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Molecular mechanisms of continuous light inhibition of Atlantic salmon parr–smolt transformation

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Abstract

Atlantic salmon (*Salmo salar*) rely on changes in photoperiod for the synchronization of the developmental events constituting the parr–smolt transformation. In the absence of photoperiod cues, parr–smolt transformation is incomplete, and such 'pseudo-smolts' normally fail to adapt to seawater. The present study addresses the endocrine and molecular mechanisms controlling the development of hypo-osmoregulatory ability and how artificial photoperiod can disrupt these changes. Juvenile Atlantic salmon reared under constant light (LL) from first feeding, were separated into two groups, and exposed to either LL or simulated natural photoperiod (LDN) from October, eight months prior to the expected completion of smoltification. Juveniles reared on LL grew well, but failed to show the smolt-related reduction in condition factor in spring. Gill mRNA levels of Na^+, K^+–ATPase (NKA) isoform α1a decreased in LDN fish through completion of parr–smolt transformation, while levels remained unchanged in the LL group. In contrast, α1b expression increased 6-fold in the LDN group between February and May, again with no change in the LL group. Further, Na^+, K^+, $2Cl^-$ co-transporter (NKCC) showed a transient increase in expression in smolts on LDN between February and May, while no changes in mRNA levels were seen in juveniles under LL. Consequently, gill NKA activity and NKA α and NKCC protein abundance were significantly lower in juveniles on LL than in smolts on LDN. LL fish in spring had lower circulating levels of thyroid hormones (THs), growth hormone (GH) and cortisol. Gill GH-receptor mRNA levels, determined by quantitative PCR, were less than 50% of controls. In contrast, circulating levels of IGF-1 and gill IGF-1 receptor expression, were comparable to controls. Our findings show that continuous light prevents the completion of parr–smolt transformation at a very basic level, disrupting the natural up-regulation of key elements of the endocrine system involved in the regulation of the parr–smolt transformation, and consequently inhibiting the smoltification-related increase in expression, abundance and activity of gill ion transport proteins.

Keywords: Na^+,K^+–ATPase; α subunit; NKCC; Growth hormone; IGF-1; Cortisol; Thyroid hormones

1. Introduction

Intensive farming of Atlantic salmon (*Salmo salar*) has become a cost-effective, season-independent industrialised animal production, with high-throughput, reduced generation time, rapid development, high

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densities, and high-energy diets. Heated water and photoperiod control, with extensive use of constant light (LL), are used to accelerate growth and development for season-independent smolt production and seawater transfer (Berge et al., 1995; Handeland and Stefansson, 2001). However, extended use of such extreme light regimes deprives the juvenile salmon of seasonal cues, critically interfering with the completion of parr–smolt transformation (Stefansson et al., 1991; Berge et al., 1995; Handeland and Stefansson, 2001). Photoperiod is recognised as the major long-term regulator of smoltification in Atlantic salmon (Stefansson et al., 1991; Handeland and Stefansson, 2001; Handeland et al., 2003). Changes in daylength provide juvenile salmon with seasonal cues, entraining postulated endogenous rhythms controlling development, and bringing them in step with the changing seasons (Duston and Saunders, 1990). Previous research has demonstrated that seasonal information is required to stimulate the development of hypo-osmoregulatory competence in smolts (see reviews by Björnsson, 1997; McCormick et al., 1998).

Perhaps the single most critical element of parr–smolt transformation is the development of hypo-osmoregulatory ability, functionally linked to differentiation of chloride cells (CC) to a marine type (McCormick, 2001), and an increase in transport proteins as part of their pre-adaptation to seawater (McCormick et al., 1998; Nilsen et al., 2003). Branchial Na^+,K^+–ATPase (NKA) activity is regulated by a number of hormones targeting the CC, including cortisol, GH, IGF-1 and TH, acting alone or in combinations (Madsen, 1990; Madsen et al., 1995; McCormick 2001). However, less is known about changes in the Na^+, K^+, $2Cl^-$ co-transporter, NKCC, and the interaction with endocrine and environmental factors. Salinity increases the immunoreactivity (Pelis et al., 2001) and expression (*in situ* hybridisation, ISH, Tipsmark et al., 2002; Ebbesson et al., unpubl. obs.) of NKCC in CCs, however, the endocrine and environmental control of these changes as part of parr–smolt transformation is generally unresolved.

The light–brain–pituitary (LBP) axis conveys information about environmental photoperiod to endocrine systems. Subsequent hormone actions regulate the functions of various tissues involved in the parr–smolt transformation, of which the gills play a major osmoregulatory role. The normal development and function of the LBP in parr is influenced by environmental light conditions, and is crucial for successful smolt-transformation. The absence of photoperiod signals during parr–smolt transformation can disrupt the development of the LBP (Ebbesson et al., 2007-this volume), which means that the endocrine system is not activated (Stefansson et al., 1991; Björnsson et al., 1995; McCormick et al., 1995). Such 'pseudo-smolts' are generally unable to adapt and perform well in seawater. The physiological consequence of such 'hypo-endocrine' status is likely to be reflected by the impaired development of gill NKA and other ion transporters with subsequent reduced hypo-osmoregulatory ability (Björnsson et al., 1995; Björnsson, 1997; McCormick, 2001). Hence, the purpose of the present study was to provide a detailed analysis of the impact of artificial photoperiod on the development of ion-regulatory mechanisms and relate these to differences in levels of circulating hormones and receptor expression.

2. Materials and methods

2.1. Fish material, rearing conditions, sampling protocol

Juvenile Atlantic salmon of the Vosso river strain (South-western Norway, see Nilsen et al., 2003) were brought from the hatchery at Voss to the Industrial and Aquatic Laboratory at the Bergen High Technology Centre in October 2001. On arrival in the lab the fish were distributed into three 1-m^2 tanks with a rearing volume of 400 l, with two tanks for the control group and one tank for continued exposure to constant light (LL). The tanks were supplied with flow-through pH-adjusted fresh water with a temperature of approximately 8 °C for the duration of the study which lasted until mid-June 2002. The control tanks received LDN photoperiod (seasonally changing light–dark cycle) of 60°N from fluorescent light tubes installed in the tank cover, while the LL tank had lights on constantly. Both groups were fed a commercial dry diet during the light hours of the control tanks. Limited number of fish were transferred to seawater on 15 May. Fish from both groups were sampled on 26 February, 15 April, 14 May in fresh water. Additional fish were sampled from the control group in fresh water and in seawater on 18 June, while gill filaments for determination of NKA activity only were sampled from the LL group in fresh water and seawater on 18 June. Due to low numbers of fish, further material was not available from the LL group in June. Fish ($n=10$) were quickly dip-netted out of the tanks, anaesthetized directly in 100 mg/l in buffered tricaine methanesulphonate (MS222; Sigma, St. Louis, MO, USA) and blood was collected from the caudal vessels using 1-ml heparinized syringes. Plasma was separated by centrifugation, frozen on dry ice and stored at −80 °C for subsequent hormone analysis. All fish were weighed (wet weight) and measured (fork length) and the condition factor (CF) was calculated ($CF = \text{body weight} \times 100 \times \text{fork length}^{-3}$). Gill tissues for determination of gene expression and protein abundance were quickly dissected out and frozen directly on dry ice. For NKA activity, the second gill arch on the left side was dissected out, immersed in SEI buffer (250 mM sucrose, 10 mM Na_2–EDTA, 50 mM imidazole at pH 7.3), frozen on dry ice and stored at −80 °C until analysis.

2.2. Analysis

2.2.1. RNA isolation and cDNA synthesis

Total RNA was extracted from ~50 mg gill tissue using TRI REAGENT (Sigma) as outlined by Chomczynski (1993) and quantified spectrophotometrically, purity assessed and integrity checked by 1% agarose/formaldehyde gel electrophoresis. Total RNA were treated with RQ1 RNase-free DNase (Promega) 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) following the manufactures instructions.

2.2.2. Real-time quantitative PCR assays

Real-time quantitative PCR (Q-PCR) TaqMan assays were used to quantify *in vitro* expression of NKA-α1a, NKA-α1b, NKA-α1c and NKA-α3 subunits, NKA-β1 subunit and NKCC in the gill. Gene Bank Accession numbers of Norwegian Salmon Genome Project expressed sequence tags clones and published sequences used to design Q-PCR primers and FAM labeled MGB probes are shown in Table 1. Gill GH-receptor and IGF-I receptor and IGF-I mRNA expression were measured using FAM labeled TAMRA probes (Table 1, Wargelius et al., 2005). Primer specificity was tested by PCR using 10 μl cDNA, 400 nM of each primer and SYBR Green Universal Master mix in a total reaction volume of 25 μl. The thermal cycling protocol consisted of 2 min at 50 °C, 10 min at 95 °C followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Melt curve analysis verified that the primer sets for each Q-PCR assay generated one single product and no primer-dimer artifacts.

All TaqMan Q-PCR assays were performed in a total volume of 25 μl on the ABI prism 7000 detection system platform (Applied Biosystems, Oslo, Norway) using 5 μl cDNA template, 900 nM forward and reverse primers, 200 nM probe and 12.5 μl TaqMan® Universal PCR Master Mix containing AmpErase® uracil *N*-glycosylase. The thermal cycling protocol consisted of 2 min at 50 °C, 10 min at 95 °C, followed by 45 cycles at 95 °C for 15 s and 60 °C for 1 min. Omission of reverse transcriptase in the RT reaction resulted in a shift in C_t values of >13 cycles in all assays, which indicated that interference from residual DNA in RNA samples after DNase treatment were unimportant. Validation experiments (ABI User Bulletin #2) using cDNA generated from 2-fold serial dilutions of total RNA gave log input cDNA versus C_t plots with $R^2 > 0.99$ and $\Delta C_t < 0.1$ for all target genes (NKA-α1a, NKA-α1b, NKA-α1c, NKA-α3, NKA-β1, and NKCC) in relation to elongation factor 1A ($EF1A_A$). See Wargelius et al. (2005) for validation of GH-R, IGF-IR and IGF-I Q-PCR assays. Results are presented as relative expression according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) using $EF1A_A$ as an internal control and anadromous parr (February 26) as calibrator. All Q-PCR assays were used within a C_t range where the log input cDNA (0.5 μg RNA template) versus C_t plots were found to be linear over 5 log phases with $R^2 > 0.98$. Non-template controls were included on all plates. For further details on cloning and validation of salmon NKA α-subunit isoforms, see Nilsen et al. (2007).

Table 1
Real-time quantitative PCR (Q-PCR) TaqMan assays used to quantify gene expression in gills of Atlantic salmon

Q-PCR assay	Acc. no	Forward primer (5′→3′)	Reverse primer (5′→3′)	TaqMan probe (5′→3′)	Size (bp)
NKA α-1a	CK878443	CCAGGATCACTCAATGTCACTCT	GCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA	ACGATTACATTATAAGGCAATACT	93 bp
NKA α-1b	CK879688	GCTACATCTCAACCAACAACATTACAC	TGCAGCTGAGTGCACCAT	ACCATTACATCCAATGAACACT	91 bp
NKA α-1c	CK 885259	AGGGAGACGTACTACTAGAAAGCAT	CAGAACTTAAAATTCCGAGCAGCAA	ACAACCATGCAAGAACT	85 bp
NKA α-3	CK170270	GGAGACCAGCAGAGGAACAG	CCCTACCAGCCCTCTGAGT	AAGACCCAGCCTGAAATG	58 bp
NKA β1	CK886866	CGTCAAGCTGAACAGGATCGT	CCTCAGGGATGCTTTCATTGGA	CCTTGGCCTGAAGTTG	68 bp
NKCC	AJ417890	GATGATCTGCGGCCATGTTC	AGACCAGTAACCTGTCGAGAAAC	CTCCAGAAGGCCCAACTT	68 bp
$EF1A_A$	AF321836	CCCCTCCAGGACGTTTACAAA	CACACGGCCCACAGGTACA	ATCGGTGGTATTGGAAC	57 bp
GH-R	AY462105	TGGGAAGTTGAGTGCCAGACT	CACAAGACTACTGTCCTCCGTTGA	TGGGAGAGCCAGCCAGCCTGC	72 bp
IGF-IR	AY049954	TGAAGAGCCACCTGAGGTCACT	TCAGAGGTGGGAGGTTGAGACT	CGGGCTAAAGACCCGTCCCAGTCC	72 bp
IGF-I	M81904	GTGTGCGGAGAGAGAGGCTTT	TGTGACCGCCGTGAACTG	TTTCAGTAAACCAACGGGCTATGG	68 bp

2.2.3. Western blotting

NKA and NKCC protein abundance was determined by Western blotting largely following the procedure of Pelis et al. (2001), with some modifications outlined below. Frozen gill tissue was thawed, rinsed briefly in SEI buffer and blotted on paper towel. The gill arch was removed and gill epithelia weighed and placed in 10 volumes of homogenisation buffer (1 complete-mini tab, Roche, dissolved in 10 ml SEI). Tissue was lightly homogenised using a teflon pestle in a 1.5 ml eppendorf tube, centrifuged at 2000 g for 7 min and the supernatant discarded. The pellet was re-suspended in 5 volumes of homogenisation buffer +-0.1% deoxycholate, homogenised on ice in a glass homogeniser and centrifuged at 2000 g for 6 min. Supernatant was combined with Laemmli sample buffer (Pelis et al., 2001), heated at 60 °C for 15 min, and stored at −80 °C. Prior to electrophoresis, protein concentration of all samples was adjusted to 10 μg per 20 μl using Laemmli buffer. For NKA, samples and standards were loaded on 7.5% SDS-PAGE gels with 10 μg protein per lane and run for approximately 2 h. For NKCC, 6% gels were used. Gels were quickly rinsed in transfer buffer and blotted overnight on ice onto Immobilion P (PVDF) membranes (Millipore, Bedford, MA). Membranes were quickly rinsed in distilled water and incubated in blocking buffer (PBS containing 0.05% Triton X-100 and 2% skim milk) for 1 h at room temperature. Membranes were rinsed three times with PBS-T (PBS containing 0.05% Triton X-100) and incubated for 1 h with the primary antibody diluted in PBS-T containing 0.1% BSA. Antibodies used were α5 at 1:2000 dilution for NKA and T4 at 1:1000 dilution for NKCC. Membranes were rinsed three times in PBS-T and incubated for 1 h with 1:1000 peroxidase-conjugated antibody diluted in PBS-T containing 0.1% BSA. Membranes were rinsed again as described and developed with DAB solution until bands were visible, after which colour development was stopped with deionised water. Membranes were allowed to dry before digital photographs were taken. Band staining intensity was quantified using the ImageJ image processing and analysis software (http://rsb.info.nih.gov/ij/), adjusting the threshold of each picture to account for slight differences in background intensity.

2.2.4. Na^+,K^+–ATPase activity

Gill filaments were thawed on the day of assay, the storage buffer discarded, and gill NKA activity analysed according to McCormick (1993). Briefly, gill tissue was homogenised in 150 μl SEID (SEI buffer containing 0.1% deoxycholic acid) and centrifuged at 5000 g for 60 s. Ten μl of supernatant was added in duplicate wells of a 96-well microplate containing 200 μl assay medium, with and without 0.5 mM ouabain, and read at 340 nM for 10 min at 25 °C. Na^+,K^+–ATPase activity was determined as the ouabain sensitive fraction of the enzymatic coupling of ATP dephosphorylation to NADH oxidation, expressed as μmol ADP mg prot.$^{-1}$ h^{-1}.

2.2.5. Hormones

Plasma growth hormone (GH) levels were quantified from duplicate 50 μl samples by a specific double-antibody salmon GH radioimmunoassay according to Björnsson et al. (1994). Plasma IGF-I was measured by homologous radioimmunoassay as described by Moriyama et al. (1994). Plasma cortisol, L-thyroxine (T_4) and triiodothyronine (T_3) were extracted with ice-cold methanol (99%), centrifuged (1600 × g, 15 min, 4 °C), the supernatant transferred to new vials and pellet re-extracted twice with ice-cold methanol. The pooled supernatant was vacuum dried (4 °C) over night and re-constituted in EIA buffer (0.1 M phosphate buffer containing 0.15 M NaCl and 0.1% BSA).

Plasma T_4 levels were quantified from duplicate 50 μl samples by an EIA according to Kulczykowska et al. (2004). Plasma T_3 levels was quantified by a competitive immunoassay (EIA) as outlined by Cerdá-Reverter et al. (1996) with a few modifications. Briefly, the assay was performed in 96-well microtiter plates pre-coated with monoclonal mouse anti-rabbit IgG (Cat # 400007, SPIBIO, France) using either 50 μl rabbit anti-T_3 antibody (Cat # T2777, Sigma; diluted 1:2000)+50 μl T_3-AchE (Cat # A04130, SPIBIO) and 50 μl sample or T_3 standard (Cat # T2877, Sigma) in each well. Color development was done with Ellmann's reagent (Cat # 400050, SPIBIO) and absorbance read at 405 nm by a temperature-controlled (25 °C) plate reader. Maximum binding (B_0; 50 μl of EIA buffer+50 μl T_3-AchE+-50 μl anti-T_3) and non-specific binding (NSB; 150 μl EIA buffer) were determined on each plate. All standards and samples were added in duplicates.

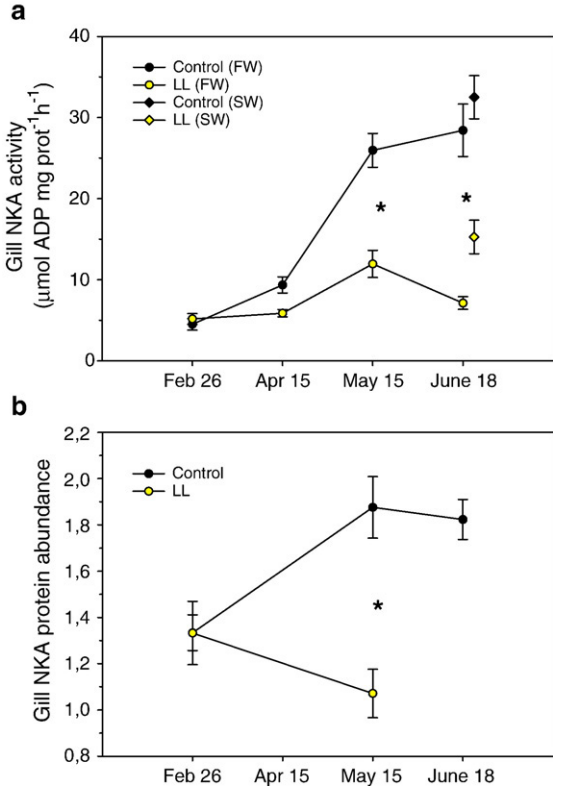

Fig. 1. (a) Enzyme activity and (b) protein abundance of Na^+,K^+–ATPase α subunit in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudo-smolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

Plasma cortisol levels were quantified by an EIA as outlined by Carey and McCormick (1998). Briefly, 96-well microtiter plates were coated with polyclonal rabbit anti-cortisol antibody (Cat # F3-314, Endocrine Science Products; diluted 1:16 000) and incubation performed using either 100 µl cortisol-horseradish peroxidase conjugate (Coralee Munro, University of California, Davis, CA, USA; diluted 1:120 000) +- 2.5 µl sample or cortisol standard (Cat # T0440, Sigma) in each well. Color development using 3,3′,5,5′-tetramethylbenzidine (Cat # T0440, Sigma) was terminated with 0.5 M HCl and absorbance read at 405 nm by a temperature-controlled plate reader. Maximum binding (B_0; 2.5 µl of EIA buffer +- 100 µl cortisol-horseradish peroxidase) and non-specific binding (NSB; 102.5 µl EIA buffer) were determined on each plate. All standards were run in triplicate and samples in duplicate.

2.3. Statistics

All statistical analyses were performed with Statistica 6.0 (StatSoft, Inc., Tulsa, OK, USA, http://www.statsoft.com). Slight and random deviations from normality of distributions (Kolmogorov–Smirnov test; Zar, 1996) and homogeneity of variances (Levene's *F*-test) were observed in some cases, however these were not considered significant for the robust parametric models used (Zar, 1996). Hence, all parameters were analysed using a two-way ANOVA, followed by a Newman–Keuls test in case of significant ANOVAs. A significance level of 0.05 was used. Data are presented as mean ± standard error of the mean (se).

3. Results

3.1. Growth, condition, morphology

Mean lengths in February, May and June were not significantly different (LDN: 14.0, 16.0, 17.1 cm; LL: 14.9, 16.7, 17.1 cm). Overall, condition factor was significantly higher in LL than in LDN fish, with a condition factor in May and June of 1.16 and 1.17, respectively, compared with 1.08 in LDN fish in May, decreasing further to 1.07 in June. Morphological changes associated with parr–smolt transformation were completed in the LDN group, with smolts showing complete silvering, dark dorsal side, dark fin margins and no visible parr-marks. In contrast, LL fish still showed distinct parr-marks, incomplete silvering, and no distinct dark fin margins nor dorsal side, indicating a pseudo-smolt status.

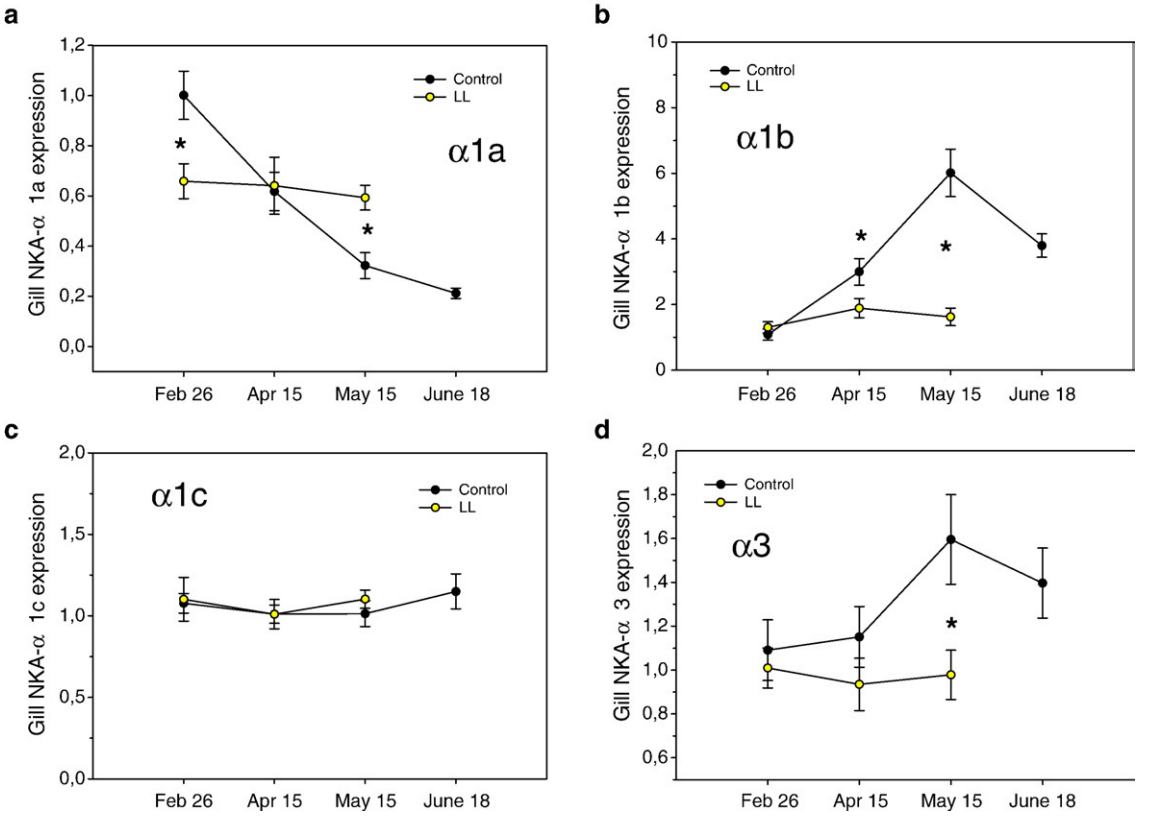

Fig. 2. a–d. Expression (mRNA levels) of Na^+,K^+–ATPase α subunit isoforms in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudo-smolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

3.2. Na^+,K^+–ATPase; activity, protein abundance, subunits and isoforms

3.2.1. Enzyme activity

Gill NKA activity of the control group showed a 5-fold increase between February and May (Fig. 1a) reaching levels of approximately 25 μmol ADP mg prot.$^{-1}$ h^{-1}, and remained elevated in June. In contrast, NKA activity did not increase between February and April in the LL group, with only a moderate increase to approximately 12 μmol ADP mg prot.$^{-1}$ h^{-1} in May, less than 50% of control levels. In June, gill NKA activity of the LL group was back to parr levels. Exposure to seawater between mid-May and mid-June did not induce any further increase in control smolts, nor did it stimulate any increase in activity of the LL fish above fresh water peak levels.

3.2.2. Protein abundance

NKA protein abundance increased by approximately 50% in control smolts between February and May, with no further changes between May and June (Fig. 1b). Initial protein abundance in fish on LL was the same in February; however, protein abundance decreased about 50% between February and May, significantly lower than in controls.

3.2.3. Expression patterns, isoforms

Expression patterns of α and β subunits differed between groups, reflecting the differences in development of hypo-osmoregulatory ability during the study. Expression of NKA α1a decreased significantly in LDN fish through completion of parr–smolt transformation, while expression levels remained unchanged in the LL group (Fig. 2a). In striking contrast, α1b expression increased 6-fold in the LDN group between February and May, again with no change observed in the LL group (Fig. 2b). Expression of NKA α1c (Fig. 2c) did not differ among groups nor over time. However, NKA α3 expression showed a similar pattern as observed for α1b, although of a lower magnitude (Fig. 2d). NKA α2 expression was not detected in gill tissue of Atlantic salmon. Expression pattern of the β1 subunit resembled those of the α1b and α3, with a distinct increase in expression between February and April, and high expression in May in controls (Fig. 3). Again, LL fish showed only minor changes in expression, with levels significantly lower than LDN fish in April and May.

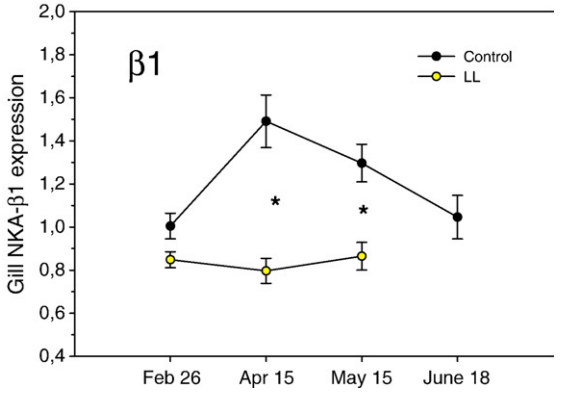

Fig. 3. Expression (mRNA levels) of Na^+,K^+–ATPase β subunit in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudosmolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

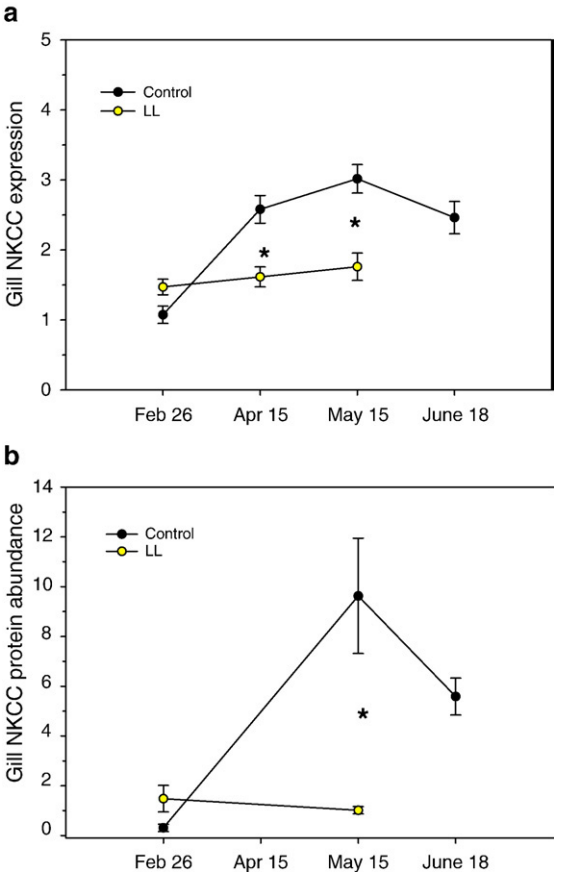

Fig. 4. (a) Expression and (b) protein abundance of $Na^+,K^+,2Cl^-$ co-transporter (NKCC) in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudo-smolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

3.3. NKCC; protein abundance and expression

3.3.1. Protein abundance

NKCC protein abundance increased approximately 30-fold in control smolts between February and May, and was reduced by approximately 50% between May and June (Fig. 4b). Initial protein abundance in February was higher among fish on LL, however, this group showed no further change in NKCC abundance with levels only about 10% of controls in May.

3.3.2. Expression

In line with protein levels, NKCC expression increased 3-fold in control smolts between February and May, with a slight reduction in June (Fig. 4a). Initial NKCC expression in

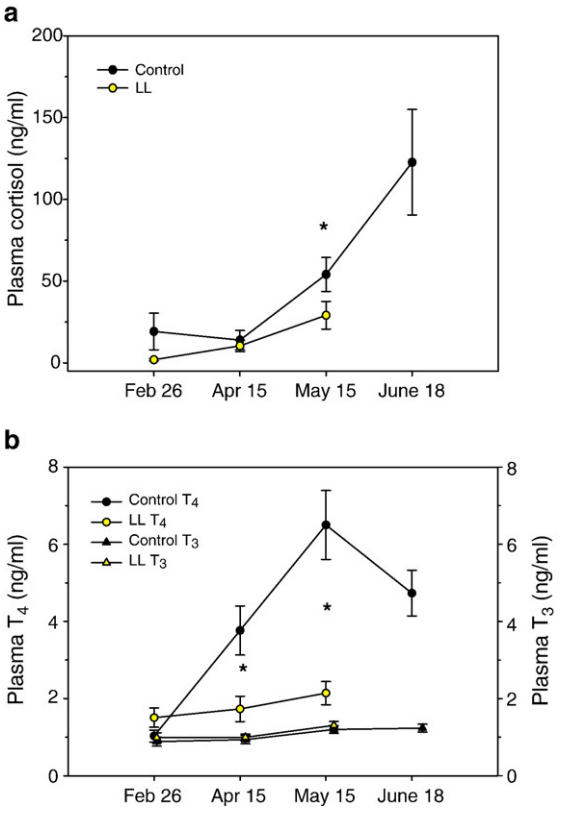

Fig. 5. Circulating levels of cortisol (a), thyroxine, T_4 and triiodothyronine, T_3 (b), in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudo-smolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

February was approximately 50% higher among fish on LL, however, no further change was observed and expression was only about 50% of control levels in May.

3.4. Endocrine changes; circulating levels and receptors

3.4.1. Cortisol

Circulating levels of cortisol increased in both groups between February and May, with a further increase in the control group until June (Fig. 5a). Overall, cortisol levels were higher in the control group, with May levels of 54.0 ng ml^{-1} significantly higher than levels of 29.1 ng ml^{-1} in the LL group.

3.4.2. Thyroid hormones; T_4, T_3

Circulating T_4 levels in controls increased from 1.0 ng ml^{-1} in February to peak levels in May of 6.5 ng ml^{-1}, decreasing to approximately 4.5 ng ml^{-1} in June (Fig. 5b). A slight (not significant) increase in T_4 levels was observed in LL, although levels never exceeded 2 ng ml^{-1}. Plasma T_3 levels showed little variation in either group throughout the study, with levels remaining between 1 and 1.4 ng ml^{-1} (Fig. 5b).

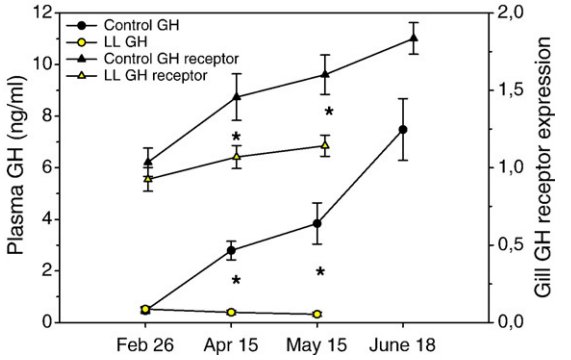

Fig. 6. Circulating levels of growth hormone, GH, and GH-receptor expression in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudo-smolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

3.4.3. Growth hormone; circulating levels, receptor expression

Circulating GH levels increased significantly in the LDN smolts between February and May, and further through June, reaching levels in May of 3.8 ng ml^{-1} (Fig. 6). In contrast,

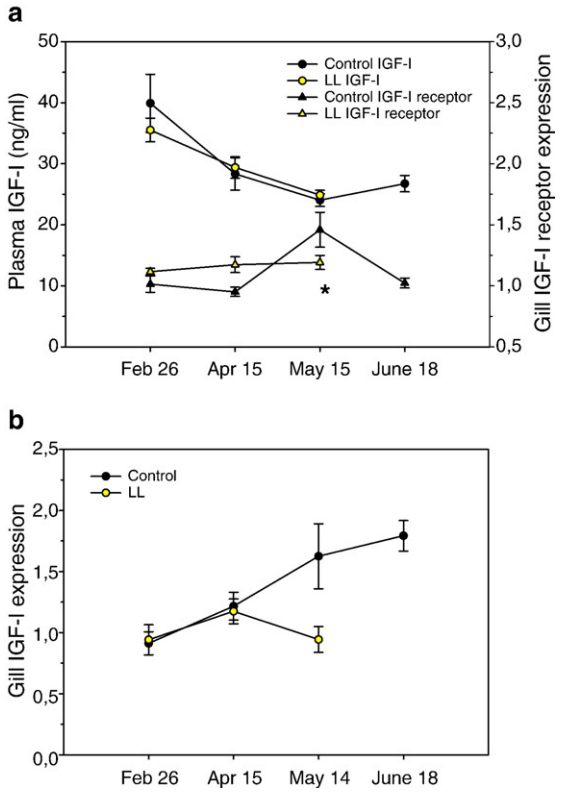

Fig. 7. Circulating levels of insulin-like growth factor-1, IGF-1, branchial IGF-1 receptor expression (a) and branchial IGF-1 expression (b) in Atlantic salmon smolts on simulated natural photoperiod (SNP, Control) and pseudo-smolts on constant light (LL). Asterisks (*) indicate significant differences ($p<0.05$) between fish on LDN and LL.

decreasing GH levels were observed in juveniles on LL, with levels remaining below 0.6 ng ml^{-1} throughout the study. A similar pattern was observed for branchial GH-receptor mRNA levels (Fig. 6), with LDN smolts showing a distinct increase while expression in the LL group did not change and was significantly below LDN in April and May.

3.4.4. IGF-1; circulating levels, receptor expression, gill IGF-1 expression

In contrast to the other hormones studied, no major differences were observed between photoperiod groups in circulating levels of IGF-1 (Fig. 7a). Levels decreased throughout the study from approximately 35–40 ng ml^{-1} in February to 24–25 ng ml^{-1} in May, with no further change in controls in June. In contrast, branchial IGF-1 receptor expression showed a transient increase in expression in controls in May, with no changes in expression observed on LL (Fig. 7a). Overall IGF-1 mRNA levels in gill tissue were higher in controls than LL, with a continuous increase in smolts contrasting the decrease in LL between April and May (Fig. 7b).

4. Discussion

The present findings on the effects of the constant light regime, including the lack of spring increase in gill NKA activity, low plasma cortisol, GH and thyroid hormone levels, along with the poorly developed morphological characters, are in general agreement with previously published results on the effects of LL on parr–smolt transformation in Atlantic salmon (Stefansson et al., 1991; Berge et al., 1995; Björnsson et al., 1995; McCormick et al., 1995; Handeland et al., 2003). This, together with the novel data presented on receptors and ion-transporter gene expression, makes it possible to elucidate the molecular basis of the failure of such pseudo-smolts to develop seawater tolerance. We show that branchial NKA α subunit isoforms, with the apparent exception of α1c, are differentially regulated as part of preparatory adaptation to seawater, and further, that these changes are abolished by continuous light, acting through the neuroendocrine system (see also Ebbesson et al., 2007-this volume).

Pseudo-smolts on LL failed to show anything but a slight increase in gill NKA activity in spring. These findings confirm several previous reports of failure of such pseudo-smolts to increase gill NKA activity (Berge et al., 1995; McCormick et al., 1995; Handeland and Stefansson, 2001). The present results on expression of subunits and their isoforms shed light on the biochemical and genetic mechanisms underlying this failure. Firstly, overall NKA α protein abundance is significantly reduced. Secondly, the lack of seasonal changes in photoperiod prevents changes in α-subunit isoforms. As a novel finding of this study, α1a expression decreases severalfold in LDN fish, while expression levels remains high on LL, which indicates a differential regulation of α-subunit isoforms as part of the preparatory adaptations of smolts. The α1a has previously been suggested as a fresh water-adaptive form in rainbow trout (*Oncorhynchus mykiss*) transferred between fresh water and seawater (Richards et al., 2003). However, no increase in α1a expression has been noted in killifish (*Fundulus heteroclitus*) transferred from 10 ppt saltwater to fresh water (Scott et al., 2005). Although further detailed biochemical and physiological studies are needed to confirm the physiological role of specific isoforms to varying salinities in different species, our results are in agreement with those of Richards et al. (2003), with high expression of α1a in pseudo-smolts failing to show the smolt-related preparatory increase in NKA activity. Further, an up-regulation of α1b, postulated as the major SW adaptive form (Richards et al., 2003), was observed in the LDN fish of the present study, while LL light regime prevented this pre-adaptive regulation of α subunit isoform expression. However, in contrast to findings in rainbow trout (Richards et al., 2003), a distinct increase in NKA α3 isoform expression was observed during parr–smolt transformation of Atlantic salmon, but not in pseudo-smolts, again suggesting that this increase is significant in preparatory adaptations to SW. The contrasting results for rainbow trout and Atlantic salmon may be true species differences and/or be related to the unique capacity of the smolt stage of the salmon to be prepared for to seawater entry while still maintaining hyper-osmoregulatory ability in fresh water (McCormick and Saunders, 1987). Our present approach also included studies of the expression of α1c and β1. The function of the β subunit in the mature NKA enzyme is generally related to anchoring the enzyme within the cell membrane, and it is believed not to be involved in ion transport or ATP processing. Overall, β1 expression reflected the changes observed in α1b expression, overall α protein levels and enzyme activity, both over time and among groups, suggesting a co-ordinated regulation of expression and synthesis of the two enzyme subunits. In contrast, α1c showed a stable expression, both over time and among groups, suggesting a different role of this isoform in branchial tissue of salmonids, probably related to 'housekeeping' functions and not seawater adaptation, in line with findings in rainbow trout by Richards et al. (2003).

Limited data exist on changes in NKCC as part of parr–smolt transformation in Atlantic salmon. NKCC is a critical transporter in the function of mitochondria rich cells, co-transporting Cl^- ions together with Na^+ and K^+ into the MRC through the basolateral membrane, with Cl^- exiting the MRC through ion-specific channels in

the apical membrane (see reviews by McCormick, 2001; Evans et al., 2005). The present results demonstrate a major up-regulation of the synthesis and abundance of NKCC in preparation for seawater entry of Atlantic salmon smolts. This is in agreement with Pelis and McCormick (2001) and Tipsmark et al. (2002) who reported an increase in NKCC abundance in smolts compared with parr and post-smolts in fresh water, and concluded that the increase was stimulated by the combined actions of cortisol and GH. The present results demonstrate for the first time the inhibitory actions of LL on the up-regulation of branchial NKCC protein expression, with mRNA levels significantly below controls in April and May. In line with low expression levels, gill homogenates of Atlantic salmon pseudo-smolts showed low NKCC protein abundance, with protein levels decreasing to only 10% those of controls in May. Recent data on tilapia (*Oreochromis mossambicus*) also point to the potential role of an apically-located NKCC isoform in ion absorption (Hiroi et al., 2005), and this possibility needs further studies on salmonids.

Working with intact animals, we cannot resolve in detail the specific hormone actions on ion transporters. However, the endocrine data obtained give insight into the endocrine stimulation of hypo-osmoregulatory ability as part of parr–smolt transformation. The significant increase in circulating cortisol levels in smolts from April through May and June are consistent with the role of cortisol as a key hormone in differentiation of mitochondria rich cells and the regulation of ion transport proteins (see McCormick, 2001; Evans et al., 2005 for reviews). Similarly, higher levels of plasma cortisol were seen in LDN than LL fish, consistent with higher gill NKA activity in the LDN group.

Thyroid hormones have a wide range of functions in vertebrates, often acting in synergy with other endocrine factors, controlling cell proliferation and differentiation. The present results largely confirm previous studies of thyroid hormones in smolting Atlantic salmon, with circulating T_4 levels showing a peak in smolts in May coinciding with the peak in smolt status, while the biologically more active T_3 show little or no variation during the course of parr–smolt transformation (Boeuf, 1993). Thyroid hormones are thought to play an important role in regulating metabolic changes during smoltification (Sheridan, 1989). However, the possible role of thyroid hormones in controlling ion-regulatory mechanisms remains unresolved (McCormick, 2001). In the present study the T_4 peak in smolts in May is in clear contrast to the static circulating levels in the LL group, again showing that critical parts of the endocrine system remained undeveloped in the pseudo-smolts.

Growth hormone is recognised as a key regulator of cellular and physiological processes in adaptation of smolts to seawater (Björnsson et al., 2002; McCormick, 2001). GH is central in stimulating the differentiation of seawater type (secretory) mitochondria rich cells, characterized by high abundance and activity of NKCC and NKA (Sakamoto et al., 2001). In line with previous studies (see Björnsson, 1997), circulating GH levels increased in smolts in preparation for seawater entry. In contrast to controls, yet in line with the low expression and abundance of key ion transporters, circulating GH levels in pseudo-smolts on LL remained at parr levels, significantly below controls both in April and May, reaching the lowest observed levels in May of around 0.5 ng ml^{-1}. This confirms the LL-induced suppression of GH levels in Atlantic salmon (Björnsson et al., 2000). In addition, a similar pattern was observed for the GH-receptor expression. Branchial GH-R mRNA levels increased in LDN smolts throughout the study, whereas only minor changes occurred in the pseudo-smolts on LL. Thus, not only does continuous light inhibit the increase in plasma GH levels, but LL also inhibits the peripheral expression of the GH receptor. Whether or not the low GH-receptor expression is directly linked to low plasma GH levels remains to be elucidated, however, it appears likely that the LL-induced suppression of the GH system, at both the hormone and the receptor level, represents a key mechanism for the subsequent failure of synthesis and function of major ion transport proteins.

In the general vertebrate model, IGF-1 is produced peripherally under the stimulation by GH, and is considered both an endocrine and paracrine factor, often targeting cells within the same tissue (for a recent discussion see Reinecke et al., 2005). Circulating IGF-1 levels largely reflect the production and release from the liver, and may therefore not reflect the tissue-specific paracrine actions of IGF-1 (see Björnsson et al., 2002). In contrast to other parts of the endocrine system discussed above, circulating IGF-1 levels were not significantly influenced by photoperiod, nor did they reflect changes in circulating GH levels or gill GH-R expression. The present findings contrast those of Agustsson et al. (2001) and McCormick et al. (2002), all showing increasing IGF-1 levels during smolting and seawater entry. Absence of information on critical aspects of IGF-1 regulation, e.g., hepatic GH-receptor expression, hepatic IGF-1 synthesis and IGF-binding proteins, makes it difficult to draw firm conclusions on the endocrine role of IGF-1 in regulating the

development of hypo-osmoregulatory mechanisms. However, the expression of the IGF-1 receptor in the gill increased in LDN fish above LL fish, suggesting that local IGF-1 production may be important in stimulating the pre-adaptive changes in ion-regulatory capacity during smolting, despite reduced circulating levels. Our findings are in general agreement with Sakamoto and Hirano (1993) and Sakamoto et al. (2001) and in line with the general model outlined above; the increased branchial IGF-1 expression in smolts through spring reflects circulating GH levels, GH-R expression and changes in NKA subunit expression, suggesting an important paracrine role of IGF-1. This proposed mode of action of IGF-1 is further supported by the low expression in LL, again reflecting the low circulating GH levels, low GH-receptor expression and poorly developed ion transport mechanisms. Viewed more as a paracrine than endocrine factor, our results on IGF-1 are in line with previous findings of IGF-1 stimulating NKA in *Salmo* (e.g. Seidelin et al., 1999; Seidelin and Madsen, 1999; McCormick, 2001).

Together with the findings of Ebbesson et al. (2007-this volume) we argue that LL inhibits the normal smolt developmental program at the brain–pituitary level, inhibiting normal signalling of the pituitary and other parts of the endocrine system. Further downstream, in the absence of endocrine stimulation, ion-regulatory mechanisms remain undeveloped. Pseudo-smolts on LL remain in a parr state, and generally fail to show any of the preparatory changes associated with development of hypo-osmoregulatory ability and normal MRC function. The fact that fish on LL failed to increase their NKA activity levels even when exposed to seawater further suggests that these pseudo-smolts lacked the molecular and cellular capacity for acclimation to seawater.

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