, is more of a postsmolt growth phase where differences in the endocrine systems between strains are expanded as growth strategies between strains are inherently different, most likely due to selection pressure differences between life history strategies (Johnston et al., 2005; McDowall, 1988, 1997).

4.2.1. Circulating GH and branchial GHR mRNA

Similar to previous studies on salmonids, plasma GH levels increased in both strains following SW transfer (Björnsson, 1997; Björnsson et al., 2002), a response which may be due to increased GH synthesis (Ágústsson et al., 2003), altered GH secretion and/or clearance rates (Sakamoto et al., 1993). However, the capacity to increase GH levels in response to SW is less pronounced in the landlocked salmon than the anadromous salmon. A parallel can possibly be drawn between this and data indicating that Atlantic salmon show highest GH-elevation when exposed to SW at the peak of their smoltification (Arnesen et al., 2003). After a month in SW, the anadromous salmon of the present study maintain relatively high GH levels, similar to earlier studies where smolts on simulated natural photoperiod had higher SW GH levels than fish kept on continuous light, an environmental condition known to be detrimental to smolt development (Stefansson et al., 1991, in press).

In the present study, GHR mRNA in the gill increased after 4 days in SW and remained high after one month in SW. Recently, the seasonal response of gill GHR mRNA to 1-day SW-acclimation showed an increase early in the smoltification period and then decreased to the lowest levels at peak smolt status (Kiilerich et al., 2007). These differences in GHR mRNA levels, following either 1 or 4 day SW exposure, may arise from temporal changes in expression levels. In rainbow trout, GH receptor binding studies did not see a change in GHR abundance in gills following SW transfer (Sakamoto and Hirano, 1991), indicating that receptor mRNA levels may not necessarily reflect protein levels after SW transfer. GHR mRNA levels were also upregulated in the landlocked salmon following 4 days in SW, however after one month in SW the GHR mRNA levels had returned to basal FW levels. Together with data on circulating GH levels, these data suggest that the GH system is involved in the plasticity surrounding the short-term partial upregulation of ion transporters upon SW entry in landlocked salmon (Nilsen et al., 2007).

4.2.2. Circulating IGF-I and gill IGF-I and IGF-IR mRNA

Plasma IGF-I levels increased in both the anadromous and landlocked salmon after 4 days in SW. However, the plasma IGF-I levels returned to their initial basal FW levels after one month in SW, with significantly higher IGF-I in the landlocked salmon. IGF-I promotes an increase in ion-secretion upon SW transfer in salmonids and several other euryhaline teleosts (McCormick, 2001). IGF-I levels

increase at 1 and 14 days following SW transfer in steelhead trout (Liebert and Schreck, 2006), and gradual exposure to higher salinity increases circulating IGF-I levels in rainbow trout (Shepherd et al., 2005). The increase in local gill IGF-I mRNA levels during 4-day SW exposure in landlocked, but not anadromous salmon, may be a mechanism to further increase the responsiveness to IGF-I in fish that is not fully ready/developed for SW (Nilsen et al., 2007). In rainbow trout, branchial IGF-I mRNA levels transiently increase at 1 day following SW exposure, but not after 4 and 21 days (Sakamoto et al., 1993). In the present study, after one month in SW, gill IGF-I mRNA levels were similar to their initial FW levels which were significantly higher in the anadromous strain. Although the present study does not provide direct evidence for a role of IGF-I in the gill during SW acclimation in salmon, its actions on proliferation and NKA activity from *in vitro* and *in vivo* studies, as well as the increased IGF-I levels in both strains after 4 days in SW, implicate this hormone in the SW stimulated plasticity as previously described in anadromous salmonids (McCormick, 2001; Sakamoto and McCormick, 2006) and shown in landlocked salmon (Nilsen et al., 2007).

Apart from a recent study by Tipsmark et al. (2007), showing increasing gill IGF-IR mRNA levels in striped bass after 24 h of SW exposure, little is known about the changes in IGF-IR expression during SW acclimation. Unlike in striped bass, gill IGF-IR mRNA levels remained unchanged in both anadromous and landlocked salmon after 4 days of SW exposure. These differences may either be due to species, developmental stage and/or duration of SW exposure. On the other hand, after one month in SW, gill IGF-IR mRNA levels were higher in the anadromous than the landlocked juveniles. The fact that no changes in gill IGF-IR mRNA levels were observed during SW acclimation, indicate that the receptor levels may remain constant and the activity of the IGF-I system may be regulated by the presence of the hormone and/or IGF-I binding proteins (Shepherd et al., 2005).

4.2.3. Circulating cortisol GR, MR and 11 β -HSD2 mRNA levels

No significant increases in cortisol levels were found following transfer to SW in either strain, similar to data on chum salmon fry after exposure to SW, which show no increase in cortisol or gill CR mRNA levels (Uchida et al., 1998). In rainbow trout, both circulating cortisol (Sakamoto et al., 1993) and gill GR mRNA but not protein levels (Singer et al., 2007) transiently increase following SW exposure. In Atlantic salmon, GR mRNA levels increase after 24 h SW exposure in the middle of smoltification, but not at other developmental stages (Kiilerich et al., 2007). Despite differences in cortisol levels between strains during SW acclimation, gill GR, MR and 11 β -HSD2 mRNAs increased in both strains. These increases during short-term SW acclimation were not observed by Kiilerich et al. (2007) at peak smoltification, although earlier in the spring when challenged with SW GR and 11 β -HSD2

mRNA levels increased while MR mRNA decreased. These differences most likely result from temporal changes in developmental responsiveness in these systems upon SW exposure as demonstrated in anadromous salmon (Kiilerich et al., 2007). In SW acclimated fish, GR and 11 β -HSD2 mRNA were higher in the anadromous strain than landlocked strain, whereas MR mRNA levels decreased to similar FW levels in both strains. These differences in the corticoid systems between landlocked and anadromous salmon further demonstrate fundamental differences in their endocrine responsiveness to SW.

4.2.4. Gill PRLR mRNA

During SW acclimation, gill PRLR mRNA did not change, and were at their lowest levels after one month in SW in both strains. This is in line with data indicating no change in gill PRLR mRNA after 24 h SW challenge at the peak of smoltification (Kiilerich et al., 2007). These data emphasize the differences between early SW acclimation plasticity and that of a fully SW-acclimated state, where the former process, with increases or unchanged endocrine systems, allows for more flexibility in gill ion regulatory development in the short-term allowing for excursions into other environments, whereas in the acclimated state development and plasticity is no longer in play, as the post-smolt enters a rapid marine growth phase.

As with many anadromous fish, salmon display a remarkable plasticity when it comes to adjusting ion homeostasis in response to changes in environmental salinity (Evans et al., 2005). In salmon, this plasticity may arise as part of a developmental event, or in response to salinity exposure (McCormick, 2001; Hiroi and McCormick, 2007). The present study demonstrates that circulating hormones and gill receptor mRNA levels are lower during spring development and SW acclimation in landlocked relative to anadromous salmon. However, it appears that various aspects of the GH-IGF-I, corticoid and prolactin systems are activated during short-term SW acclimation in the landlocked salmon. Overall, the endocrine data presented here, along with the known actions of these systems in regulating ion transporters (McCormick, 2001; Prunet et al., 2006; Sakamoto and McCormick, 2006), corresponds well with the degree of ion regulatory changes where the landlocked salmon appears to have lost some of the preparatory increases in key ion-secretory proteins but have retained some of the ion regulatory plasticity when challenged with SW (Nilsen et al., 2007). This partial upregulation of key ion transporters and the maintenance of low plasma ion levels in SW in the landlocked strain are similar to changes seen in the anadromous strain during FW preparatory development. Further, the activation of the GH-IGF-I system during the initial stages in SW in the landlocked strain parallels the preparatory changes observed in this system during the FW development of the anadromous salmon, most likely a compensatory action on the underdeveloped SW preparation (Nilsen et al., 2007). The apparent loss of the preparatory osmoreg-

ulatory changes in FW in landlocked salmon are likely the result of natural selection, since these changes are no longer necessary, as energy may be wasted in processes that reduce their overall fitness (McDowall, 1988), as seen in other traits such as the evolutionary loss of maximal muscle fiber number in this landlocked strain (Johnston et al., 2005). In contrast, the ability to respond to SW, as a protective mechanism, has been retained, due to its importance in the capacity for exploiting other habitats. This plasticity most likely is under less selection pressure, as the trait is only energy demanding upon SW stimulation.

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