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Intestinal incomplete process on the osmoregulation system during *Salmo salar* smoltification in a productive conditions

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ABSTRACT

Smoltification under intensive aquaculture conditions involves important physiological changes to intestinal structures that facilitate acclimation to hyperosmotic environments. This study evaluated if genic expression of primary contributors during the smoltification process in the intestine are in synchrony with gill Na^+/K^+-ATPase activity. Discordance was found between gill Na^+/K^+-ATPase activity levels and intestinal genic expressions. In particular, the intestine showed increased mRNA expression for prolactin, a hormone that participates in hypoosmotic environments, and in mRNA expression of the growth hormone, which mediates the hydromineral balance in hyperosmotic environments. These findings indicate a lack of sufficient intestinal maturation in the fish at the moment when they were transferred to seawater environments in the normal aquaculture practices. This intestinal immaturity is a possible explanation for the high rates of desadapted individuals in the seawater phase in Chilean salmon aquaculture.

1. Introduction

Prior to migrating to the sea, Atlantic salmon (*Salmo salar*) undergo a developmental transition termed smoltification also called parr-to-smolt transition. After this process, smolts will be able to migrate from a hypoosmotic to a hyperosmotic environment (Robertson and McCormick, 2012). During this period, Atlantic salmon have significant morphological, behavioral, and physiological changes (Stefansson et al., 2008). The smolting process is conditioned by endocrine factors as well as by environmental stimuli, including photoperiod, nutrition, temperature, and water flow. In synchronization, these variables promote a complete parr-to-smolt transition (Björnsson et al., 2011).

Salmon possess diverse osmoregulatory strategies to adapt and maintain fluid/electrolyte homeostasis over the course of their lifetime (Greenwell et al., 2003). The mechanisms to increase osmoregulatory capacity in anadromous fish during the transition from a hipo- to hyperosmotic environment include changes in the gills, kidney, and intestine, all of which are principal organs involved in osmoregulation (McCormick and Saunders, 1987). Important morpho-physiological changes occur in the ultraestructural branchial during smolting. These modifications are particularly associated with changes in the expression and activity levels of ion transporters in chloride cells, also termed ionocytes or mitochondria-rich cells (Pisam et al., 1988). One such activity is the Na^+/K^+-ATPase (NKA) pump (Lemmetyinen et al., 2013).

The NKA pump is located in the basolateral membrane of cells and allows the excretion of excess plasmatic Na^+ and Cl^- into the marine environment (Manzon, 2002; Wilson and Laurent, 2002). Other transporters also help maintain osmotic processes, such as the membrane Na-K-Cl cotransporter (NKCC) and cystic fibrosis transmembrane receptor I (CFTR) in the apical region of chloride cells, which allow the excretion of excess salts into the marine environment (McCormick, 2013). Osmoregulatory mechanisms also include a change in the type and quantity of aquaporins and claudins, which allows a change in the transport of paracellular water in fresh water to transcellular in sea water. This process is strongly linked to greater salinity tolerance during smolting (Pelis and McCormick, 2001; Sundell and Sundh, 2012)

All of these physiological adaptations are carried out through hormonal components induced by signals from the hypothalamus-

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hypophysis axis, which in turn are triggered by the previously described environmental stimuli (Stefansson et al., 2008; McCormick, 2009, 2013). These hormonal components fulfill essential roles in the adaptation of fish to both hypo- and hyperosmotic environments. Noteworthy among the hormones involved in these adaptations are prolactin (Prl), cortisol, the growth hormone (GH), and thyroid hormones (TH) (Ágústsson et al., 2001; Manzon, 2002; Sakamoto and McCormick, 2006; Kiilerich et al., 2011)

Prolactin is fundamental for adapting to hypoosmotic environments. In particular, this hormone promotes the development of α cells useful to hypoosmotic environments by limiting the size and quantity of chloride β cells in gill, which have a high NKA expression (Shikano and Fujio, 1998; Perry, 1997). Prolactin additionally decreases NKA activity while promoting the activity of other pumps such as H^+-ATPase, which allows the entry of Na^+ ions. In the intestine, during stay in the hypoosmotic environment prolactin decreases intestinal permeability through induction in the production of mucus (Sakamoto et al., 2005a, 2005b). In a hyperosmotic environment, prolactin regulates bicarbonate excretion and indirectly, allows a water flow through the intestinal barrier (Ferlazzo et al., 2012). As the smolting process advances, increased salinity tolerance is required. This need results in a synergistic participation of cortisol and the GH in increasing the presence of NKCC (Sakamoto and McCormick, 2006) and of the NKA α1b subunit in the basal membrane of chloride cells (McCormick et al., 2013). Higher NKCC and α1b levels allow the gills to excrete salts once in a hyperosmotic environment. In turn, the GH induces water entry into the intestines to compensate for liquid passively lost to the external medium (Veillette et al., 1993; Tipsmark et al., 2010), this is mainly achieved by aquaporins in the apical membrane of the enterocyte (Madsen et al., 2011). Finally, thyroid hormones, such as 3–5′ triiodothyronine (T_3) and thyroxine (T_4), not only fulfill metabolic functions, but can also induce migration and increase gill-based NKA activity. This final role of increasing NKA activity is made possible through synergies between the GH and cortisol (Shin et al., 2014; McCormick, 2009).

All of the prior holds true for smoltification in a natural environment. However, authors such as McCormick and Björnsson (1994) and Björnsson et al. (2011) indicate that differences in development and endocrine control could exist between hatchery-raised and wild larvae/smolts. Related to this, smoltification has been widely industrialized using the photoperiod as the principal inductor. NKA pump activity in the gills is quantified in farmed salmon to establish the occurrence of smoltification and, therefore, the opportune moment at which fish should be transferred from fresh- to saltwater. Nevertheless, Zydlewski and Zydlewski (2012) indicate that this practice may not work as a good predictor of smoltification in farmed fish since analyses examine only one tissue (i.e. gills) and only one of the many involved transporters. Therefore, an important range of uncertainty exists over if the fish is fully smolted and prepared for induced migration.

The unclear nature of current common practices for establishing smoltification drives the need for complementary determinations and assessments in more tissues, with the aims of which would be to more clearly identify the smolting process. Therefore, the objective of this study was to test if a complete osmoregulation process of farmed fish is in synchrony with gill NKA activity in smolts produced in a fish farm, at the moment when specimens are transferred to a marine environment. Premature transfer would mean a high quantity of non-adapted fish in the seawater phase and, consequently, notable economical losses for the aquaculture industry.

2. Materials and methods

2.1. Animals and sampling

The fish were kept in natural light and temperature conditions at the calabozo farming center (Marine Harvest, Puerto Montt, Chile). Before induction of smolification by photoperiod and guided by morphological characteristics and NKA values, 10 specimens (parr) were obtained. These usually have vertical bands and dotted spots (McCormick, 2013). Subsequently, from the same group, a few days before the transfer to sea water, 10 smolts were collected, which during the smoltification process showed normal morphological and physiological signs of smolting including silvery scales, dark fin margins, high gill $Na+/K+$-ATPase (NKA) activity (McCormick, 1993), in addition to a noticeable increase in size, but with weight loss, which implied that the fish condition factor was reduced from 1,34% to 1,07% (McCormick, 2013).

All individuals were fasted 24 h prior to sampling. Prior to tissue removal, the sampled fish were euthanized by an overdose of 2-Phenoxyethanol (77699-L, Sigma), and spinal sectioning was performed. To establish the developmental stage, particularly in relation to smoltification, NKA activity was assessed as in the aquaculture industry. For this, the second branchial arch was excised and two to four gill filaments were collected for posterior NKA activity determinations. Furthermore, the intestine (from end of pyloric blind to rectum) was extracted and divided into foregut, midgut, and hindgut sections. Finally, the intestinal epithelium was obtained by scraping with a scalpel blade.

Tissues for NKA activity analyses (i.e. gills and foregut/midgut/hindgut sections) were suspended in SEI buffer (150 mM sucrose, 10 mM EDTA, and 50 mM Imidazole, pH 7.3) and stored at −80 °C until analysis (McCormick, 1993). In turn, tissues for gene expression assessments (i.e. foregut/midgut/hindgut sections) were directly stored in liquid nitrogen at −80 °C until analyses.

All experimental protocols for animal sampling were conducted in accordance with guidelines established by the Chilean National Commission for Scientific and Technological Research (CONICYT, Spanish acronym) and the Universidad Austral de Chile.

2.2. Na^+/K^+-ATPase activity (NKA)

Kinetic assays for evaluating gill-based NKA activity were performed according to McCormick (1993). In brief, this assay determines the difference in ATP hydrolysis in the absence vs. presence (340 nm, 10 min at 25 °C) of ouabain, a specific inhibitor of the NKA pump. Values were normalized to the protein content of the homogenate. To establish intestinal NKA activity, the lumen of foregut, midgut, and hindgut sections was scraped with a scalpel to remove cells. Kinetic assays were then performed following the same procedure used for gill samples.

2.3. Total RNA preparation and cDNA synthesis

Total RNA was extracted with the column (Total mini Kit RNA, Geneaid®) following the manufacturer's instructions. The obtained samples were treated with DNase I (1 U/g RNA, Invitrogen). RNA concentration and purity were assessed via A260/280 and A260/230 ratios on a spectrometer (Nano, Maestrogen®). Samples were then stored at −80 °C until use.

Complementary DNA (cDNA) was synthetized by the reverse transcription of RNA using a cDNA synthesis kit (Promega) following the manufacturer's recommendations. Then, cDNA concentration and the A260/280 and A260/230 ratios were measured, and samples were diluted to 100 ng/μL.

2.4. Real-time quantitative PCR

Primers were designed from the Atlantic salmon transcriptome and previous publications (Table 1). Melting curve analysis and sequencing were used to test for non-specific amplifications and primer-dimer formations. Real-time quantitative PCR (qPCR) analysis was carried out using the AriaMx Real-Time PCR System (Agilent, Stratagene). A total reaction volume of 12 μL was obtained using SYBR Green (Thermo Fisher®). Reaction runs were as follows: incubation for 10 min at 95 °C,

Table 1
Primers used for real-time qPCR analysis of mRNA expressions in Atlantic salmon (*Salmo salar*) parr and smolts.

Gene	Forward	Reverse	Reference
NKA α 1a	CCAGGATCACTCAATGTCACTCT	GCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA	Nilsen et al., 2007
NKA α 1b	GCTACATCTCAACCAACAACATTACAC	TGCAGCTGAGTGCACCAT	Nilsen et al., 2007
PRL	CCCTCCTCCCAGTACATTTCTT	CATGTTTCTGGTCGCATTTTGG	Romero et al., 2012
PRLr	CTCGAGTCCAAGAGCCAGTC	CCACACTTCTCCATCAGCAA	Kiilerich et al., 2007
GH	CCGTCTGATTGAATCCTGGGAGTA	GATGAGCAGGTTGATGCCCACTTT	Accession Number
GHr	TGACTTTAAATGCCAGCACAAGGA	TGGTCACCAAATACTTCCCTCTTGA	Kiilerich et al., 2007
18S	GTCCGGGAAACCAAAGTC	TTGAGTCAAATTAAGCCGCA	Pontigo et al., 2016

Abbreviations: GH, growth hormone; GHr, growth hormone receptor; NKA, Na^+/K^+ATPase; PRL, prolactin; PRLr, prolactin receptor, 18S, 18 subunit ribosomal.

followed by 40 cycles of 10 s at 90 °C and 30 s at 60 °C, and a final melting curve of 30 s at 95 °C, 30 s at 65 °C, and 30 s at 95 °C. mRNA gene expressions were normalized to the Atlantic salmon ribosomal gene *18S* using the comparative $^{\Delta\Delta}$Ct method (Livak and Schmittgen, 2001). Each real-time qPCR was performed with triplicate samples.

2.5. Statistical analyses

All obtained data are presented as the mean ± standard error (S.E.). The student's *t*-test was used to establish statistical differences ($p < 0.05$) between parr and smolt.

3. Results

3.1. Na+/K+-ATPase activity

To confirm smoltification in the collected *S. salar* specimens, gill NKA activity was assessed, as per current practices employed by aquaculture centers. Smolt specimens significantly differed as compared to parr specimens, presenting a 4 μmol ADP/mg prot/h comparative increase (Fig. 1). This activity was as would be expected for fish finishing smoltification, according to company-established protocols. Similarly, NKA pump activity in the intestinal foregut was higher in smolt than in parr specimens (Fig. 2a), whereas the genic expression of NKAα-1a was significantly lower in smolts than in parr (Fig. 2b). In turn, the genic expression of NKAα-1b did not significantly differ between smolt and parr groups (Figs. 2c). In the intestinal midgut, NKA activity was increased nearly 9 units in smolt specimens as compared to parr counterparts (Fig. 2d). However, the genic expressions of NKAα-1a (Fig. 2e) and NKAα-1b (Fig. 2f) were significantly decreased in smolts. Finally, in the intestinal hindgut, no significant differences in NKA activity were found (Fig. 2g), but NKAα-1a (Fig. 2h) and NKAα-1b (Fig. 2i) gene expressions were significantly lower in smolt than in parr specimens.

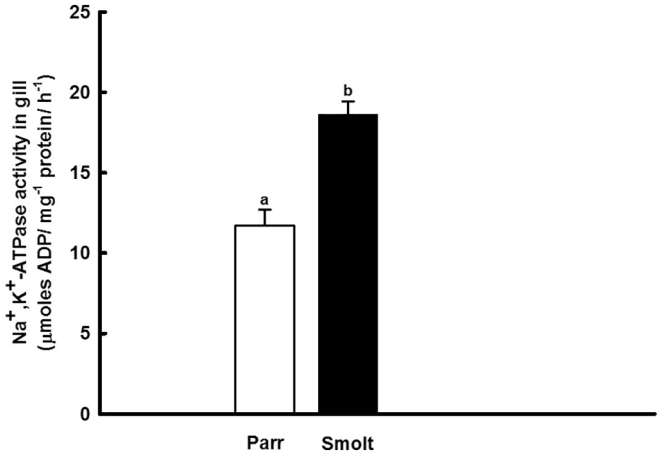

Fig. 1. Gill NKA activity in Atlantic salmon specimens parr and smolt during smoltification process. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

3.2. Intestinal endocrine expression

Both prolactin and the prolactin receptor were significantly increased in the foregut, midgut and hindgut sections of smolt specimens (Figs. 3, 4, 5). The intestinal midgut section presented the greatest change (8-fold) in prolactin gene expression, whereas the intestinal hindgut evidenced the greatest change (4-fold) in expression for the prolactin receptor.

Regarding GH and GH receptor expressions, both were significantly increased in all intestinal sections of smolt specimens (Figs. 6, 7, 8). The foregut (Fig. 6) and hindgut (Fig. 8) presented the greatest expressional change (7-fold) in the GH. In turn, the greatest expressional change (11-fold) for the GH receptor was detected in the intestinal midgut (Fig. 7).

4. Discussion

Smoltification, a process of metamorphosis in the osmoregulatory and endocrine systems, occurs in anadromous and catadromous fish that must adapt to new environmental conditions (i.e. changes in salinity) (Stefansson et al., 2008). The present study focused specifically on assessing the role played by the intestine, an organ that, together with the kidney, presents a complex network in which the neuroendocrine, osmoregulatory, and immune systems participate (Tort, 2011; Yada et al., 2002).

The obtained results evidenced changes in both NKA activity and the expression of genes associated with smoltification in the intestine (Figs. 2, 3, 4). These findings coincide with prior research (Collie and Bern, 1982; Veillette et al., 1993; Grosell, 2006; Sundh et al., 2014) reporting that the intestine is a key organ for acclimatizing to hyperosmotic environments. However, the salmon culture industry induces smoltification through an artificial photoperiod and measures the preparedness of fish for a seawater transition by measuring NKA activity in the gills. While the presently obtained results indicated sufficient *S. salar* maturity on the basis of NKA activity in the gills, this finding did not correlate with adequate intestinal maturity, thus indicating an inability of fish to optimally adapt to a hyperosmotic environment. Zydlewski and Zydlewski (2012) coincide in this point, specifically postulating that gill-based NKA activity does not ensure the successful acclimation of specimens once transferred to seawater. This assumption is founded on NKA activity in the gills not necessarily reflecting the maturity of other osmoregulatory organs, such as the intestine and posterior kidney.

Intestinal NKA activity did not significantly differ between the foregut and hindgut sections (Fig. 2a, g), which respectively transport salts and water-salts (Loretz, 1995). The genic expressions of both NKA subunits significantly decreased in the intestinal foregut and hindgut sections of smolt specimens (Fig. 2b, c, h, i). In turn, NKA activity increased by 9 units in the midgut (Fig. 2d), the primary function of

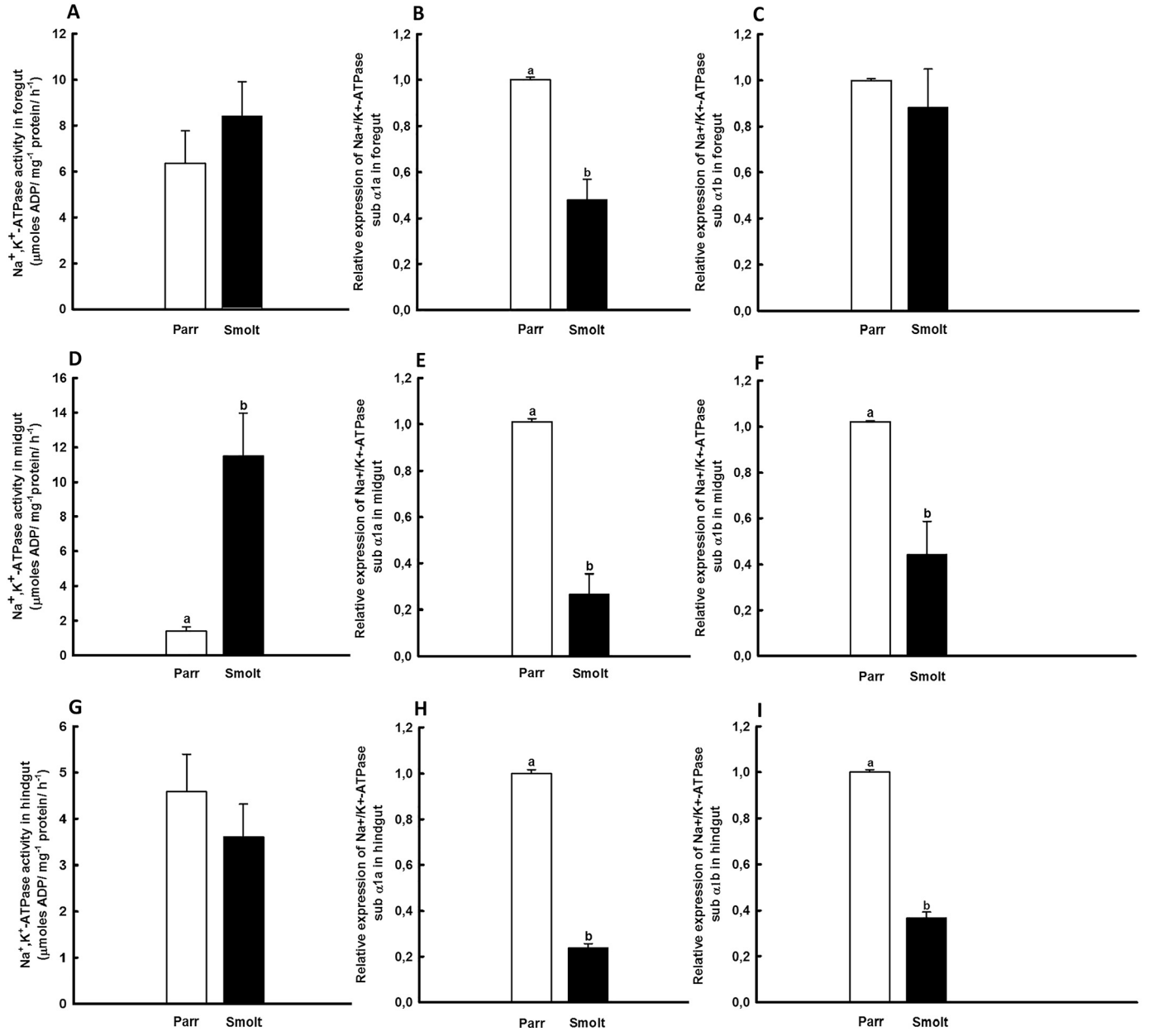

Fig. 2. (A) Determinations of NKA activity (A, D, G) and genic expression of the (B, E, H) α1a and (C, F, I) α1b subunits in foregut, midgut and hindgut of parr and smolt specimens. NKA activity was determined according to McCormick (1993). Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

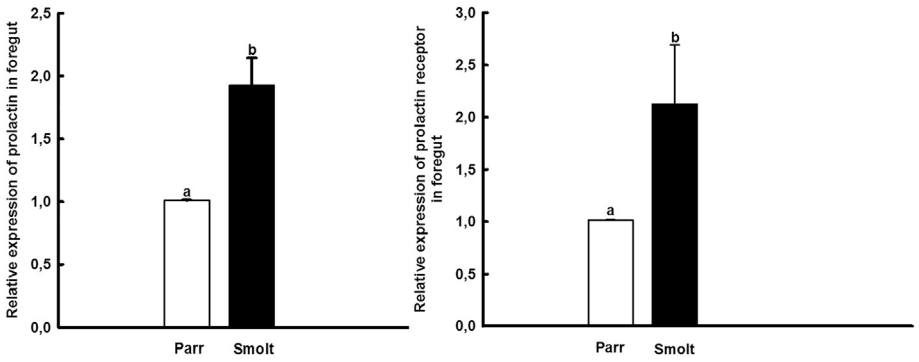

Fig. 3. Gene expression analysis of (Left) prolactin and (Right) the prolactin receptor in the intestinal foregut of parr and smolt specimens. Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

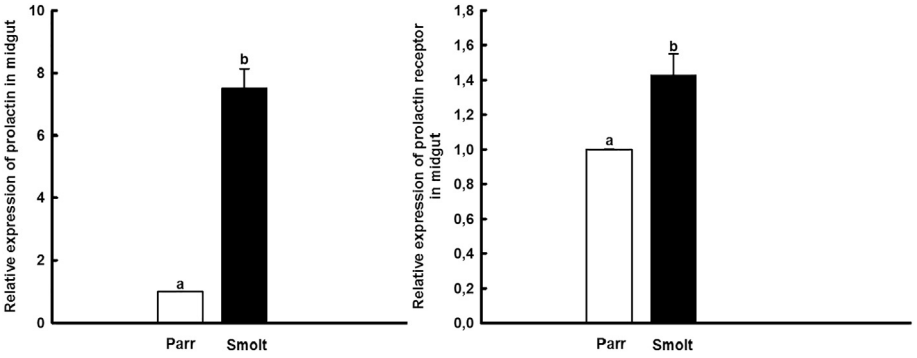

Fig. 4. Gene expression analysis of (Left) prolactin and (Right) the prolactin receptor in the intestinal midgut of parr and smolt specimens. Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

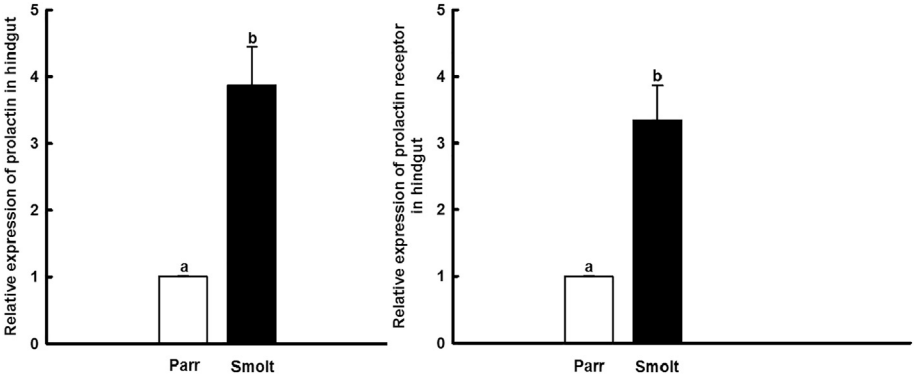

Fig. 5. Gene expression analysis of (Left) prolactin and (Right) the prolactin receptor in the intestinal hindgut of parr and smolt specimens. Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

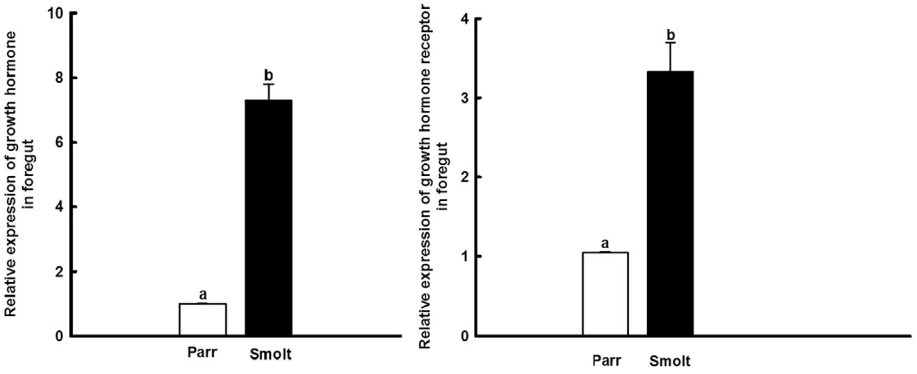

Fig. 6. Gene expression analysis of the (Left) growth hormone and (Right) growth hormone receptor in the intestinal foregut of parr and smolt specimens. Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

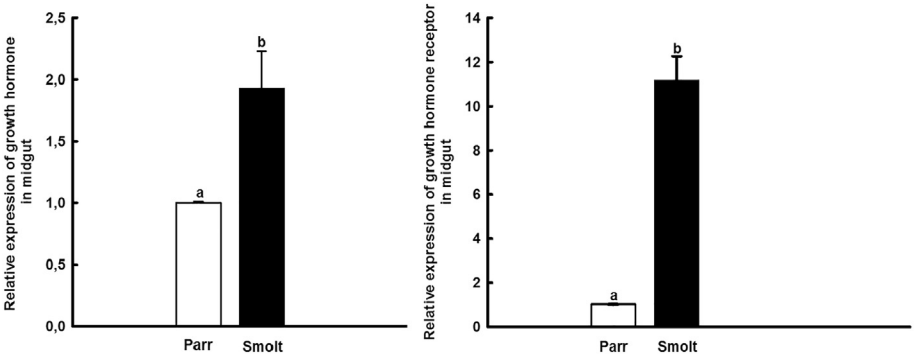

Fig. 7. Gene expression analysis of the (Left) growth hormone and (Right) growth hormone receptor in the intestinal midgut of parr and smolt specimens. Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

which is water transport. The decrease in NKA activity in the foregut and hindgut sections might be due to an inhibitory effect exercised by prolactin, as has been described in gill ionocytes (Tipsmark and Madsen, 2005). Nevertheless, it would appear that prolactin content was insufficient to inhibit NKA activity in the intestinal midgut. Furthermore, cotransporters such as NKCC and H-APTase could be actively participating in the entrance of salts to the foregut and hindgut portions (Grosell, 2006).

Prolactin and prolactin-receptor mRNAs were overexpressed in all three intestinal sections of smolt specimens (Figs. 3, 4, 5). Our results are in disagree to the osmoregulatory function described for intestine-located prolactin in hyperosmotic environments (Hirano, 1986; McCormick, 2009). For example, Ferlazzo et al. (2012) reported that prolactin regulates the luminal secretion of bicarbonate in the intestine

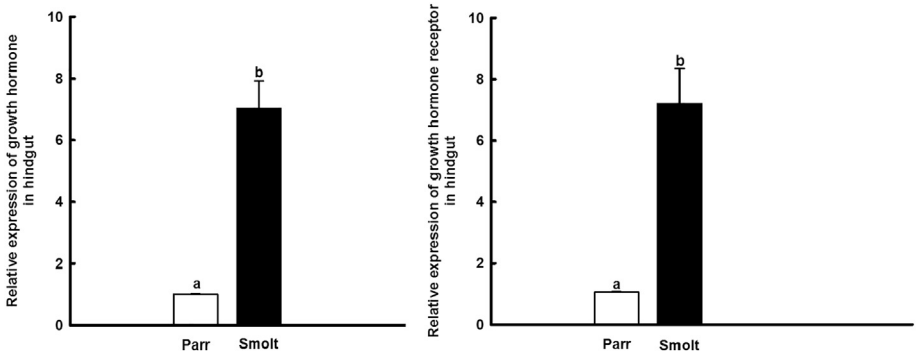

Fig. 8. Gene expression analysis of the (Left) growth hormone and (Right) growth hormone receptor in the intestinal hindgut of parr and smolt specimens. Expressional analysis of the messengers was carried out using real-time qPCR and 18S for normalization. Letters over the bars indicate statistical relationships, with different letters indicating statistically significant differences. A Student's *t*-test was used for analyses ($p < 0.05$; $n = 10$).

of gilt-head bream (*Sparus aurata*), thus creating an alkaline environment that allows for the formation of Ca^{++} aggregates in the intestine. This facilitates subsequent water absorption (Grosell, 2006). Furthermore, Sandra et al. (2001) observed that intestinal prolactin-receptor expression increases in the intestine of Nile tilapia (*Oreochromis niloticus*) when in hyperosmotic environments. This increase primarily occurs in the hindgut portion, which has been described as immunologically active (Wu et al., 2016). The genic expressions of prolactin and the prolactin receptor were increased in the intestinal hindgut of currently assessed *S. salar* specimens. This finding might be due to a greater quantity of immune cells, as has been described in mammals presenting the prolactin receptor (Leite de Moraes et al., 1995). Prolactin and prolactin receptor expressions in the intestine of *S. salar* might trigger these additional processes/expressions, as supported by the measured increased in mRNA. Nevertheless, further determinations, such as of plasmatic prolactin, are needed to corroborate the presence of this protein, while labelling assays are needed to confirm location.

A genetic overexpression of the GH was found in all three intestinal sections of smolt specimens (Figs. 6, 7, 8). Of these sections, the greatest increase in the GH receptor occurred in the intestinal hindgut (Fig. 8). These results corroborate prior reports in which the foregut and hindgut sections have key roles in acclimation to hyperosmotic environments (Collie and Bern, 1982; Stefansson et al., 2008). Among other hormones, the GH triggers acclimation by both directly and indirectly (e.g. IGF-I) (Sakamoto and Hirano, 1993; Mancera and McCormick, 1998a) stimulating increased NKA activity (Veillette et al., 1993; Madsen et al., 1995; Mancera and McCormick, 1998b; Xu et al., 1997; McCormick, 2001). This increase implicates morphological changes in the gastrointestinal tract (Nonnotte et al., 1995) that ultimately lead to decreased permeability at the esophageal level or increased permeability at the intestinal level (Sakamoto and McCormick, 2006). These changes are facilitated by an increased ingestion of water prior to seawater transference (Fuentes and Eddy, 1997).

While NKA activity did not significantly increase in the intestinal foregut or hindgut sections (Fig. 2a, g), this result might be due to the recorded increase in prolactin and the prolactin receptor in these sections of the intestine. Prolactin and the prolactin receptor might inhibit the actions exercised by the GH, as has been demonstrated in other salmonids by authors such as Madsen and Bern (1992), Boeuf et al. (1994), and Seidelin and Madsen (1997, 1999).

Based on the evidence obtained by the present study, greater attention should be given to osmoregulatory organs, such as the intestine, when assessing the smoltification process. The intestine is essential for ion and water homeostasis in marine environments, with expressions of the growth hormone, prolactin and the NKA subunits being potential markers for determining the maturity of this organ, this results align with research conducted by Zydlewski and Zydlewski (2012), a study in which artificial photoperiod manipulations it does not produce complete maturation of the osmotic and endocrine system, this status could translate into animals that are not osmotically prepared for transfer to seawater. A high percentage of inadequately adapted farmed fish consequently develop diseases as a result of premature transfer, and this situation subsequently results in high economic losses for the aquaculture industry (Sernapesca, 2015). Proper assessments of *Salmo salar* maturity prior to seawater transference will aid in reducing monetary losses in the aquaculture industry.

5. Conclusions

In summary, our determinations of gene expression of growth hormone, prolactin and NKA show the intestinal incomplete process of the fish at the moment of being transferred under culture conditions and therefore, the lights of the motive of the losses in the industry once they are found in the hyperosmotic environment, this despite the determination of the NKA at the branchial level indicated that these were in optimal conditions for their transfer.

Finally, it is understood that an organism in the natural environment and optimal growth conditions with natural cycles respond in a way and, on the other hand, individuals exposed to intense induction processes suffer from dysregulation disorders. That is why the need to implement a method complementary to the determination of the branchial activity of NKA, such as evaluating the genetic and protein expression in the intestine and the posterior kidney.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2018.03.022.

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