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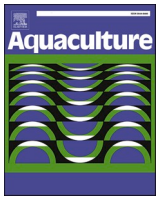

Molecular responses in the intestine of Atlantic salmon (*Salmo salar*) following light and diet stimulation of smoltification: Potential molecular markers for a seawater-ready smolt

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ABSTRACT

The transfer to seawater (SW) represents a critical stage in the production of Atlantic salmon. The success of the transfer links with the optimal development of hypo-osmoregulatory capacities during smoltification. While various strategies are adopted in aquaculture to stimulate smoltification, considerable fish loss still occurs after transfer to sea cages. Therefore, we investigated the molecular responses in the anterior and posterior intestine of Atlantic salmon, following 1) a photoperiod treatment (24 h light (L):0 h dark (D) → 24 L:0D vs. 7 L:17D → 24 L:0D) and 2) dietary treatment (regular feed or feed enriched with a salt mix/tryptophan), combined with, or without a photoperiodic treatment in freshwater (FW), to evaluate how intestinal osmoregulatory mechanisms are modulated by these treatments, and to identify potential intestinal markers indicative of a SW-ready smolt. Using quantitative real-time PCR (qPCR), we investigated transcript levels of transporters and channels involved in ion movements through the enterocytes, tight junction components, and receptors (i.e., calcium-sensing receptor and prolactin receptor). The two intestinal regions showed different gene profiles and responsiveness towards the experimental treatments. In the anterior intestine, the exposure to short photoperiod (7 L:17D) upregulated Na^+/K^+ – ATPase subunit alpha 1c (*nkaα1c*), $Na^+/K^+/2Cl^-$ cotransporter 1 (*nkcc1*), $Na^+/K^+/2Cl^-$ cotransporter 2 (*nkcc2*), Cl^-/HCO_3^- exchanger Slc26a6 (*slc26a6*), and cystic fibrosis transmembrane conductance regulator I (*cftrI*), in FW and SW. Also, Na^+/K^+ – ATPase subunit alpha 1b (*nkaα1b*), occludin (*ocln*), and prolactin receptor (*prlr*) were upregulated in FW and claudin 15 (*cldn15*) in SW groups exposed to this photoperiod. The posterior intestine was less responsive to the experimental treatments, although upregulation of *nkcc1, nkcc2*, *slc26a6,* and *cftrI* was observed in FW in the short photoperiod groups. Hence, our findings show that exposure to a winter signal in FW more effectively activates hypo-osmoregulatory mechanisms in the intestine of Atlantic salmon, where a coordinated and complementary role of the anterior and posterior intestine ensures optimal SW processing. Dietary treatment had a positive but more marginal effect on the regulation of the genes investigated, mainly enhancing the impact of short photoperiod when the two treatments were combined. Overall, we propose the apical $Na^+/K^+/2Cl^-$ cotransporter, *nkcc2,* and the apical Cl^-/HCO_3^- exchanger, *slc26a6*, as potential FW molecular markers in the anterior intestine to assess "SW-readiness" in Atlantic salmon smolts.

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1. Introduction

The life cycle of anadromous Atlantic salmon (*Salmo salar*) includes a migratory phase of juveniles from freshwater (FW) to seawater (SW) following the parr-smolt transformation (also smolting or smoltification). Morphological, physiological, endocrine, and behavioral changes occur during smoltification to prepare the fish for downstream migration and SW entry (Hoar, 1988; Stefansson et al., 2008). Besides being a fish with a complex life cycle, Atlantic salmon is a widely and successfully farmed aquaculture species. Despite the well-established production techniques and the high technology applied at all production levels, the industry still faces considerable fish losses following the transfer to sea cages due to suboptimal smolt quality (Aunsmo et al., 2008; Hjeltnes et al., 2019). In this context, priority is given to stimulating smoltification by acting on rearing conditions and using functional feeds (e.g., enriched with salt and amino acids) to produce smolts with prime salinity tolerance, thus leading to high survival rates and good growth upon SW transfer (Handeland et al., 2013; Sigholt et al., 1995; Staurnes and Finstad, 2000). For wild Atlantic salmon, the critical environmental factor determining the onset and completion of smoltification is triggered by the increase in photoperiod in spring (McCormick, 2013). Therefore, the aquaculture industry artificially manipulates light conditions by exposing the juveniles to a short (winter) photoperiod ($\leq$ 12 h light) followed by a long (spring) photoperiod to achieve a smolt ready for SW transfer (Duncan and Bromage, 1998; Handeland and Stefansson, 2001; Sigholt et al., 1995). Enhancement of the hypo-osmoregulatory capacity and survival rates in SW have also been observed in salmonids using feed enriched with NaCl during the FW phase (Perry et al., 2006; Salman and Eddy, 1990; Staurnes and Finstad, 2000), as well as in other species such as tilapia (Al-Amoudi, 1987). The positive results obtained with such feeds and the ability of Atlantic salmon to develop hypo-osmoregulatory capacity regardless of photoperiod treatment (Handeland et al., 2013; Sigholt et al., 1995) open the possibility for alternative production strategies involving the use of continuous light photoperiod in combination with dietary stimulation through the FW phase.

Osmoregulatory processes in fish are under endocrine control (Takei and McCormick, 2013). A successful SW entry is linked to the action of endocrine factors in organs like the gill and the gastrointestinal tract, allowing to switch from the hyper-osmoregulatory mechanisms required in FW to reduce water gain and ions loss to hypo-osmoregulatory mechanisms necessary in SW to favor water absorption and ion excretion (Marshall and Grosell, 2006). In SW, fish actively drink to compensate for the passive water loss (Fuentes and Eddy, 1997). The gastrointestinal tract fulfills the function of processing the ingested SW, and it is accountable for 70–85 % of water absorption (Genz et al., 2008; Wilson et al., 1996) through aquaporin channels (AQPs) located in the enterocyte membrane (Cerdà and Finn, 2010; Tipsmark et al., 2010b), and/or through tight junctions between adjacent enterocytes (Zihni et al., 2016). After desalination in the esophagus (NaCl absorption) (Hirano and Mayer Gostan, 1976; Parmelee and Renfro, 1983) and passage through the stomach, the ingested SW enters the intestine as a fluid with different chemistry and a highly reduced osmotic pressure (Grosell, 2014). In the intestine, water uptake is strictly linked to NaCl absorption. The basolateral Na^+/K^+– ATPase (NKA) provides the electrochemical gradient needed for Na^+ and Cl^- uptake (Skou and Esmann, 1992) through apical cotransporters, like Na^+/Cl^- (NCC), and $Na^+/K^+/2Cl^-$ cotransporters (NKCC2) (Musch et al., 1982; Watanabe et al., 2011). Additionally, a fraction of Cl^- enters the enterocytes through apical Cl^-/HCO_3^- exchangers (AEs) belonging to the solute carrier family 26 (Slc26) (Grosell et al., 2009; Kurita et al., 2008). The functioning of the Cl^-/HCO_3^- exchangers promote Cl^- absorption but also leads to high luminal HCO_3^- concentrations and alkalinization of the intestinal fluid, which cause $CaCO_3$ and $MgCO_3$ formation as insoluble precipitates, also known as ichthyocarbonates (Grosell and Oehlert, 2023; Walsh et al., 1991). The carbonate precipitation further reduces the osmotic pressure of the intestinal fluid, favoring water absorption along the whole intestine (Grosell and Oehlert, 2023; Wilson et al., 2002). In the basolateral membrane, the K^+/Cl^- cotransporter (KCC) (Takei, 2021) and chloride channels, such as cystic fibrosis transmembrane conductance regulator (CFTR) and Clc (Marshall and Singer, 2002; Miyazaki et al., 1999), represent possible routes for Cl^- transit from the enterocyte into the plasma, and its subsequent removal across the gill by secretion (Evans et al., 2005).

Different intestinal regions specifically contribute to imbibed water processing. The molecular pathways involved in the ion movement across the intestinal epithelium vary along the intestine in various species, such as European sea bass (*Dicentrarchus labrax*) (Alves et al., 2019), sea bream (*Sparus aurata*) (Gregório et al., 2013), Mozambique tilapia (*Oreochromis mossambicus*) (Ruiz-Jarabo et al., 2017), rainbow trout (*Oncorhynchus mykiss*) (Grosell et al., 2007), and Atlantic salmon (Sundh et al., 2014).

Gill developmental changes have been extensively studied during smoltification and after SW transfer in Atlantic salmon. The increase in NKA activity and the switch in the expression of NKA subunits α1a and α1b are considered good molecular markers of smoltification and development of hypo-osmoregulatory capacities (Christensen et al., 2018; Nilsen et al., 2007; Richards et al., 2003). On the contrary, only a few studies have focused on the osmoregulatory changes in the intestine. Therefore, the objective of this study was to characterize at the molecular level the intestinal responses of Atlantic salmon following photoperiod manipulation alone or in combination with a transition diet (regular feed added a salt mix and free tryptophan) to stimulate smoltification and salinity tolerance (Striberny et al., 2021 for details). We investigated genes putatively involved in fish intestine osmoregulatory mechanisms such as Na^+/K^+ – ATPase subunit alpha 1a, 1b, and 1c (*nkaα1a, nkaα1b*, and *nkaα1c*), $Na^+/K^+/2Cl^-$ cotransporter 1 and 2 (*nkcc1 and nkcc2*), Cl^-/HCO_3^- exchangers Slc26a3 and Slc26a6 (*slc26a3* and *slc26a6*), cystic fibrosis transmembrane conductance regulator I and II (*cftrI* and *cftrII*), occludin (*ocln*), claudin 15 (*cldn15*), calcium-sensing receptor (*casr*), and prolactin receptor (*prlr*), focusing in the anterior and posterior regions of the intestine. Moreover, we aimed to identify potential intestinal markers indicative of a SW-ready smolt.

2. Materials and methods

2.1. Animals and experimental design

This study is part of a larger experimental design described in detail by Striberny et al., 2021. In brief, fertilized Atlantic salmon eggs (*Salmo salar*) were obtained from the AquaGen strain (AquaGen, Trondheim, Norway) and hatched and raised to parr at the Aquaculture Research Station in Tromsø (Norway). From mid-March 2017, at start-feeding, fish were kept under continuous light (24 L:0D) and in FW at 4 °C till two weeks before the start of the experiment (February 6th, 2018). The temperature was increased by 0.5 °C /day to 10 °C for two weeks.

On February 6th, 2018, 1400 fish (body mass of ~40 g; referred as small fish in Striberny et al., 2021) were divided into eight circular tanks (300 L/tank) and kept in FW at 10 °C. Four tanks were subjected to six weeks of short photoperiod with 7 h of light and 17 h of darkness (7 L:17D, "SP" groups), and the others remained under 24 h of light (24 L:0D, "LL" groups). At the end of the six weeks under a short photoperiod for SP groups (March 21st), all fish were brought back to continuous light (24 L:0D). At this time-point, the water temperature was increased to 12 °C and two tanks of each light condition were fed with pellet supplemented with a salt mix and the amino acid tryptophan ("LL-LL + diet" and "SP-LL + diet" groups). The remaining tanks were fed with the usual commercial pellet ("LL-LL C" and "SP-LL C" control groups). The dietary treatment was carried out in the last six weeks in FW before the SW transfer. The composition of the two feeds is shown in Table 1. On May 11th, 50 fish from each treatment were transferred to 33 ‰ SW at

Table 1
Diet composition (Striberny et al., 2021).

Diet composition	Control (%)	Salt (%)
Wheat	15.00	9.90
Wheat gluten	10.00	12.00
Sunflower meal	5.00	2.00
Soy protein concentrate	15.50	15.00
Fababean dehulled	4.80	2.00
Fish meal	31.30	32.30
Rapeseed oil	8.50	8.60
Fish oil	8.50	8.60
Water	0.30	1.00
Vitamin and mineral premixes	1.10	1.10
Sodium chloride	0.00	6.00
Calcium chloride	0.00	0.75
L-tryptophan	0.00	0.40
Magnesium chloride	0.00	0.25
Total	100.00	100.00
Moisture	8.30	8.30
Protein	43.55	43.24
Fat	21.99	21.99
Ash	6.98	13.36
Gross energy (MJ)	22.17	21.21

8 °C in circular tanks (300 L/tank).

Fish were fed in excess and continuously by automatic systems with pellets suitable for the life stage (Skretting AS, Stavanger, Norway). During the short photoperiod for SP-LL groups, all groups were fed during the 7 h of light. Feeding was withheld 24 h before fish sampling to prevent undigested food in the intestine. However, some undigested food was present in the intestine of FW fish, while SW fish intestines were devoid of food.

For this study, fish were sampled in FW at the end of the dietary treatment and after seven days in SW (average body mass of ~105 g at the moment of the samplings). For each experimental condition, ten fish were sampled. Animals were anesthetized with an overdose of benzocaine (160 ppm), blood was collected, and fish were sacrificed by decapitation. The intestine was isolated and divided into two regions: the anterior intestine (2–3 cm caudal of the point of insertion of the last pyloric caeca) and the posterior intestine (2–3 cm posterior to the ileorectal sphincter). Samples from both regions were collected from individual fish, stored in RNAlater at 4 °C for 24 h (Sigma-Aldrich), and frozen at −20 °C until RNA extraction.

The experiment was conducted following guidelines provided in Norwegian and European legislation related to animal research. The Norwegian Food Safety Authority formally approved the experimental protocol, FOTS ID 13891.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted with the Total RNA Kit I (E.Z.N.A., Omega Bio-tek, Norcross, GA, USA), complemented with the on-column DNase treatment (RNase-Free DNase I Set, Omega Bio-tek, Norcross, GA, USA) following the manufacturer instructions. RNA quantity and quality were assessed using the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA quantities were measured at 260 nm, and the purity was evaluated with 260/280 and 260/230 absorbance ratios (≥ 1.8). Also, RNA integrity was determined by electrophoresis. All the samples showed sharp 28S and 18S rRNA bands on 1 % agarose gel. Samples were stored at −80 °C till further use. Reverse transcription of RNA into cDNA was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, UK) with 500 ng of total RNA in a final reaction volume of 20 μL. Samples were stored at −20 °C for further use.

2.3. Selection of candidate reference genes and primer design

A previous study by Sundh et al. (2014) highlighted the difficulty of proper qPCR data normalization in the intestine of Atlantic salmon because of intrinsic differences in the reference genes between regions. Under the experimental conditions used in this study, reference genes commonly used for qPCR data normalization in salmon and other species, like 18S ribosomal RNA (*18S*), elongation factor 1 alpha (*ef1α*), and beta actin (*actb*) (Alves et al., 2020; Breves et al., 2020b; Campinho et al., 2010; Gilmour et al., 2012; Striberny and Jørgensen, 2017; Tipsmark et al., 2008) were inadequate as single reference gene for anterior and posterior intestine comparisons. Therefore, transcriptomic data available for the anterior and posterior intestine from the same experiment (unpublished data) were analyzed to identify genes showing low variations between the experimental groups. Following this analysis, beta-2-microglobulin (*b2m*), actin-related protein 2/3 complex subunit 1b (*arpc1b*), and ubiquitin-associated protein 2 (*ubap2*) were selected as potential reference genes (see Table 2 for details).

The cDNA sequences of reference genes and target genes were extracted from the EST collection database at the National Center of Biotechnology (NCBI, https://blast.ncbi.nlm.nih.gov) using tBLASTn queries of known protein or deduced protein sequences from other salmonid species and compared with the sequences of the Atlantic salmon transcriptome database (unpublished data). Primer pairs were designed using the web interface Primer3Plus (Untergasser et al., 2012) or extracted from previous publications, as reported in Table 2. Primer specificity was validated by PCR amplification, and product size was checked on 1.5 % agarose gel. After cloning into pGEM-T Easy Vector (Promega, Madison, WI, USA), each amplicon identity was confirmed by the Sanger sequencing (Sanger and Coulson, 1975) in the Molecular Biology platform of CCMAR (Centre of Marine Sciences, Faro, PT) and using the NCBI nucleotide BLAST software (https://blast.ncbi.nlm.nih.gov).

2.4. Quantitative real-time PCR (qPCR)

Real-time PCR amplifications were performed in duplicate for all the samples in a final volume of 6 μL with 1× SensiFAST SYBR Hi-ROX (Bioline), optimal concentrations of forward and reverse primers (from 200 to 300 nM, Table 2), and 1 μL of 1:6 diluted cDNA. For *18S*, *ef1α*, and the remaining reference genes, cDNA was used at 1:1000, 1:100, and 1:50 dilutions, respectively. No-template controls (NTC) were included in the amplification of all the genes investigated. Amplifications were performed in 384-well plates using the CFX384 Touch Real-Time PCR System (Bio-Rad Laboratories, Hercules, CA, USA) with the following program: denaturation and enzyme activation step at 95 °C for 3 min, followed by 44 cycles of 95 °C for 10 s and primer pair specific annealing temperature (T_a, Table 2) for 15 s. After the amplification phase, a dissociation step was carried out to confirm the specificity of each reaction. The temperature was increased gradually by 0.5 °C every 5 s, from 60 °C to 95 °C. Amplification efficiencies (E) and correlation coefficient (R^2) were estimated for each gene, generating a standard curve for each primer pair from 10-fold serial dilution of DNA cloned into a plasmid (Table 2). The range of acceptance for the amplification efficiency was set between 90 % and 110 %.

2.5. Data analysis

The reference and target genes data were obtained from the CFX Maestro Software (Bio-Rad Laboratories, Hercules, CA, USA) as quantification cycles (C_q) and in linear scale (starting quantities, SQ) using a standard curve for each gene. To evaluate the stability of the candidate reference genes, the Excel add-in tool NormFinder was used (Andersen et al., 2004). Its algorithm determines the expression stability in the selected reference genes, considering the estimated intra- and inter-group variation in the investigated groups. Aside from ranking the set of reference genes, NormFinder also suggests the best combination of two genes for target normalization. An advantage of NormFinder's model base is that the possible presence of co-regulated genes does not

Table 2
Nucleotide sequences (5′ to 3′) of forward (F) and reverse (R) primers used for real-time PCR (qPCR) with corresponding amplicon size in base pairs (bp), final primers concentration (nM), annealing temperature (T_a), qPCR efficiency (E), correlation coefficient (R^2), and NCBI accession number or reference.

Gene symbol	Gene name	Sequence (5′ to 3′)	Size (bp)	Primer conc. (nM)	Ta (°C)	E (%)	R^2	NCBI acc. n./ Refer.
18S rRNA	18S ribosomal RNA	F: CCTGCGGCTTAATTTGACTC R: AACCAGACAAATCGCTCCAC	139	200	58	94.2	0.994	AJ427629.1
arpc1b	Actin-related protein 2/3 complex subunit 1b	F: CGCGTATGTCTGGTCTCTGAA R: GCAGATGGATATGAGGCGTGA	145	200	60	95.5	0.995	XM_045690514.1
actb	Beta actin	F: ACACCTTCTACAACGAGCTGAG R: TGATCTGGGTCATCTTCTCCCT	105	200	60	96.6	0.980	AF012125.1
b2m	Beta-2-microglobulin	F: CAAATGGCACAGCTTCTGATG R: GTGTTCAAATCTAATGGTACCA	125	200	60	95.5	0.999	NM_001123699.1
casr	Calcium-sensing receptor	F: AATGGGAGCACCAGTTTCAG R: AATGTCCTGTAGGGCCTGTG	147	200	60	98.3	0.999	NM_001123571.1
slc26a3	Cl^-/HCO_3^- exchanger	F: CTTCCTTGGCACTTCCAGAC R: ACTCTCTGCTCATCGCTGGT	150	250	60	91.2	0.992	XM_021577256.1
slc26a6	Cl^-/HCO_3^- exchanger	F: GCTGAGCACTTTTCCTGTCC R: GAGCATAGGCCATACCCTGA	125	250	60	91.9	0.993	XM_014134693.1
cldn15	Claudin 15	F: GAGGCAACCGGATTCTTCATGT R: GTTCTCATACTGTCGGGTGGAG	126	300	60	99.3	0.998	XM_014139145.2
cftrI	Cystic fibrosis transmembrane conductance regulator I	F: ACGACCCCTGTCCAAAGATAG R: TGTAACGCAGGTAGGTGTTCC	131	250	60	93.6	0.997	AF155237.1
cftrII	Cystic fibrosis transmembrane conductance regulator II	F: TGCTTAAGGTTAGTGCCTCAGG R: AAGGCTACTTCAGGTTAATCAC	111	250	60	97.4	0.999	NM_001123534.1
ef1α	Elongation factor 1 alpha	F: AGGCATTGACAAGAGAACCATT R: TGATACCACGCTCCCTCTC	119	200	60	100.7	0.986	Striberny and Jørgensen, 2017
nkaα1a	Na^+/K^+ – ATPase subunit alpha 1a	F: CCAGGATCACTCAATGTCACTCT R: TCTATCAAAGGCAAATGAGTTTAATATCATTGTAAAA	93	250	60	100.0	0.997	Striberny et al., 2021
nkaα1b	Na^+/K^+ – ATPase subunit alpha 1b	F: GAGGTTGGGTGGAACAGGAG R: AGCTGAGTGCACCATCGCAG	162	250	60	102.1	0.999	Striberny et al., 2021
nkaα1c	Na^+/K^+ – ATPase subunit alpha 1c	F: GGTTGTCTTGCTGCTCGGAATT R: GGAGCTGTGTCTTGGTTCATCA	113	250	60	102.1	0.995	AY319389.1
nkcc1	$Na^+/K^+/2Cl^-$ cotransporter 1	F: ACGTGTCCCACATCTCAG R: GAGGGCTTGGATGAGTCT	121	200	60	95.5	0,991	Kiilerich et al., 2007b
nkcc2	$Na^+/K^+/2Cl^-$ cotransporter 2	F: AAAGACCAACCTCCAGGTCA R: AACCAGGCCATGTAGAGGTG	129	200	60	106.1	0.991	XM_014127389.1
ocln	Occludin	F: AAGGCAGAGATGTGAAGGACTG R: GATGCTATTGGCTGAGGCATTG	119	300	60	99.9	0.995	XM_014123589.2
prlr	Prolactin receptor	F: CCAGATTCATCAGATGGGAGGG R: GGATCTGCTGAGATGGTACTCG	135	200	60	99.6	0.997	DQ508436.1
ubap2	Ubiquitin-associated protein 2	F: GGAATGAGATGTTCGAGCAGG R: GCTAACAAGTCTGATTTGGGAAGG	130	200	60	94.2	0.998	XM_014142130.2

significantly interfere with the stability assessment of the reference genes. Using the data in linear scale as input (averages from the technical duplicates), three separate analyses were carried out in NormFinder: one for each intestinal region alone (anterior intestine and posterior intestine) and another integrating both intestinal regions. After stability analysis, the geometric mean between *18S and actb*, the two most stable reference genes based on the combined expression stability analysis of the anterior and posterior intestine, was used to normalize the target genes investigated.

2.6. Statistical analysis

All results are shown as mean ± SEM. Outliers were identified using the ROUT method (Motulsky and Brown, 2006), and the homogeneity of variances and normality was assessed before statistical analysis. When necessary, data were transformed to comply with ANOVA assumptions. In FW and SW sampling, and for each intestinal region, differences in mRNA levels were assessed by two-way ANOVA, considering light (LL-LL and SP-LL) and diet (control and dietary treatment) as main factors, followed by the Bonferroni *post-hoc* test when significant interaction between light and diet was detected. For each experimental group, subsequent statistical analysis of the data was carried out using the unpaired *t*-test to assess differences between the anterior and posterior intestine. All statistical analyses were performed using GraphPad Prism 8.4.0 (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at $p < 0.05$.

3. Results

3.1. Phenotype characterization

The light and dietary treatments employed in this study resulted in the development of distinct phenotypes. A description of the phenotypic changes observed throughout the entire experimental period was previously detailed in our publications (Duarte et al., 2023; Gaetano et al., 2023; Striberny et al., 2021). Briefly, we report here some of the highlights.

At the end of the experimental treatments in FW (May), fish exhibited uniform silvering across all treatments (stage 3). Fish exposed to continuous light (LL-LL) showed a higher mass gain, while a reduction in the condition factor was observed in the short photoperiod groups (SP-LL) (Striberny et al., 2021). After seven days in SW, fish displayed good hypo-osmoregulatory abilities regardless of treatment. Still, after two months in SW, higher specific growth rates and appetite were observed in the SP-LL groups and the LL-LL + diet (Striberny et al., 2021). At the intestinal level, enhancement in intestinal function was observed due to the light and dietary treatments (Gaetano et al., 2023). Furthermore, distinct developmental dynamics and morpho-functional

specialization were observed between anterior and posterior intestine (Duarte et al., 2023; Gaetano et al., 2023).

3.2. Ranking of the candidate reference genes

The mRNA abundance of six candidate reference genes *18S*, *ef1α, actb*, *b2m, arpc1b,* and *ubap2* was determined by qPCR in the anterior and posterior intestine of Atlantic salmon exposed to different light and dietary treatments at the end of the smolting period in FW and after SW transfer. In the anterior intestine, the stability ranking for the reference genes (from the most stable to the least stable) was *actb* > *arpc1b* > *18S* > *ef1α* > *ubap2* > *b2m*, while in the posterior intestine was *ubap2* > *ef1α* > *arpc1b* > *b2m* > *actb* > *18S* (Suppl. Table 1). The best combination of two genes for the anterior intestine was represented by *18S* - *actb*, while for the posterior intestine by *ef1α* - *arpc1b* with stability values of 0.100 and 0.194 respectively (Suppl. Table 1). When the anterior intestine data were analyzed together with the posterior intestine, to define common genes suitable for the normalization of qPCR data in both intestinal regions, NormFinder ranked the candidate genes as followed *arpc1b* > *ubap2* > *ef1α* > *actb* > *b2m* > *18S*. The best combination of two genes for the anterior and posterior intestine was *18S* - *actb* with a stability value of 0.182 (Suppl. Table 1).

3.3. mRNA abundance of target genes

3.3.1. Light and diet effects

In the anterior intestine of Atlantic salmon, light treatment in FW significantly regulated the mRNA levels of *nkaα1b* (Fig. 1C), *nkaα1c* (Fig. 1E), *nkcc1* (Fig. 2A), *nkcc2* (Fig. 2C), *slc26a6* (Fig. 3C), *cftrI* (Fig. 4A), *ocln* (Fig. 5A), and *prlr* (Fig. 6C), and in SW the levels of *nkaα1a* (Fig. 1A), *nkaα1c* (Fig. 1E), *nkcc1* (Fig. 2A), *nkcc2* (Fig. 2C), *slc26a6* (Fig. 3C), *cftrI* (Fig. 4A), and *cldn15* (Fig. 5C) (two-way ANOVA). For these genes, higher mRNA levels were observed in the groups exposed to the SP-LL photoperiod, except for *nkaα1a*, which showed lower transcript levels in the SP-LL groups than in the LL-LL groups (Fig. 1A). In the posterior intestine, *nkcc1* (Fig. 2B), *nkcc2* (Fig. 2D), *slc26a6* (Fig. 3D), and *cftrI* (Fig. 4B) were significantly affected by light in FW (two-way ANOVA), showing higher mRNA levels in the SP-LL groups than in the LL-LLs. Light also significantly regulated *slc26a3* mRNA levels in the posterior intestine of Atlantic salmon, where lower mRNA levels were detected in the SP-LL groups in both FW and SW (two-way ANOVA; Fig. 3B).

Dietary treatment significantly affected the mRNA levels of the genes investigated only in FW fish. In the anterior intestine, dietary treatment affected *nkaα1b* (Fig. 1C), *nkaα1c* (Fig. 1E), *nkcc2* (Fig. 2C), and *ocln* (Fig. 5A) (two-way ANOVA), with higher mRNA levels observed for the groups treated with the experimental diet. Increased mRNA levels were

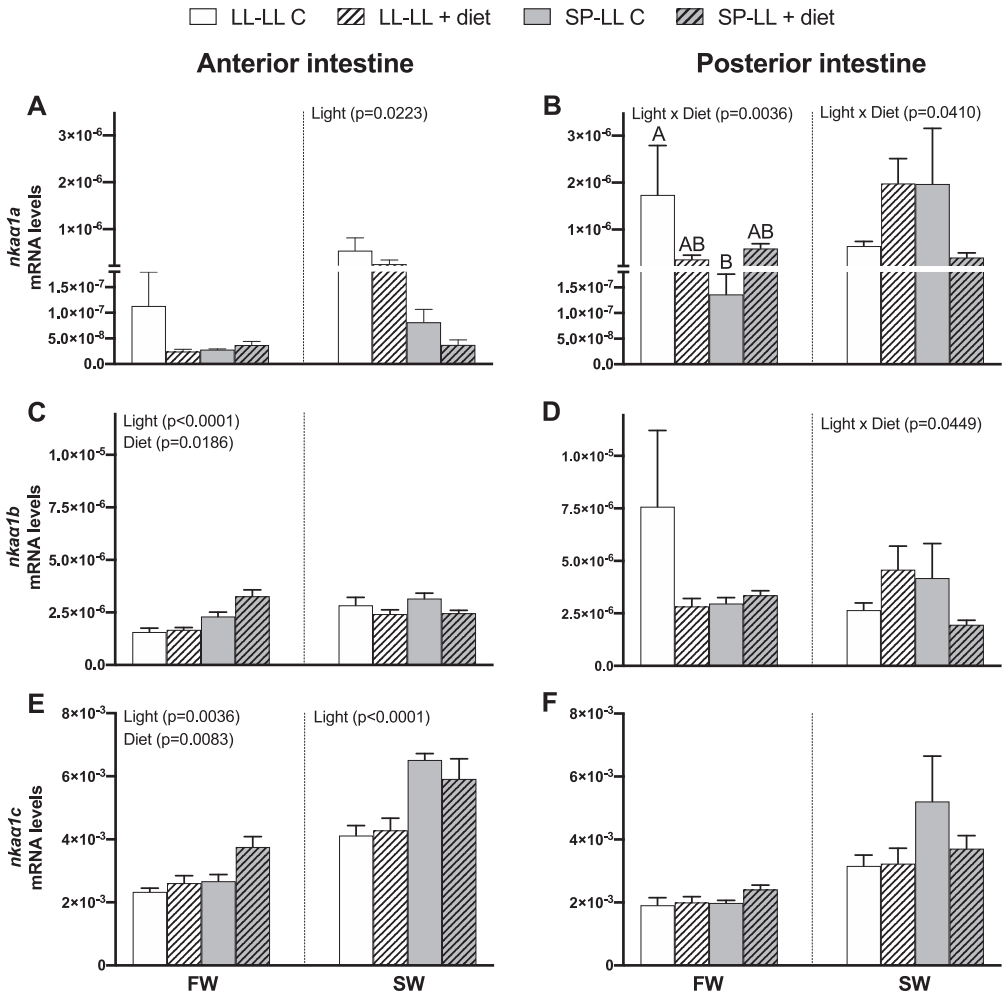

Fig. 1. Relative *nkaα1a* (A, B), *nkaα1b* (C, D), and *nkaα1c* (E, F) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean ± SEM ($n = 6–10$). In the analysis, light and dietary treatment are the two considered factors ($p < 0.05$, two-way ANOVA). When a significant interaction between light and dietary treatment was observed, the Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Only significant effects are reported.

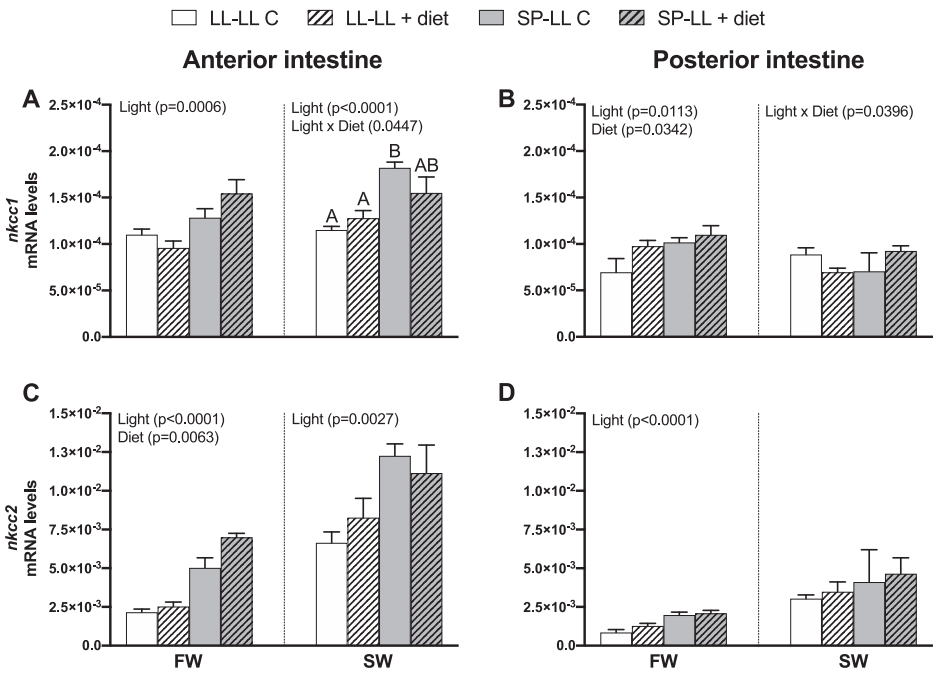

Fig. 2. Relative *nkcc1* (A, B) and *nkcc2* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean ± SEM (n = 6–10). In the analysis, light and dietary treatment are the two considered factors ($p < 0.05$, two-way ANOVA). When a significant interaction between light and dietary treatment was observed, the Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Only significant effects are reported.

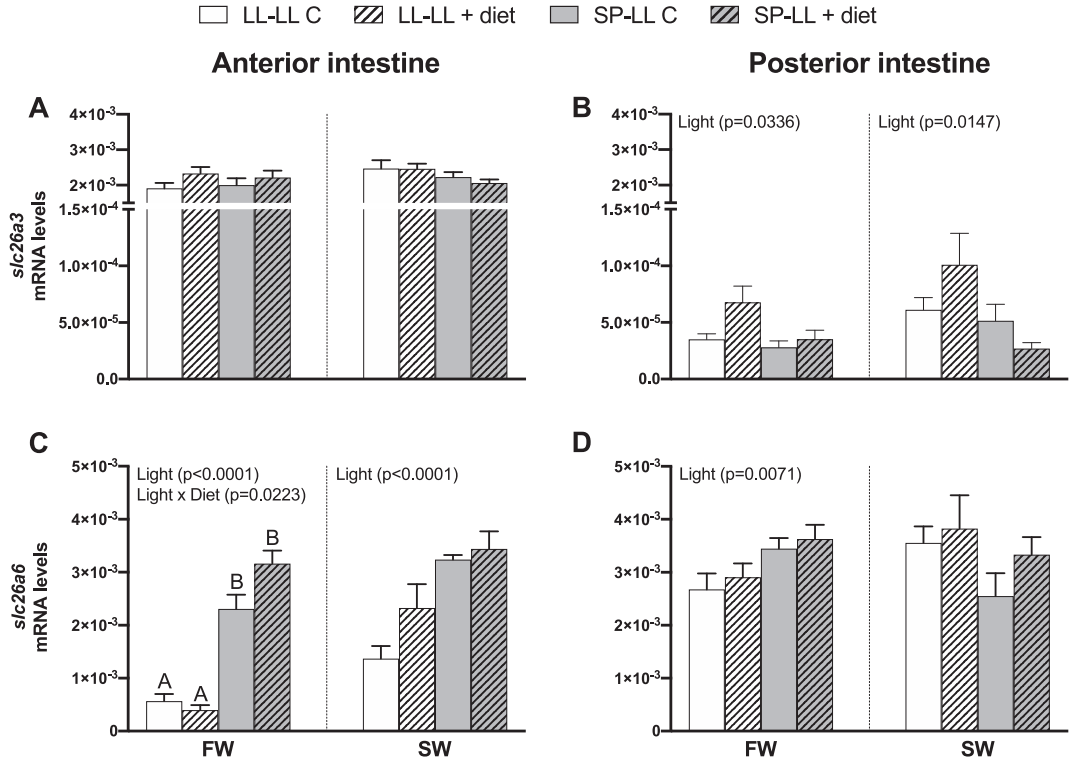

Fig. 3. Relative *slc26a3* (A, B) and *slc26a6* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean ± SEM (n = 6–10). In the analysis, light and dietary treatment are the two considered factors ($p < 0.05$, two-way ANOVA). When a significant interaction between light and dietary treatment was observed, the Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Only significant effects are reported.

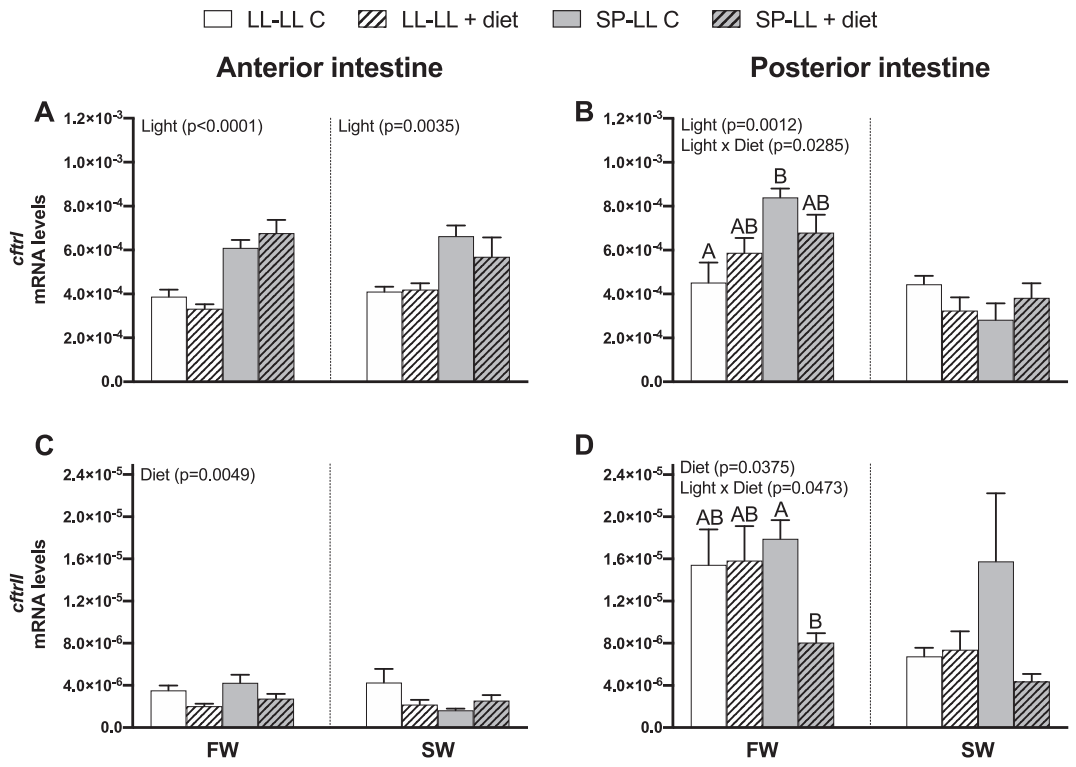

Fig. 4. Relative *cftrI* (A, B) and *cftrII* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean ± SEM (n = 6–10). In the analysis, light and dietary treatment are the two considered factors ($p < 0.05$, two-way ANOVA). When a significant interaction between light and dietary treatment was observed, the Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Only significant effects are reported.

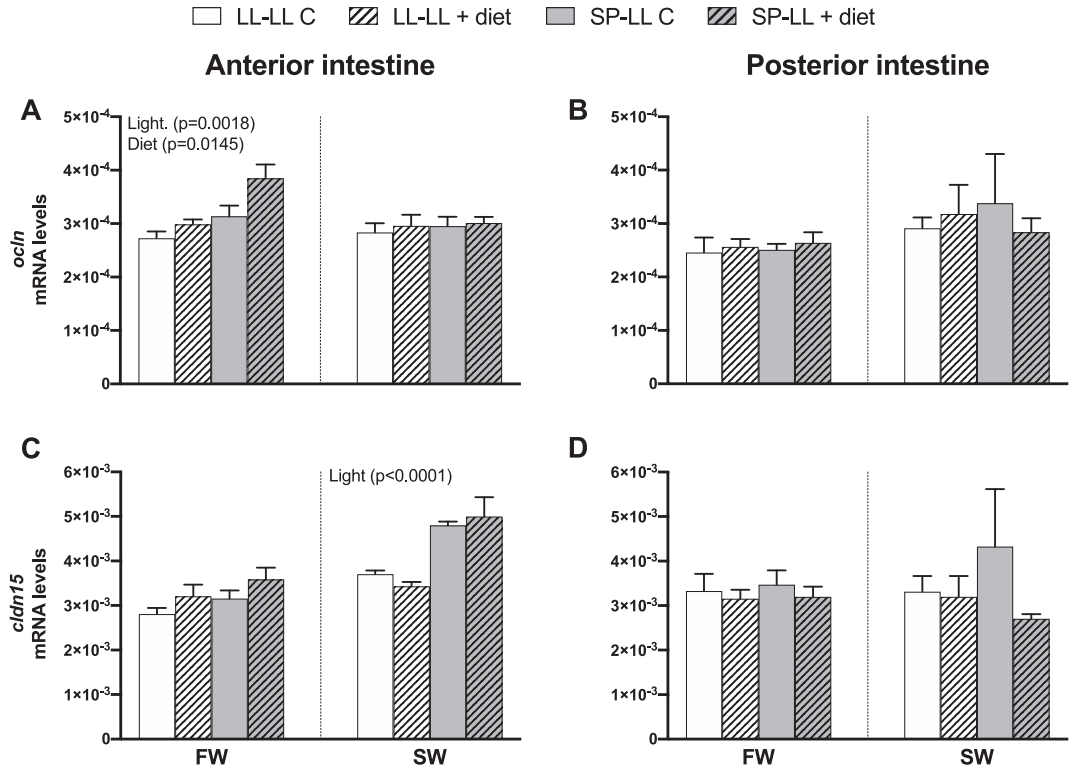

Fig. 5. Relative *ocln* (A, B) and *cldn15* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean ± SEM (n = 6–10). In the analysis, light and dietary treatment are the two considered factors ($p < 0.05$, two-way ANOVA). Only significant effects are reported.

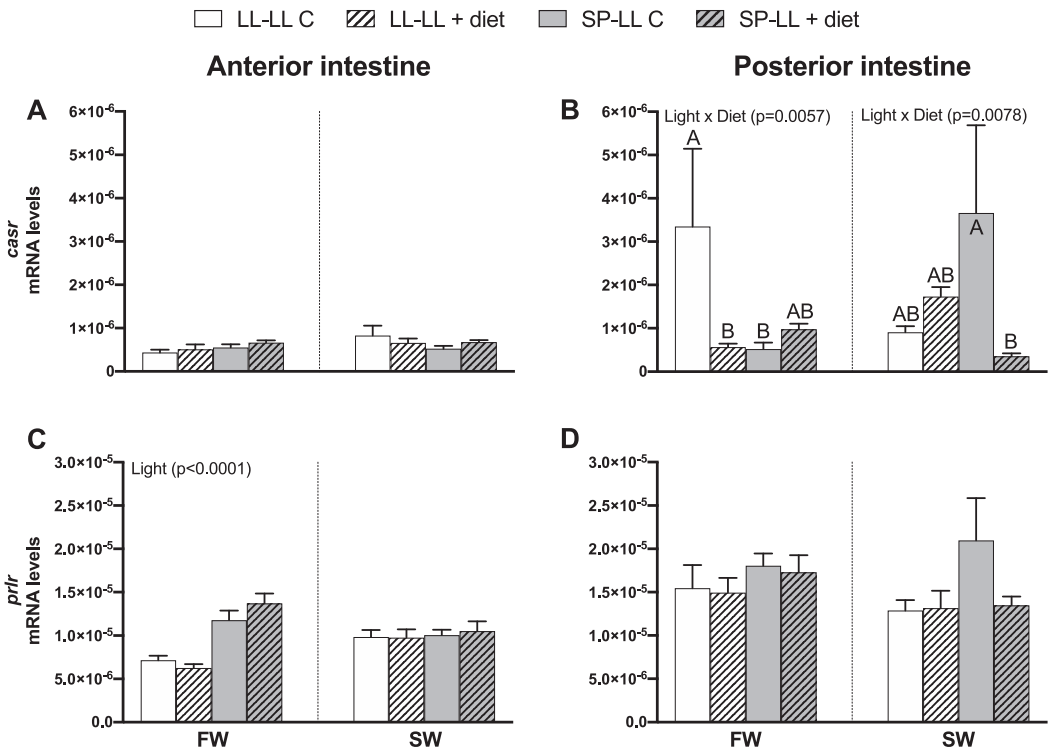

Fig. 6. Relative *casr* (A, B) and *prlr* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean ± SEM (n = 6–10). In the analysis, light and dietary treatment are the two considered factors ($p < 0.05$, two-way ANOVA). When a significant interaction between light and dietary treatment was observed, the Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Only significant effects are reported.

also observed for *nkcc1* in the posterior intestine of FW fish (two-way ANOVA; Fig. 2B). mRNA levels of *cftrII* were affected by the diet in FW, in both anterior and posterior intestine, but for this gene, the experimental diet induced a reduction of the mRNA levels (two-way ANOVA; Fig. 4C, D).

Statistical interaction between light and diet was observed. In the anterior intestine, a significant interaction was observed, in FW, for *slc26a6*, where the SP-LL groups showed significantly higher *slc26a6* mRNA levels than the LL-LL groups (two-way ANOVA followed by Bonferroni *post-hoc* test; Fig. 3C). Moreover, in SW, *nkcc1* showed interaction of light and diet, with significantly higher levels of *nkcc1* in the SP-LL C than in the LL-LL groups (two-way ANOVA followed by Bonferroni *post-hoc* test; Fig. 2A). In the posterior intestine, the interaction of light and diet was observed in FW for *nkaα1a* (Fig. 1B), *cftrI* (Fig. 4B), *cftrII* (Fig. 4D), and *casr* (Fig. 6B) (two-way ANOVA followed by Bonferroni *post-hoc* test). The Bonferroni *post-hoc* test revealed significantly higher *nkaα1a* mRNA levels in the LL-LL C group than in the SP-LL C (Fig. 1B), higher *cftrI* levels in the SP-LL C than in the LL-LL C group (Fig. 4B), higher *cftrII* levels in the SP-LL C group than in the SP-LL + diet (Fig. 4D), and higher *casr* levels in the LL-LL C group than in

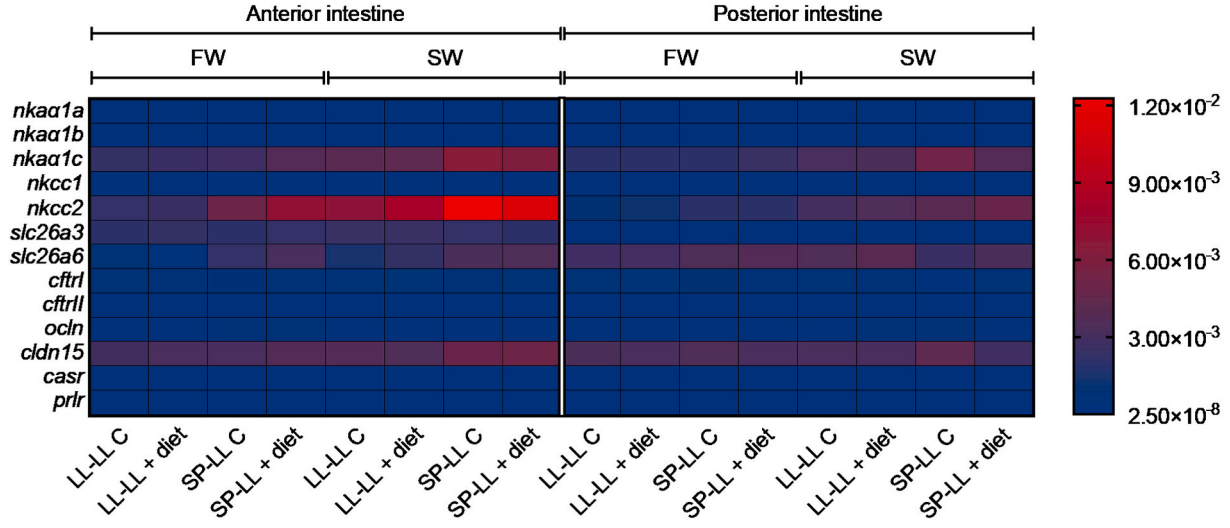

Fig. 7. Heatmap summarizing the relative mRNA levels of the genes investigated in the anterior and posterior intestine of Atlantic salmon (*Salmo salar*) after exposure to light and dietary treatment in FW and following seven days of residence in SW.

LL-LL + diet and SP-LL C groups (Fig. 6B). In SW, significant interaction between light and diet was observed in the posterior intestine for *nkaα1a* (Fig. 1B), *nkaα1b* (Fig. 1D), *nkcc1* (Fig. 2B) and *casr* (Fig. 6B) (two-way ANOVA followed by Bonferroni *post-hoc* test). For the latter, the group SP-LL C showed higher *casr* mRNA levels than SP-LL + diet (Bonferroni *post-hoc* test; Fig. 6B).

The regulation dynamics of the genes investigated in this study are summarized in a heatmap in Fig. 7.

3.3.2. Anterior intestine vs. posterior intestine

In FW, the posterior intestine of Atlantic salmon showed significantly higher *nkaα1a* and *cftrII* mRNA levels than the anterior intestine in all the experimental groups (unpaired *t*-test; Table 3). Moreover, higher mRNA levels in the posterior intestine were observed for *nkaα1b* in the LL-LL + diet group, *slc26a6* in the LL-LL groups and SP-LL C, *cftrI* in the groups LL-LL + diet and SP-LL C, *casr* in the LL-LL C and SP-LL + diet groups, and for *prlr* in the LL-LL groups and SP-LL C (unpaired t-test; Table 3). *nkcc2* and *slc26a3* levels were significantly higher in the anterior intestine of all FW groups (unpaired t-test; Table 3). Higher mRNA levels in the anterior intestine were also observed for *nkaα1c* in the SP-LL groups, *nkcc1* in the groups LL-LL C and SP-LLs, and for *ocln* in LL-LL + diet and SP-LL groups (unpaired t-test; Table 3).

When comparing the two intestinal regions in SW, significantly higher mRNA levels were observed in the posterior intestine for *nkaα1a* in the groups LL-LL + diet and SP-LL + diet, *slc26a6* in the LL-LL C groups, *cftrII* in the LL-LL + diet and SP-LL groups, and for *casr* in the group LL-LL + diet (unpaired t-test; Table 3). In SW, higher mRNA levels were observed in the anterior intestine for *nkcc1*, *nkcc2*, and *slc26a3*, in all the experimental groups (unpaired t-test; Table 3). Also, higher levels in the anterior intestine were detected for *nkaα1c* in SP-LL + diet group, *cftrI* in SP-LL C, cldn15 in SP-LL + diet group, and for *casr* in the group SP-LL + diet (unpaired t-test; Table 3).

Table 3
p-values of unpaired *t*-test for the mRNA abundance analysis in the intestine of Atlantic salmon (*Salmo salar*). Analyzed differences between anterior and posterior intestine. In yellow are highlighted the groups in which the mRNA levels are significantly lower in the posterior intestine than in the anterior intestine, and in blue the groups in which mRNA levels are significantly higher in the posterior intestine. Statistical significance was accepted at $p < 0.05$.

Freshwater (FW)

GENE	LL-LL C	LL-LL + diet	SP-LL C	SP-LL + diet
nkaα1a	0.0362	0.0007	0.0239	<0.0001
nkaα1b	0.0789	0.0111	0.0734	0.8073
nkaα1c	0.0806	0.0540	0.0098	0.0017
nkcc1	0.0103	0.8298	0.0333	0.0196
nkcc2	0.0005	0.0015	0.0003	<0.0001
slc26a3	<0.0001	<0.0001	<0.0001	<0.0001
slc26a6	<0.0001	<0.0001	0.0031	0.2354
cftrI	0.5840	0.0017	0.0005	0.9556
cftrII	0.0002	<0.0001	<0.0001	<0.0001
ocln	0.3384	0.0375	0.0167	0.0014
cldn15	0.1529	0.8857	0.4132	0.2857
casr	0.0363	0.5879	0.6024	0.0378
prlr	0.0010	<0.0001	0.0022	0.1518

Seawater (SW)

GENE	LL-LL C	LL-LL + diet	SP-LL C	SP-LL + diet
nkaα1a	0.2328	0.0035	0.0548	0.0004
nkaα1b	0.7223	0.0776	0.5195	0.0653
nkaα1c	0.0512	0.0876	0.1996	0.0095
nkcc1	0.0077	<0.0001	0.0003	0.0028
nkcc2	0.0005	0.0036	0.0056	0.0068
slc26a3	<0.0001	<0.0001	<0.0001	<0.0001
slc26a6	<0.0001	0.0544	0.0756	0.8078
cftrI	0.5053	0.1299	0.0021	0.1861
cftrII	0.0604	0.0116	0.0137	0.0447
ocln	0.7745	0.7033	0.6562	0.5703
cldn15	0.3233	0.6083	0.7592	0.0003
casr	0.5582	0.0002	0.0880	0.0041
prlr	0.0512	0.1665	0.0574	0.0645

AI > PI PI > AI

4. Discussion

The present study investigated the molecular mechanisms underlying the physiological processes evoked in the intestine during artificial stimulation of smoltification in parr of Atlantic salmon. Additionally, the study aimed to identify potential intestinal molecular markers indicative of a smolt ready to be transferred to SW.

The findings in this study reveal a region-specific profile and regulation of the genes involved in hypo-osmoregulatory mechanisms in the intestine of Atlantic salmon and highlight the significance of photoperiod and dietary treatments in optimizing SW acclimation. Based on our results, *nkcc2* and *slc26a6* emerge as potential molecular markers in the anterior intestine of Atlantic salmon, enabling the assessment of smolt hypo-osmoregulatory capacities in FW.

As observed in several teleost species, discrete regions of Atlantic salmon's intestine display morpho-functional differences (Duarte et al., 2023; Gaetano et al., 2023; Sundell et al., 2003; Veillette et al., 1993). Preliminary validation of reference genes was carried out to study the anterior and posterior intestine's osmoregulatory role at the molecular level. The different regulation in the two regions of the reference genes investigated was in line with the different developmental dynamics we previously observed in Atlantic salmon's anterior and posterior intestine (Duarte et al., 2023) and provided valuable information about the profound differences between intestinal regions. Therefore, it is essential to exercise caution when selecting reference genes for studies involving diverse regions of the intestine, and it is advisable to prioritize the use of multiple genes.

The transcript abundance of the genes investigated here demonstrates that Atlantic salmon's anterior and posterior intestine contribute differently to the processing of the ingested SW. As the fluid moves from the anterior to the posterior region of the fish intestine, its chemistry changes (Grosell, 2014), and it is expected that the relative importance of the same transporter may change in the different regions conferring to the anterior and posterior intestine complementary roles in the ions and water absorption. Functional studies conducted in the intestine of Atlantic salmon demonstrated a functional specialization of different intestinal regions (Gaetano et al., 2023; Sundell et al., 2003; Veillette et al., 1993). Furthermore, at the molecular level, differential mRNA levels of key genes involved in the osmoregulatory mechanisms were observed between intestinal regions of the Atlantic salmon (Sundh et al., 2014), as demonstrated here. Besides showing region-specific mRNA level changes (Table 3), the two regions were differently affected by the photoperiod and dietary treatments used in this study. These treatments aimed at enhancing SW tolerance strongly modulated the anterior

intestine, while the posterior intestine was less responsive.

The development of hypo-osmoregulatory capacity, regardless of photoperiod, has been previously described in Atlantic salmon (Handeland et al., 2013; Striberny et al., 2021). In agreement with this, the molecular machinery necessary to counteract dehydration in SW was sufficiently developed in the intestine of all the experimental groups investigated. However, the molecular pathways investigated, known to be involved in the movement of ions through the enterocyte, were upregulated in the groups exposed for six weeks to short photoperiods, demonstrating that the light signal is a critical factor at the intestinal level. Further, Stefansson et al., 2007 showed that fish exposed to continuous light fail to complete smoltification because of the absence of the environmental cues needed for the proper activation of the endocrine cascade mechanisms, some of which are directly involved in regulating hypo-osmoregulatory processes in the fish intestine (Takei and Loretz, 2011). Therefore, even if animals exposed to continuous light can adapt to SW to complete smoltification successfully and optimize, among others, the intestinal hypo-osmoregulatory mechanisms, exposure to a period of short days is required.

This study also used a diet enriched with salt mix and tryptophan to stimulate smoltification, which has been demonstrated to have beneficial effects on survival rates and SW performances of several salmonids [chinook salmon (*Oncorhynchus tshawytscha*) (Zaugg et al., 1983), rainbow trout (Perry et al., 2006; Salman and Eddy, 1990), Artic charr (*Salvelinus alpinus*) (Staurnes and Finstad, 2000), Atlantic salmon (Striberny et al., 2021)]. In rainbow trout, Perry et al. (2006) observed remodeling of the gill in fish fed with a salt-rich diet with the stimulation of SW-phenotype chloride cells, while in the intestine of Atlantic salmon, using the intestinal fluid composition of SW fish as an index, we previously demonstrated an improvement in the capacity to process the ingested SW following dietary treatment with salt mix and tryptophan (Gaetano et al., 2023). Although the diet had a more marginal effect than the photoperiod, here, we demonstrated that the experimental diet stimulates, already in FW and particularly in the anterior intestine, the transcript levels of some of the genes known to be involved in fish hypo-osmoregulatory mechanisms (e.g., *nkaα1b*, *nkaα1c, nkcc1, nkcc2*). As well as with salt, the diet used in this study was enriched with the amino acid tryptophan. The beneficial effects of tryptophan supplementation in the reduction of aggression and stress have been well documented in fish (Höglund et al., 2019; Hoseini et al., 2019); however, direct involvement of this essential amino acid on the hypo-osmoregulatory mechanisms in fish is still not well defined, and only a study in common carp (*Cyprinus carpio*) showed enhancement of SW tolerance in fish fed with a diet supplemented with tryptophan (Hoseini and Hosseini, 2010). Depending on extracellular Ca^{2+} concentrations, tryptophan, like other L-amino acids, activates the G-protein-coupled calcium-sensing receptor (CaSR) (Conigrave et al., 2007; Loretz, 2008), which supports calcium homeostasis and osmoregulation in fish (Gregório and Fuentes, 2018; Jury et al., 2021). Surprisingly, in this study, no effect of treatments was observed for the *casr* transcriptional state either in the anterior or the posterior intestine (Fig. 6A, B). Further studies would be needed to establish a direct role of the amino acid tryptophan in the osmoregulatory mechanisms of fish and the involvement of the CaSR activity in the osmoregulatory mechanisms in the intestine of Atlantic salmon.

The functioning of the basolateral NKA is the basis of the absorption mechanisms in the intestine of fish, both in terms of nutritional and osmoregulatory processes (Hedén et al., 2022; Kelly et al., 1999; Loretz, 1995). This study analyzed three isoforms of the α catalytic subunit of the NKA, *nkaα1a*, *nkaα1b*, and *nkaα1c*. Contrary to the observations in salmonid gills (Nilsen et al., 2007; Richards et al., 2003; Striberny et al., 2021), the mRNA levels of *nkaα1a* and *nkaα1b* in the intestine did not provide relevant information about the development of salinity tolerance in Atlantic salmon (Fig. 1A-D). However, the upregulation, already in FW, of the *nkaα1c* in the anterior intestine of the fish subjected to short photoperiod and even higher mRNA levels following SW transfer (Fig. 1E), as previously observed (Sundh et al., 2014; Tipsmark et al., 2010a), reaffirms the crucial role of the isoform α1c of NKA in the hypo-osmoregulation mechanism in the intestine of Atlantic salmon and suggests a putatively better preadaptation of the groups exposed to the short photoperiod.

A key pathway for the inward movement of ions through the apical membrane of the enterocytes is the NKCC2 cotransporter (Watanabe et al., 2011). Upregulation of *nkcc2* has been observed in the intestine of the Mozambique tilapia (Li et al., 2014), Japanese eel (*Anguilla japonica*) (Ando et al., 2014), and red drum (*Sciaenops ocellatus*) (Esbaugh and Cutler, 2016) following transfer to SW, emphasizing the central role of this cotransporter in teleosts SW adaptation. In this study, the observed increase in mRNA levels for *nkcc2* in the short photoperiod groups, particularly in the anterior intestine of both FW and SW fish (Fig. 2C), suggests a better preadaptation and the potential for improved SW performance for these fish, in agreement with our previous findings (Duarte et al., 2023; Gaetano et al., 2023; Striberny et al., 2021). Considering our results and the preparatory development of intestinal Nkcc2 demonstrated during smoltification (Duarte et al., 2023; Sundh et al., 2014), *nkcc2* emerges as a promising first potential molecular marker for evaluating hypo-osmoregulatory capacity in farmed Atlantic salmon.

Maintaining ionic and osmotic balance in teleost relies on Cl^- channels like CFTR. The essential role of CFTR in the intestine has been established in several SW-acclimated species, including killifish (*Fundulus heteroclitus*) (Marshall et al., 2002), sea bass (Bodinier et al., 2009), sea bream (Gregório et al., 2013), and Gulf toadfish (*Opsanus beta*) (Ruhr et al., 2014). In Atlantic salmon, two *cftr* isoforms are present (Chen et al., 2001), and both are expressed in the intestine (Sundh et al., 2014). *cftrI* and *cftrII* were modulated by the treatments used in this study (Fig. 4A-D), and both isoforms exhibited different mRNA abundance between intestinal regions (Table 3); however, contrary to the findings in the gill of Atlantic salmon (Nilsen et al., 2007; Singer et al., 2002) and in the intestine of other species as previously discussed, the results obtained in this study did not reveal changes in mRNA abundance consistent with an increased role of *cftr* isoforms in the intestinal hypo-osmoregulatory mechanisms of Atlantic salmon, suggesting the putative involvement of other pathways as the primary route for the Cl^- removal across the basolateral side of the enterocytes. Functional outcomes in anterior and posterior intestine suggested the presence of CftrI or CftrII in both apical and basolateral positions (Gaetano et al., 2023). A basolateral Cftr could be one of the pathways facilitating the exit of Cl^- into the bloodstream. Meanwhile, apical located Cftr, in combination with the basolateral Nkcc1, could be involved in Cl^- secretion as observed in mammals (Jakab et al., 2011), recycling chloride to the lumen, so NKCC2 cotransporter and Cl^-/HCO_3^- exchangers can use it to function and to move water inwards and bicarbonate to the lumen as imbibed SW processing progresses. The upregulation of *nkcc1* (Fig. 2A, B) may support the involvement of this secretory pathway in the short photoperiod groups occurring with a similar pattern observed for *cftrI*, particularly in the anterior intestine (Fig. 2A and 4A). The presence of two isoforms, differently regulated and expressed along the intestine, and the possible localization in either the apical or basolateral side of the enterocytes makes it challenging to understand the expression dynamics of Cftr isoforms. Immunohistochemistry studies are required to define the distribution of the two isoforms in the enterocytes and to clarify their role in developing the hypo-osmoregulatory capacities in Atlantic salmon.

A Na^+-independent route for Cl^- absorption in the enterocytes is represented by the exchangers belonging to the Slc26 family (Ohana et al., 2009). This intestinal mechanism, not yet explored in Atlantic salmon, plays a vital role in fish hypo-osmoregulation. It promotes water absorption from the intestinal fluid by facilitating the uptake of Cl^- in exchange for HCO_3^-, which in turn sustains the luminal precipitation of Ca^{2+} and Mg^{2+} as intestinal carbonates (Grosell et al., 2005; Grosell et al., 1999; Wilson and Grosell, 2003). In the present study, we investigated two of the anion exchangers of the Slc26 family: *slc26a3* and

slc26a6. Transcripts for both exchangers were detected in the anterior and posterior intestine (Fig. 3A-D), suggesting the involvement of both regions in the HCO_3^- secretion and carbonate precipitation. However, they exhibited a distinct regulation by the experimental treatments and region-specific patterns, which suggests a complementary role of *slc26a3* and *slc26a6* in the physiology of the two regions, that could also be associated with a different stoichiometry in the exchange of Cl^- and HCO_3^- (Grosell et al., 2009). Notably, a marked upregulation of *slc26a6* was observed in the anterior intestine of FW animals exposed to the short photoperiod (Fig. 3C), reaching levels matching those of SW fish and the posterior intestine (Fig. 3C, D). Given the relevant role of Cl^-/HCO_3^- exchangers in processing the ingested SW, this finding reinforces the beneficial effect of a short-day period in the production of Atlantic salmon. Moreover, it highlights *slc26a6* as a potential molecular marker in the anterior intestine of Atlantic salmon to evaluate the development of the hypo-osmoregulatory capacities during smolting-stimulation.

The intestinal epithelium of teleosts is leaky (Loretz, 1995), and it enables the passive movement of ions and molecules through tight junctions based on size and charge (Zihni et al., 2016). The specific expression of tight junction components and the molecular changes of transmembrane proteins such as occludins (Ocln) and claudins (Cldn) are suggested to be essential in the regulation of epithelium selectivity (Cummins, 2012; Günzel and Yu, 2013; Van Itallie and Anderson, 2006). In this study, we focused on examining *ocln* and *cldn15* (Fig. 5A-D), two of the tight junction components involved in regulating paracellular permeability in the intestine of Atlantic salmon (Tipsmark et al., 2010a; Tipsmark and Madsen, 2012). As observed for several transcellular pathways investigated in this study, *ocln* and *cldn15* were predominantly modulated in the anterior intestine and exhibited higher mRNA levels in the short photoperiod groups (Fig. 5A, C). Previous studies linked the upregulation of *ocln* and *cldn15* to a potential reduction in epithelial permeability in SW fish (Tipsmark et al., 2010a; Tipsmark and Madsen, 2012). However, our previous measurements of transepithelial resistance (R_t) revealed lower R_t in the groups exposed to the short photoperiod, suggesting higher permeability of the epithelium in both FW and SW (Gaetano et al., 2023), even in the posterior intestine that showed here stable levels of *ocln* and *cldn15* (Fig. 5B, D). The molecular results obtained in this study and previously obtained functional findings (Gaetano et al., 2023) suggest a complex remodeling of the paracellular pathway, particularly in response to photoperiod and salinity. This remodeling, however, may involve different tight junction components in the two regions not investigated in this study or may be related to differences in protein post-translational modifications.

Smoltification is an endocrine-regulated event, and several hormones act in concert to stimulate it, i.e., GH, cortisol, insulin-like growth factor-I (IGF—I), and thyroid hormones; prolactin (PRL), instead, is considered to have an inhibitory effect (McCormick, 2013). Prolactin plasma levels decrease during smoltification in Atlantic salmon (Prunet et al., 1989), as well as the transcript levels of prolactin receptors (*prlr*) in the gill (Kiilerich et al., 2007a). But the dynamics of the *prlr* in the intestine are still unknown. Considering this and the inhibitory action of prolactin on the intestinal bicarbonate secretion demonstrated in the sea bream (Ferlazzo et al., 2012), we investigated *prlr* in the anterior and posterior intestine. The mRNA levels of *prlr* were marginally regulated by the treatments used in this study (Fig. 6C, D). Only in the anterior intestine of FW fish *prlr* was downregulated in the groups exposed to continuous light, which showed the lowest levels (Fig. 6C). In contrast, the other groups showed entirely unchanged mRNA levels. Being prolactin a "FW-adapting hormone" (Breves et al., 2020a), higher *prlr* levels would be expected in FW than in SW; however, our results did not reveal a particular regulation, but they are in line with previous studies in the gill of Atlantic salmon where unchanged levels of *prlr* were observed after SW transfer (Nilsen et al., 2008), and even higher levels were reported during 24 h SW challenge through the smoltification period (Kiilerich et al., 2007a). Nilsen et al. (2008) observed lower *prlr* levels after one month in SW and suggested high flexibility of the osmoregulatory system during early SW acclimation.

This study represents a comprehensive investigation into the molecular mechanisms occurring in the intestine of Atlantic salmon reared under different photoperiods and dietary treatments intended to stimulate smoltification and optimize SW performance. In summary, the results provide evidence that the exposure to a winter signal, followed by an increase in day length, plays a critical role in the efficient activation of the molecular mechanisms linked with the hypo-osmoregulatory role of the intestine, and demonstrate the synergetic action of the short photoperiod with the transition diet, though this last cannot be the sole stimulating factor. Moreover, the different transcriptional profile obtained between anterior and posterior intestine reflects the coordinated and complementary role of these two regions in SW processing.

The apical $Na^+/K^+/2Cl^-$ cotransporter, *nkcc2,* and the apical Cl^-/HCO_3^- exchanger, *slc26a6,* emerge as potential FW molecular markers in the anterior intestine of Atlantic salmon, offering a means to evaluate smolt hypo-osmoregulatory capacities. Indeed, this last finding requires additional validation to understand the expression dynamic and functioning of Slc26a6 during the entire smoltification period and following SW transfer. While the expression of Nkcc2 during natural and artificial smoltification, as well as during SW acclimation, has been described (Duarte et al., 2023; Sundh et al., 2014), very little is known about Slc26a6 and the associated bicarbonate secretion, in the intestine of Atlantic salmon. Nevertheless, these results represent a significant initial stride towards a potential tool for enhancing fish welfare and optimizing aquaculture practices.

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CRediT authorship contribution statement

Pasqualina Gaetano: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Vilma Duarte:** Writing – review & editing, Methodology, Investigation. **Anja Striberny:** Writing – review & editing. **David G. Hazlerigg:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Even H. Jørgensen:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization. **Marco A. Campinho:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Juan Fuentes:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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