



INTESTINAL PHYSIOLOGY IN ATLANTIC SALMON: PREPARATORY CHANGES DURING SMOLTING AND AFTER SEAWATER ENTRY

Doctoral Thesis
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INTESTINAL PHYSIOLOGY IN ATLANTIC SALMON: PREPARATORY CHANGES DURING SMOLTING AND AFTER SEAWATER ENTRY

Memoria presentada por D^a Pasqualina Gaetano para optar al
Grado de Doctor en Ciencias

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*To my four siblings,
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Figure 4.12. Heatmap summarizing the relative mRNA levels of the genes investigated in the anterior and posterior intestines of parr and post-smolt Atlantic salmon (*Salmo salar*) **169**

LIST OF ABBREVIATIONS AND ACRONYMS

18S	18S ribosomal RNA
28S	28S ribosomal RNA
Actb	beta actin
ADP	adenosine diphosphate
AEs	anion exchangers
ANOVA	analysis of variance
Aqp(1/1a/8/10)	aquaporin (1/1a/8/10)
Arpc1b	actin-related protein 2/3 complex subunit 1b
ATP	adenosine triphosphate
Atp6v1a	vacuolar H ⁺ -ATPase subunit A
B2m	beta-2-microglobulin
BCA	bicinchoninic acid
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
BUM	bumetanide
BW	brackish water
CA	carbonic anhydrase
CA-IV	carbonic anhydrase IV
CAc	cytosolic carbonic anhydrase
cAMP	3',5'-cyclic adenosine monophosphate
CaSR	calcium-sensitive receptors
cDNA	complementary deoxyribonucleic acid
CFTR	cystic fibrosis transmembrane conductance regulator
Clc(2/3/7)	chloride channel (2/3/7)
Cldn(2/10e/15/25b/27a/30)	claudin (2/10e/15/25b/27a/30)
C _q	quantification cycle
DIDS	4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid
DMSO	dimethyl sulfoxide
E	qPCR efficiency
<i>e.g.</i>	<i>exempli gratia</i> (for example)
EDTA	ethylenediaminetetraacetic acid
Ef1 α	elongation factor 1 alpha
EST	expressed sequence tag
FK	forskolin
FW	freshwater
GC-C	guanylyl cyclase-C
GFR	glomerular filtration rate
GH	growth hormone
GIT	gastrointestinal tract
GN	guanylin
Gr1	glucocorticoid receptor 1
Gr2	glucocorticoid receptor 2
HCTZ	hydrochlorothiazide
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HPI	hypothalamic-pituitary-interrenal
<i>i.e.</i>	<i>id est</i> (in other words)
IBMX	3-isobutyl-1-methylxanthine

IF	intestinal fluid
IGF-I	insulin-like growth factor-1
I_{sc}	short-circuit current
J_v	fluid transport
K	condition factor
KCC	K^+/Cl^- cotransporter
Kirc2.1	potassium inwardly rectifying channel 2
L_f	fork length
LL-LL	continuous light groups
L_t	total length
M_b	body mass
Mr	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
Nbc1	Na^+/HCO_3^- cotransporter 1
NCBI	National Center for Biotechnology Information
NCC	Na^+/Cl^- cotransporter
ND	not determined
NHE(2/3)	Na^+/H^+ exchanger (2/3)
NKA	Na^+/K^+ -ATPase
Nka α 1(a/b/c)	Na^+/K^+ -ATPase α subunit 1 (a/b/c)
NKCC(1/2)	$Na^+/K^+/2Cl^-$ cotransporter (1/2)
NTC	no-template controls
Ocln	occludin
Osm	osmolality
p	p-value
PCR	polymerase chain reaction
PKA	protein kinase A
PL	plasma
PRL	prolactin
Prlr	prolactin receptor
PST	parr-smolt transformation
PTHrP	parathyroid hormone-related protein
qPCR	quantitative real-time PCR
R^2	correlation coefficient
RAS	recirculating aquaculture systems
RNA	ribonucleic acid
R_t	transepithelial resistance
RT	room temperature
SEI	sucrose-EDTA-imidazole
SEM	standard error of the mean
SGLT1	Na^+ /glucose cotransporter
SGR	specific growth rate
Slc4a4	solute carrier family 4 member 4
Slc9a(2/3)	solute carrier family 9 member (2/3)
Slc12a(1/3)	solute carrier family 12 member (1/3)
Slc26a(3/6)	solute carrier family 26 member (3/6)
SP-LL	short photoperiod groups
SQ	starting quantity
STC	stanniocalcin

SW	seawater
T ₃	triiodothyronine
T ₄	thyroxine
T _a	annealing temperature
tBLASTn.....	translated basic local alignment search tool nucleotides
TEP	transepithelial potential
THs.....	thyroid hormones
Ubp2.....	ubiquitin-associated protein 2
VHA.....	V-type H ⁺ -ATPase

NOTE:

Gene symbols are italicized, with all letters in lowercase. Protein symbols are not italicized, and the first letter is uppercase, with the exception of acronyms e.g. NKA (Na⁺/K⁺-ATPase).

RESUMEN GENERAL

La fisiología intestinal de los peces teleósteos es compleja, y existen múltiples factores que contribuyen a una función osmorreguladora óptima. Esta complejidad es particularmente pronunciada en especies migratorias que de forma natural experimentan variaciones en la salinidad ambiental. En consecuencia, en la presente tesis se aplicó un enfoque multifactorial, considerando factores biológicos y ambientales que de alguna manera pueden contribuir al desarrollo de la capacidad hipo-osmorreguladora en el intestino del salmón del Atlántico durante la transformación de parr a smolt (esmoltificación). Un proceso que precede a la migración de los juveniles hacia el océano. Utilizando un enfoque integrador, el trabajo tuvo como objetivo principal investigar el desarrollo de los mecanismos de transporte iónico asociados con el transporte de Na^+ y Cl^- a través del epitelio intestinal, crucial para la absorción de agua en un entorno deshidratante como el agua de mar. Con este propósito, se recrearon condiciones de acuicultura y crecimiento natural en el medio ambiente (luz). Los resultados sugieren un papel crítico de la señal del fotoperiodo en el desarrollo de la capacidad hipo-osmorreguladora intestinal y destacan al cortisol como un modulador endocrino crucial de la función osmorreguladora en el intestino del salmón Atlántico. También se investigó la cascada de secreción de HCO_3^- intestinal, que previamente había pasado por inadvertida en los estudios de fisiología intestinal de esta especie, y se obtuvieron resultados novedosos, que incluyen evidencias sobre la acción reguladora mediada por el cortisol en esta vía. Además, en las etapas de vida examinadas en esta tesis (parr, smolt y post-smolt), se estableció una clara especialización anterior-a-posterior del tejido intestinal. Asimismo, los resultados ofrecen evidencia de una vía secretora (de aniones) en el intestino del salmón aclimatado al agua de mar. En general, los resultados presentados en esta tesis brindan nuevas perspectivas sobre la fisiología intestinal del salmón del Atlántico y resaltan la función intestinal como una herramienta con potencial para la acuicultura. Una evaluación más precisa de la capacidad hipo-osmorreguladora en los smolts indudablemente sería ventajosa para optimizar la producción, y tendría implicaciones económicas, y consecuentemente, sociales y ambientales.

GENERAL ABSTRACT

The intestinal physiology of teleost fishes is complex, and multiple factors contribute to an optimal osmoregulatory function. This complexity is particularly pronounced in migratory species that experience variations in environmental salinity. Consequently, this thesis employed a multifactorial approach, considering biological and environmental factors that may contribute to the development of hypo-osmoregulatory capacity in the intestine of Atlantic salmon during the parr-smolt transformation (smolting or smoltification) that precedes migration of juveniles to the ocean. Utilizing an integrative approach, the work primarily aimed to investigate the development of the mechanisms associated with Na^+ and Cl^- movement across the intestinal epithelium, crucial for water absorption in the dehydrating environment of SW. For this purpose, both aquaculture and natural on-growing conditions were recreated. The results suggest a critical role of the photoperiodic cue in developing the intestinal hypo-osmoregulatory capacity and highlight cortisol as a crucial modulator of the osmoregulatory function in the intestine of Atlantic salmon. The intestinal HCO_3^- secretion cascade pathway, previously overlooked in the physiology of this species, has also been investigated. Novel results were obtained, including evidence about the regulatory action mediated by cortisol on this pathway. A clear anterior-to-posterior specialization of the intestine also emerged in the life stages examined in this thesis (parr, smolt, and post-smolt). Furthermore, the results offer evidence of a secretory pathway in the intestine of Atlantic salmon acclimated to SW. Overall, the findings presented in this thesis provide new insights into the intestinal physiology of Atlantic salmon and highlight the intestinal function as a potential tool for aquaculture. A more precise evaluation of the hypo-osmoregulatory capacity in smolts would undoubtedly be advantageous for optimizing the production, with economic, social, and environmental implications.

CHAPTER

1

General introduction

1.1. Ionic and osmotic regulation in teleosts

1.1.1. Ionic and osmotic imbalance

Teleosts constitute the largest and most diverse group of vertebrates, with over 29,500 known species (Nelson *et al.*, 2016). They are distributed worldwide in habitats characterized by different salinities, including freshwater (FW), seawater (SW), ion-poor water, brackish water, and hypersaline water.

Regardless of the specific habitat characteristics, teleosts are immersed in a medium with ionic and osmotic properties that significantly differ from their extracellular body fluids. These differences create an ionic and osmotic gradient between the two compartments (body and environment), primarily across the gill. Indeed, the gill's structure, which enhances gas exchanges, simultaneously exposes a large body area to osmotic and ion movements between the environment and the extracellular fluids (Falanghe Carneiro *et al.*, 2007; Grosell, 2014).

The osmotic gradient between the fish body and the environment is detected by osmosensors and transduced by intracellular signaling pathways that trigger the set of cellular responses required to maintain constant the electrolytes composition of intra- and extracellular fluids (e.g. K^+ , Na^+ , Cl^- , Mg^{2+} , Ca^{2+} , Zn^{2+}) to prevent variations in cellular volume and macromolecules damages that would compromise cellular functions (Evans and Kültz, 2020; Takei and Hwang, 2016). This link between the environment and the fish, along with a complex endocrine system, directs epithelial transporters and permeability in the osmoregulatory organs, ensuring whole body homeostasis. All teleosts are, indeed, ion- and osmo-regulators and they employ two distinct osmoregulatory strategies: hyper-osmoregulation in dilute environments and hypo-osmoregulation in SW (Fig. 1.1). Both strategies foresee the coordinated but distinctive functioning of the osmoregulatory organs, particularly the gill, gastrointestinal tract (GIT), and kidney, as discussed in more detail in the sections below. Though believed to play a minor and species-specific role, skin, urinary bladder, and operculum are also implicated in the osmoregulatory mechanisms of teleosts (Marshall and Grosell, 2006).

1.1.2. Osmoregulation in freshwater

The extracellular fluids of FW teleosts are hyper-osmotic compared to the surrounding environment, causing an osmotic tendency for water gain and diffusive

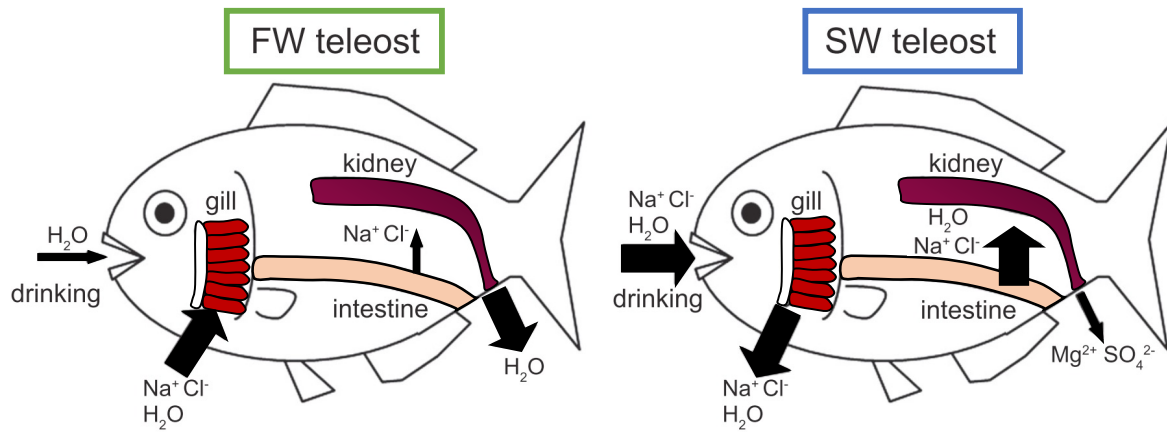


Figure 1.1. Schematic representation of ion and water movement in FW and SW teleosts (Modified from: Takei *et al.*, 2014).

ion loss principally across the large gill epithelium. However, hyper-osmotic mechanisms allow fish to counteract this tendency by the active uptake of ions across the gills and from the diet while excreting large volumes of highly dilute urine (Marshall and Grosell, 2006). Osmoregulatory mechanisms in FW are complex, and many differences can be observed among species, making difficult the conceptualization of models like in SW fish. This variability is likely due to the high heterogeneity of the FW habitats (ion composition, pH, hardness, etc.), and to evolutionary history. FW mechanisms may be evolved many times in different ways (Edwards and Marshall, 2013; Hwang and Lin, 2014).

In FW, the diffusive ions loss is compensated by the active uptake of ions from the environment, mainly Na⁺ and Cl⁻, across the tighter gill epithelium of FW teleosts (Evans *et al.*, 2005; Hwang and Hirano, 1985). Multiple subtypes of ionocytes with distinct characteristics and transport capacities were identified in FW teleosts and differences also appear to be species-specific (Dymowska *et al.*, 2012; Galvez *et al.*, 2002; Hiroi *et al.*, 2005; Hwang, 2009). In zebrafish (*Danio rerio*), for instance, four subtypes of ionocytes have been identified, each with putatively distinct functions for Na⁺, Cl⁻, and Ca²⁺ uptake, K⁺ secretion, and acid-base balance (Abbas *et al.*, 2011; Hwang, 2009).

To counteract the water inward trend, ingestion of water is limited in FW teleosts and associated with feeding events (Eddy, 2007). Most osmoregulatory studies have been conducted on fasted fish, which limits the knowledge about the contribution of the GIT to osmoregulation mechanisms in FW fish. However, growing evidence

suggest an important contribution of the diet in the ion homeostasis in FW teleosts, depending on the species and medium characteristics. In rainbow trout (*Oncorhynchus mykiss*), following a meal of a commercial feed, net absorption of Na^+ along the GIT was negligible, while significant net absorption of Cl^- (81%), K^+ (89%), Ca^{2+} (28%), and Mg^{2+} (60%) was observed (Bucking and Wood, 2007, 2006). Moreover, some species, such as mummichog (*Fundulus heteroclitus*) and bluegill (*Lepomis macrochirus*), show minimum or absent capacity for Cl^- uptake through the gill, making dietary uptake the primary or even only source of Cl^- (Patrick *et al.*, 1997; Tomasso and Grosell, 2005).

The kidney has a central role in maintaining body homeostasis in FW. Through the kidney, water is removed, and ions are reabsorbed. The high glomerular filtration rates (GFR $\sim 4\text{--}16\text{ mL/kg/h}$), together with the low tubular reabsorption of water and the high ion reabsorption, lead to the production of large amounts of dilute urine ($\sim 20\text{--}50\text{ mOsm/L}$) (Takvam *et al.*, 2021). Up to 97% of NaCl from the primary urine is reabsorbed (Martinez, 2017) leaving behind concentrations of Na^+ and Cl^- below 20 mM (Hickman and Trump, 1969). Divalent ions (Ca^{2+} , Mg^{2+} , and SO_4^{2-}), as well as K^+ , HCO_3^- , and a small percentage of water are also reabsorbed (Beyenbach, 2000; Takvam *et al.*, 2021).

1.1.3. Osmoregulation in seawater

In SW, teleosts encounter the opposite osmotic challenge than in FW. Their extracellular fluids are hypo-osmotic compared to the environment, and as a consequence, fish tend to lose water osmotically mainly through the gill. Dehydration is counteracted by ingesting SW and subsequent NaCl-coupled water absorption in the intestine. Additionally, small volumes of isotonic urine are excreted to minimize water loss. The salt load is then eliminated mainly across the branchial epithelium (Marshall and Grosell, 2006).

Osmoregulatory mechanisms in the gill of SW teleosts are better understood than in FW species. The current SW model identified only one type of ionocytes with similar set of transporters among teleosts (Evans *et al.*, 2005). Ionocytes form multicellular complexes with other ionocytes and accessory cells with a common apical crypt (Kato *et al.*, 2001; Sardet *et al.*, 1979). Through these complexes, the excess of ions absorbed from the ingested SW (mainly Na^+ and Cl^-) is removed. The apical

exit of Cl^- through secondary active transport generates a serosa positive transepithelial potential across the branchial epithelium, which drives the paracellular exit of Na^+ through the leaky tight junctions located between accessory cells and ionocytes (Edwards and Marshall, 2013; Evans *et al.*, 2005).

In SW teleosts, the GIT plays a central role in water balance. To offset the osmotic loss of water, teleosts actively drink SW with rates in the order of 1 to 5 ml/kg/h (Fuentes and Eddy, 1997; Marshall and Grosell, 2006; Perrott *et al.*, 1992). The ingested SW is then processed along the GIT where >95% of NaCl is absorbed (Grosell, 2014) followed by up to 85% of water (Genz *et al.*, 2008; Wilson *et al.*, 1996). SW processing starts in the esophagus with active and passive absorption of Na^+ and Cl^- . The esophageal epithelium is barely permeable to water and other ions so there is a great reduction of osmolality of the imbibed SW allowing subsequent absorption of water in the intestine (Esbaugh and Grosell, 2014; Hirano and Mayer Gostan, 1976; Parmelee and Renfro, 1983). After minor processing in the stomach, mostly dilution following gastric secretion, the ingested SW arrives in the intestine with a greatly reduced osmolality. Combined absorption and secretion processes in the intestine allow further osmolality reduction and NaCl-coupled water absorption via transcellular and/or paracellular pathways (Grosell, 2014; Madsen *et al.*, 2015a). A minor fraction of the ingested SW is excreted as rectal fluid characterized by low concentrations of Na^+ and Cl^- and high levels of Mg^{2+} , SO_4^{2-} and HCO_3^- (Genz *et al.*, 2008; Grosell, 2014). High levels of Mg^{2+} and SO_4^{2-} are due to the selective absorption of Na^+ , Cl^- and K^+ and water, while high HCO_3^- is the consequence of the secondary active secretion in the intestine (Grosell, 2006). Despite the concentration gradient across the intestinal epithelium, Mg^{2+} and SO_4^{2-} are absorbed at very low levels and the excess is eliminated by the kidney (Beyenbach, 2004; Grosell and Taylor, 2007; Hickman, 1968). The intestinal absorption of Ca^{2+} is also limited, and the Ca^{2+} imbibed with SW is mainly excreted as CaCO_3 precipitates, together with a fraction of Mg^{2+} as MgCO_3 precipitates (Perry *et al.*, 2011; Walsh *et al.*, 1991).

The role of the kidney in SW fish is more limited than in FW, but certainly it limits water loss and contributes to excrete part of the ions absorbed, especially Mg^{2+} and SO_4^{2-} (Beyenbach, 2004). SW kidney shows GFR ~0.5-2.0 ml/kg/h, much lower than in FW (Takvam *et al.*, 2021). Due to the absence of Henle's loop, the fish kidney is incapable of producing hypertonic urine like mammals. However, urine is made

almost isotonic to the plasma by the reabsorption of water coupled with NaCl, and the secretion of ions excreted in a small volume of urine (Beyenbach, 2004; Hickman and Trump, 1969). Mg^{2+} , SO_4^{2-} and Cl^- are the major components, but also some Ca^{2+} and Na^+ are present (Hickman and Trump, 1969; Takvam *et al.*, 2021).

1.1.4. Stenohaline and euryhaline species

Salinity is a major environmental factor exerting selective pressure on aquatic organisms. The ability of teleosts species to thrive in different habitats depends on their ability to cope with the salinity of the surrounding medium (Falanghe Carneiro *et al.*, 2007). Based on the capacity of adaptation to different salinity levels, teleosts, and the other aquatic animals are classified as stenohaline and euryhaline. Most teleosts are stenohaline, as they are restricted to specific habitats due to their ability to survive within a limited range of salinity. For example, the FW stenohaline goldfish (*Carassius auratus*) and the SW stenohaline tiger puffer (*Takifugu rubripes*). In contrast, a small percentage of teleosts (~1%) are classified as euryhaline and can successfully adapt and survive in a wider range of environmental salinity.

Euryhalinity is a physiological phenotype distributed in different orders of fishes, that represents an evolutionary advantage (Schultz and McCormick, 2013). Euryhaline fishes, indeed, include species that can tolerate frequent and/or high salinity variations, as in the case of intertidal and estuarine species like tidepool sculpin (*Oligocottus maculosus*) and Nile tilapia (*Oreochromis niloticus*), but also migratory species, such as the anadromous Atlantic salmon (*Salmo salar*), and the catadromous European eel (*Anguilla anguilla*), which move between FW and SW at specific moments of their life cycle associated with reproduction or foraging. Euryhaline fishes also encompass species capable of adapting to extreme environments, such as hypersaline, alkaline, and ion-poor waters (Brauner *et al.*, 2013).

Euryhaline species can remodel their osmoregulatory mechanisms to meet new or changing environments through the highly integrated functioning of osmosensors, osmoregulatory organs, and the endocrine system. When salinity conditions change, many structural and functional adaptations occur in the osmoregulatory organs. While these changes can be species-specific, they generally include alterations in drinking rates, water absorption capacity in the intestine, and regulation in the expression of several key ion transporters in osmoregulatory epithelia.

1.2. The intestine and its osmoregulatory function in seawater

1.2.1. Morphology

The intestine is a unique organ capable of performing secretory and absorptive functions. Its morphology can considerably change in fish in relation to the species, and depending on diet habits, salinity, developmental stage, and health (Whittamore, 2012). In gastric species, the intestine starts after the pyloric sphincter. Depending on the species, it is possible to identify a variable number of blind-ended appendages, the pyloric caeca, as well as discrete intestinal regions (proximal/anterior, middle, distal/posterior, and rectum) (Olsson, 2011; Wilson and Castro, 2011). The intestine is formed of several tissue layers, of which the mucosa (comprising the columnar epithelium, lamina propria, and muscularis mucosa) is the one in direct contact with the lumen and responsible for several functions, including ion and fluid movements (Banan Khojasteh, 2012; Olsson, 2011). The principal cellular type in the intestinal epithelium is the enterocyte (Wilson and Castro, 2011). The enterocytes are polarized cells, with microvilli forming a characteristic brush border at the apical side, specialized for the transport of molecules across the epithelium (Fig. 1.2). The apical side of enterocytes faces the intestinal lumen, while the basolateral side interfaces with the circulation. Adjacent enterocytes are connected at the apical edge by tight junctions, the structure of which defines the epithelium's paracellular selectivity, and present in between a hyper-osmotic compartment in the lateral intercellular space (lis) (Larsen *et al.*, 2009).

1.2.2. Na^+/K^+ -ATPase

The Na^+/K^+ -ATPase (NKA) is a central element in the osmoregulatory mechanisms of SW teleosts. NKA is an electrogenic pump that extrudes 3Na^+ in exchange for 2K^+ , establishing a strong cytosolic-negative membrane potential and low Na^+ intracellular concentrations (Skou and Esmann, 1992).

In the intestine, the basolateral NKA drives the ion movement across the epithelium, energizing the absorption Na^+ , Cl^- and K^+ and affecting HCO_3^- secretion, as revealed by functional studies employing serosal application of ouabain, a specific NKA inhibitor (Grosell and Genz, 2006; Trischitta *et al.*, 1992). The key role of the NKA in the processing of the ingested SW is further demonstrated by the increase in NKA

expression and activity frequently observed in the intestine of euryhaline fishes when transferred from FW to SW (Barany *et al.*, 2020; Fuentes *et al.*, 1997; Sundh *et al.*, 2014).

1.2.3. Na⁺, Cl⁻ and K⁺ transport

The intestine of SW fishes (stenohaline and euryhaline species) is the major site of water absorption, which is linked to Na⁺ and Cl⁻ uptake from the intestinal fluid (Ando *et al.*, 2003; Grosell, 2014). The electrochemical gradient generated by the NKA energizes the absorption of Na⁺, Cl⁻, and K⁺ by the two parallel apical cotransporters Na⁺/K⁺/2Cl⁻ (NKCC) and Na⁺/Cl⁻ (NCC). The absorptive NKCC in the intestine of fishes is the apical NKCC2 (Slc12a1). Removing four ions from the lumen (1Na⁺:1K⁺:2Cl⁻), NKCC2 efficiently supports water uptake in hyper-osmotic environment (Musch *et al.*, 1982). *slc12a1* transcript levels increase in euryhaline teleosts when transferred from FW to SW (Esbaugh and Cutler, 2016; Seale *et al.*, 2014). The functioning of NKCC2 relies on the presence of K⁺, which is present at low concentrations in SW compared with Na⁺ and Cl⁻. K⁺ is likely recycled through apical K⁺ channels, of which Kir2.1 is a potential candidate in the eel intestine (Takei, 2021). The involvement of the NCC cotransporter (Slc12a3) in the intestinal osmoregulatory mechanisms is species-specific and region-dependent (Cutler and Cramb, 2008; Li *et al.*, 2014; Madsen *et al.*, 2015b). In eel, *slc12a3* mRNA levels increase in the more distal regions of the intestine, particularly in the rectum, where inhibitions of NCC by the hydrochlorothiazide (HCTZ) reduces Na⁺, Cl⁻ and water absorption in sac preparations of SW animals (Watanabe *et al.*, 2011).

Na⁺ uptake across the apical membrane may also involve the Na⁺/H⁺ exchangers (NHE) belonging to the Slc9 solute carrier family, specifically, the NHE2 (Slc9a2) and NHE3 (Slc9a3) (Gawenis *et al.*, 2002). In rainbow trout, *slc9a3* mRNA levels increased in the anterior intestine after exposure to 65% SW (Grosell *et al.*, 2007). However, no changes after transfer to SW were observed in the eel intestine for both *slc9a2* and *slc9a3*, and in Gulf toadfish (*Opsanus beta*), their abundance was very low or absent in SW fish, suggesting interspecies variability (Esbaugh and Grosell, 2014; Takei, 2021).

In the intestine of SW fish, Cl⁻ absorption takes place at higher rates than Na⁺ (Grosell, 2006). *In vitro* experiments revealed the presence of a Na⁺-independent pathway for Cl⁻ uptake, which relies on apical anion exchangers (AEs) from the Slc26

solute carrier family, facilitating the absorption of Cl^- in exchange for HCO_3^- (Grosell *et al.*, 2001, 1999).

Across the basolateral membrane, Na^+ exits the enterocytes through the NKA, while Cl^- can exit via channels like the cystic fibrosis transmembrane regulator (CFTR) (Marshall *et al.*, 2002) and Clc channels like Clc2, Clc3, and Clc7 (Sundh *et al.*, 2014; Takei, 2021). Additionally, Cl^- can leave the enterocyte via basolateral K^+/Cl^- co-transport, which is also a potential pathway for K^+ recycling (Halm *et al.*, 1985; Takei, 2021).

1.2.4. Intestinal bicarbonate secretion and carbonate precipitation

The intestinal bicarbonate (HCO_3^-) secretion is a common feature among all marine teleosts and is essential for osmoregulation in SW (Grosell, 2006). The pathways implicated in the HCO_3^- secretion cascade contribute to a reduction in the osmotic pressure of the intestinal fluid by up to 190 mOsm Kg^{-1} enabling additional water absorption in the intestine of SW-acclimated fish (Grosell and Oehlert, 2023).

HCO_3^- secretion via apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers was suggested by early studies (Ando and Subramanyam, 1990; Shehadeh and Gordon, 1969) and later confirmed and characterized by functional and molecular approaches (Alves *et al.*, 2019; Grosell *et al.*, 2005, 1999; Kurita *et al.*, 2008). The two members of the Slc26 family proposed to mediate the absorption of Cl^- in exchange for HCO_3^- in the teleost enterocytes are the Slc26a3 and the Slc26a6 (Gregório *et al.*, 2013; Grosell *et al.*, 2009a; Kurita *et al.*, 2008; Ruiz-Jarabo *et al.*, 2017). The secreted HCO_3^- originates from the hydration reaction of the endogenous CO_2 , catalyzed by the action of the cytosolic carbonic anhydrase (CAc), as well as from extracellular HCO_3^- entering the enterocyte through the basolateral $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC1, Slc4a4) (Grosell and Genz, 2006; Taylor *et al.*, 2010).

In SW-acclimated fish, the apical V-type H^+ -ATPase (VHA) is relevant for the intestinal physiology (Grosell *et al.*, 2009b). VHA extrudes H^+ accumulated in the enterocyte during the CO_2 hydration reaction, preventing cytosol acidification, and facilitating the AEs functioning despite the high luminal HCO_3^- concentration. Facilitated by the activity of the membrane-bound carbonic anhydrase IV (CA-IV) and possibly other CA isoforms (Grosell *et al.*, 2009a), H^+ titrates HCO_3^- . The titration reaction reduces the HCO_3^- gradient favoring secretion and produces CO_2 , which can

diffuse back into the enterocytes and undergo hydration to form HCO_3^- available for the AEs (Grosell *et al.*, 2007; Guffey *et al.*, 2011).

The high secretion rates of HCO_3^- in SW teleosts result in high luminal HCO_3^- concentration ($\sim 70 \text{ mmol L}^{-1}$) and alkalinization of the intestinal fluid (up to pH 9 in some species) (Gregório *et al.*, 2013; Grosell *et al.*, 2001; Wilson *et al.*, 2002). These conditions lead to the precipitation of Ca^{2+} and Mg^{2+} , present in the ingested SW and concentrated along the GIT, as insoluble carbonate precipitates (ichthyocarbonates) (Grosell and Oehlert, 2023; Walsh *et al.*, 1991). Approximately 30 to 65% of the ingested Ca^{2+} is precipitated as CaCO_3 , while a small and variable fraction of the Mg^{2+} precipitates as MgCO_3 (Marshall and Grosell, 2006; Perry *et al.*, 2011; Walsh *et al.*, 1991). Precipitation reaction remove ions from the intestinal fluid, thus promoting water absorption. Carbonate precipitation results from favorable chemical conditions within the intestine of SW fish. Still, recent studies in Gulf toadfish and a few other marine teleost species have also outlined the participation of an organic matrix, composed of ~ 150 proteins, in the regulation of Ca(Mg)CO_3 precipitation in the fish intestine (Schauer *et al.*, 2018, 2016).

The significance of the carbonate precipitates goes beyond the strictly physiological aspects of fish. Once expelled along with the rectal fluid, they become integral part of the global carbon cycle, contributing 3-15% of the total ocean carbonate production (Wilson *et al.*, 2009).

1.2.5. Water movement

The intestine is the primary site for water absorption in SW teleosts. Present models of water absorption include both transcellular and paracellular pathways. However, the specific routes of water movement across the intestinal epithelium and the relative contribution of each are yet not fully elucidated (Thiagarajah and Verkman, 2018; Whittamore, 2012).

The transcellular route may involve diffusion through the lipid bilayer, transport with ions and nutrients (e.g., Na^+ /glucose cotransporter – SGLT1), and passage through the water channels aquaporins (Loo *et al.*, 2002; Preston *et al.*, 1992; Sundell and Sundh, 2012). Aquaporins represent a rapid and selective route for the transcellular movement of water in both apical and basolateral membranes (Cerdà and Finn, 2010). Of the many aquaporins identified in fishes, Aqp1, Aqp8, and Aqp10 were identified as potentially relevant pathways for water movement in the intestines of SW-

acclimated fish. Their expression is upregulated in numerous euryhaline species, such as Atlantic salmon (Engelund *et al.*, 2013; Tipsmark *et al.*, 2010b), European eel (Martinez *et al.*, 2005), Japanese eel (*Anguilla japonica*) (Aoki *et al.*, 2003; Kim *et al.*, 2010), sea bass (*Dicentrarchus labrax*) (Giffard-Mena *et al.*, 2007), silver sea bream (*Sparus sarba*) (Deane *et al.*, 2011).

Movement of ions and molecules, including water, across the intestinal epithelium can occur via the paracellular pathway, depending on charge and size (Zihni *et al.*, 2016). The permeability of this pathway is proposed to be regulated by the structure of the tight junctions, particularly by the presence of channel-forming claudins. Specifically, claudin-2 (Cldn2) was suggested to function as water channel, facilitating the transport of water across leaky epithelia (Rosenthal *et al.*, 2010).

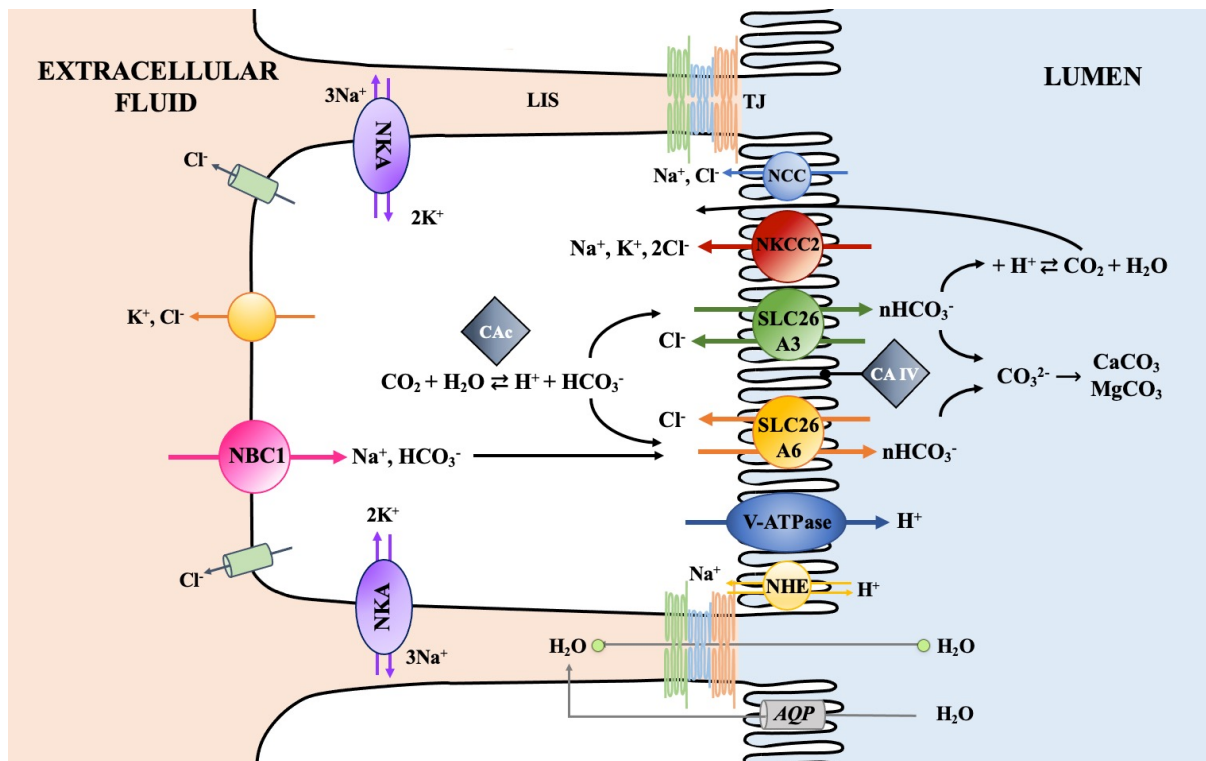


Figure 1.2. Schematic representation of ion and water movement across SW fish enterocytes (Readaptation from: Grosell 2014 and Sundell and Sundh 2012).

1.2.6. Endocrine regulation

The endocrine regulation of the hyper-osmoregulatory mechanisms in teleost intestine is complex. Ions and water uptake across the intestinal epithelium of SW teleosts are regulated by several hormones, e.g. somatostatin-related peptides, neuropeptide Y, urotensin II, and catecholamines. Others, instead, have an inhibitory

action, like natriuretic peptides, guanylin, vasointestinal peptide, acetylcholine, serotonin and histamine (Takei and Loretz, 2011). Of these, guanylin (GN) is a peculiar hormone. The binding of GN to its guanylyl cyclase-C (GC-C) receptor activates apical CFTR, stimulating Cl^- and the subsequent fluid secretion, considered counterproductive in SW fish (Takei and Loretz, 2011). Functional studies in the intestinal epithelium of eel demonstrated the inhibitory action of GNs on the intestinal ion absorption (Yuge and Takei, 2007). However, the expression of GNs and GC-C is upregulated in SW eels compared with FW animals (Yuge *et al.*, 2003). It has been proposed that the activation of CFTR by GN could provide Cl^- for the functioning of NKCC2 and $\text{Cl}^-/\text{HCO}_3^-$ exchanger to ensure ion absorption and aggregate formation in case of Cl^- deficit in the intestinal fluid (Takei and Loretz, 2011). Also, the stimulation of fluid secretion by GN has been proposed to facilitate the excretion of solid, including carbonate precipitates, from the intestine (Ruhr *et al.*, 2014).

Regulation of HCO_3^- secretion and aggregates precipitation comes from calciotropic hormones like parathyroid hormone-related protein (PTHrP) and stanniocalcin (STC). *In vivo* experiments in sea bream (*Sparus aurata*) showed the role of these hormones on the carbonate aggregates formation and demonstrated the inhibitory role of the hyper-calcemic PTHrP and the stimulatory role of the hypo-calcemic STC (Gregório *et al.*, 2014).

Prolactin (PRL), growth hormone (GH), and cortisol, classified as slow-acting hormones are all involved in the intestinal osmoregulatory mechanisms (Takei and McCormick, 2013). PRL is considered the “FW-hormone” due to its action on promoting phenotypes supportive of FW acclimation and to inhibit SW phenotypes (Breves *et al.*, 2020). Functional studies in the intestine of rainbow trout and Japanese eel, showed that exogenous PRL reduced Na^+ , Cl^- and fluid absorption (Morley *et al.*, 1981; Utida *et al.*, 1972). Also, PRL exerts an inhibitory action on the intestinal HCO_3^- secretion of sea bream (Ferlazzo *et al.*, 2012), when analyzed *in vitro*.

GH and cortisol are commonly referred to as the “SW-hormones” because of their stimulatory effect on the hypo-osmoregulatory mechanisms of teleosts. GH promotes salinity tolerance in teleosts (Takei and McCormick, 2013), and its action is in part mediated by interaction with insulin-like growth factor-1 (IGF-I) (McCormick, 2001). Morphological changes, including increased intestinal length and thickness, and absorptive area of the epithelium were observed following GH treatment

(Nonnotte *et al.*, 1995; Walker *et al.*, 2004). Furthermore, increased transcriptional levels of *nka α 1c* were observed in different segments of Atlantic salmon intestine (Tipsmark *et al.*, 2010a).

Cortisol stimulates several mechanisms associated with hypo-osmoregulatory strategy of teleosts. Cortisol implants stimulate drinking rates and water transport capacity in Atlantic salmon (Cornell *et al.*, 1994; Fuentes *et al.*, 1996), and increase intestinal NKA activity in chinook salmon (*Onchorhynchus tshawytscha*) (Veillette and Young, 2004). Also, upregulation of *aqp1* and *nka α 1c* were observed in eel and Atlantic salmon intestine respectively (Martinez *et al.*, 2005; Tipsmark *et al.*, 2010a). Cortisol may interact with GH to regulate hyper-osmoregulatory mechanisms, but it can also promote FW acclimation acting alone or together with the PRL (McCormick, 2001).

1.2.7. Intestinal regions

The osmoregulatory mechanisms described above take place in all the intestinal segments, which contribute differently depending on the species and environmental salinity.

When present, the pyloric caeca of teleosts can represent up to 70% total intestinal surface area (Wood and Bucking, 2011). Although often overlooked, it plays an important role in osmoregulatory processes in SW, as demonstrated by high conductance and HCO_3^- secretion observed in trout, as well as the upregulation of mRNA levels of *nbc1*, *vha*, *calV*, *cac* (Grosell *et al.*, 2009a, 2007). In chinook salmon, increased NKA activity and fluid absorption capacity were also observed (Veillette *et al.*, 2005).

The anterior region is considered in several teleost species the major site of ion and water absorption, with elevated NKA activity, Cl^- uptake rates, HCO_3^- secretion rates and water absorption (Alves *et al.*, 2019; Carvalho *et al.*, 2012; Gregório *et al.*, 2013; Grosell, 2006). Similar relevance can be suggested also for the mid intestine (Alves *et al.*, 2019; Ruiz-Jarabo *et al.*, 2017; Taylor *et al.*, 2010).

The posterior intestine also participates in the osmoregulatory mechanisms and in salmonids represents an important site for water absorption (Sundell and Sundh, 2012). The role of the rectum is also relevant as demonstrated in eel (Kim *et al.*, 2008), and its involvement in osmoregulation increases in hyper-saline environments, like

also for the posterior intestine (Gregório *et al.*, 2013; Guffey *et al.*, 2011; Li *et al.*, 2014).

Functional differences between regions are linked to the different expression patterns of osmoregulatory pathways observable in teleosts at both transcriptional and protein levels. These differences are often species-specific (Cutler and Cramb, 2008; Duarte *et al.*, 2023; Li *et al.*, 2014; Watanabe *et al.*, 2011).

Also, the endocrine factors differently modulate the intestinal regions like observed in brown trout (*Salmo trutta*) (Seidelin *et al.*, 1999), Atlantic salmon (Tipsmark *et al.*, 2010a), in Gulf toadfish (*Opsanus beta*) (Ruhr *et al.*, 2014), and eel (Takei *et al.*, 2019).

The mechanisms in act in the different regions of the intestine leave behind a fluid with chemical characteristics significantly distinct from the ingested SW. The composition of the intestinal fluid greatly changes along the intestine with increased concentration of Mg^{2+} , SO_4^{2-} , HCO_3^- resulting from the selective absorption of monovalent ions and water, and from the intestinal secretion of HCO_3^- (Genz *et al.*, 2008; Grosell, 2014). The analysis of the intestinal fluid collected from discrete intestinal regions further substantiates the anterior-to-posterior specialization present in the intestine of numerous teleosts investigated to date (Alves *et al.*, 2019; Grosell *et al.*, 2001).

From the literature it becomes clear that the organization of the intestine changes from species to species. It could be argued that the anterior-to-posterior organization/specialization of fish intestine has a general pattern. However, given the large number of species and habitats they populate, the intestine tends to mirror specific lifestyles.

1.3. Atlantic salmon (*Salmo salar*)

1.3.1. Distribution and life cycle

Atlantic salmon (*Salmo salar*) is a carnivorous teleost naturally present in the temperate and subarctic regions of the North Atlantic Ocean and in rivers flowing into it (Thorstad *et al.*, 2011). This species is anadromous and begins the life cycle in FW where spends a variable period as juvenile before migrating into the ocean for feeding and growth. However, some populations, known as “landlocked” populations can complete their life cycle without migrating to the ocean (Berg, 1985).

Like other diadromous species, Atlantic salmon has a complex and fascinating life cycle, many aspects of which are not yet fully elucidated (Fig. 1.3). Sexually mature salmon spawn in the rivers between September and February. Eggs are deposited in gravel nests (redds) and hatch during the following spring. The hatched alevins remain in the gravel until the yolk-sac is absorbed, after which they emerge as fry able to feed independently. Over the autumn, fry develop into parr with dark vertical bands (parr marks) and red spots along the body. Parr remain in FW for 1-8 years before going through several adaptive changes, known as parr-smolt transformation, smolting or smoltification, that transform them into smolts, ready for downstream migration and sea entry. In SW, young post-smolts grow and sexually mature (1-5 years) before they migrate back to the natal rivers for spawning (Thorstad *et al.*, 2011; Webb *et al.*, 2007).

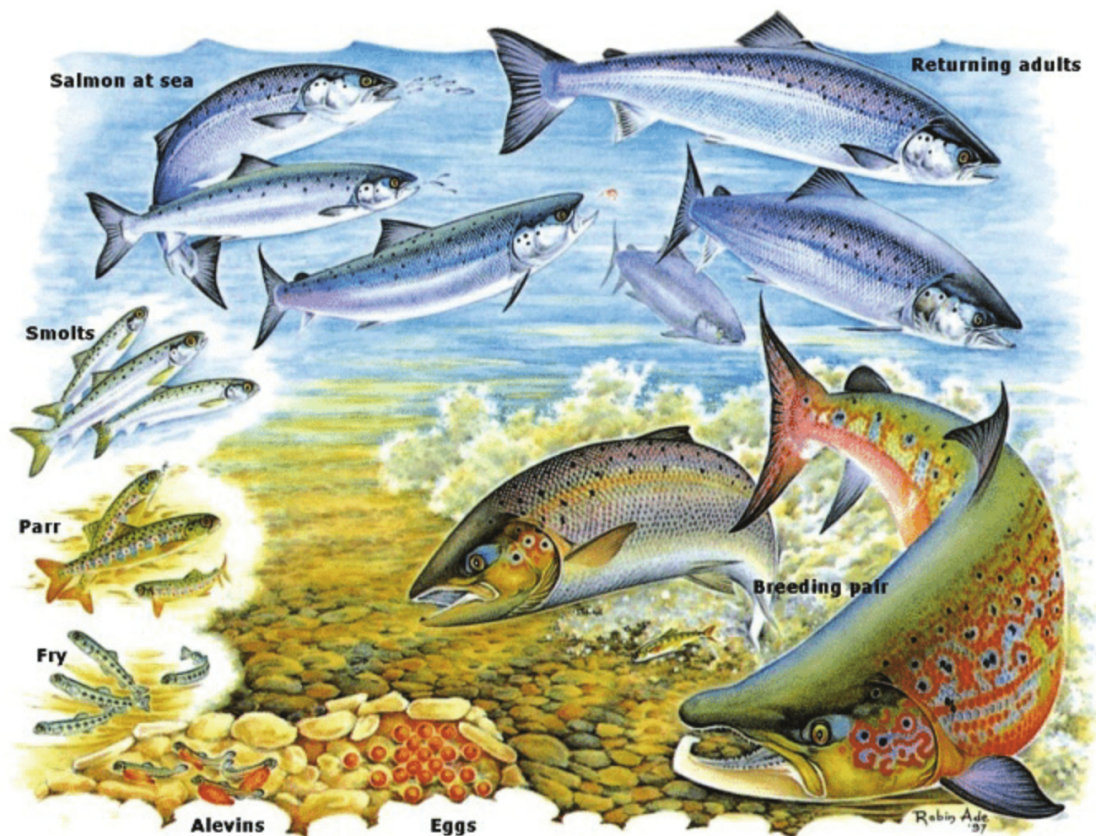


Figure 1.3. Life cycle of Atlantic salmon (Source: © Robin Ade and Atlantic Salmon Trust).

1.3.2. Smoltification

The migration of Atlantic salmon (and other anadromous salmonids) from the rivers to the open ocean is preceded by a phenotypic developmental phenomenon

known as parr-smolt transformation (PST), smolting, or smoltification (Boeuf, 1993; Hoar, 1988). In Atlantic salmon, smoltification is a seasonal event occurring in late spring in fish that reached a minimum threshold size (12-15 cm) (McCormick, 2013), and it includes a set of independent but coordinated developmental changes (biochemical, physiological, morphological, and behavioral) preparatory for the successful downstream migration and marine life (Stefansson *et al.*, 2008).

Smoltification is driven by several environmental cues, including photoperiod, temperature, river flow and water turbidity (McCormick *et al.*, 1998), and endogenous rhythms (Eriksson and Lundqvist, 1982).

Photoperiod is recognized as the major environmental factor regulating the timing of the physiological and behavioral changes linked with smoltification in Atlantic salmon (Berge *et al.*, 1995; Björnsson *et al.*, 1995). Changes in day length, specifically the increase in day length following the short-days of the winter period, represent the seasonal cue that stimulates the light-brain-pituitary axis promoting the neuroendocrine mechanisms that synchronize smolting-related changes (Ebbesson *et al.*, 2003). Photoperiod may also act to increase activity (migratory restlessness or Zugunruhe), rendering the fish responsive to other environmental factors, like water temperature, flow, and turbidity, that act as releasing factors to start the migration (McCormick *et al.*, 1998).

Temperature is an important modulator of smoltification, which influences juvenile salmon's growth and developmental rates, determining the age at which they reach the threshold size required for smoltification (Stefansson *et al.*, 2008). Correlation between migration timing and temperature was also found and some authors reported a temperature of about 10 °C to initiate downstream migration (McCormick, 2013). However, temperature thresholds can vary between populations, and degree-days (d°C) were suggested to indicate migration timing in Atlantic salmon smolts better than the threshold temperature (Zydlewski *et al.*, 2005). The duration of the smolt-window has been calculated in the range of 300-400 d°C (Stefansson *et al.*, 2008).

Downstream migration of Atlantic salmon has been associated with higher water flow in the rivers, and it includes both passive and active movements of smolts in the current. The increased river flow has also been correlated with increased fish

survival, attributed to the higher migration speed and water turbidity, which can reduce predation (McCormick, 2013).

1.3.3. Smolting-related changes

During smoltification in salmonids, several morphological and metabolic changes occur, preparing smolts for pelagic life. The typical parr-marks and red spots disappear, covered by purines (guanine and hypoxanthine) deposition in skin and scales, conferring a silvery color to the smolts (Johnston and Eales, 1967; Leclercq *et al.*, 2010). Simultaneously, the fin margins become darker due to changes in the melanophores (Chen *et al.*, 2014; Mizuno *et al.*, 2004). A characteristic decrease in the condition factor (K) is also observed during smoltification due to the faster growth in length compared to the weight, which results in a slimmer body shape in smolts (McCormick, 2013; Stefansson *et al.*, 2008). The K reduction may also be associated to higher energetic demands and changes in the lipid metabolism that cause the reduction of body lipids during smoltification (McCormick, 2013; Wedemeyer *et al.*, 1980).

Smoltification includes behavioral changes. While parr are agonistic and territorial in an environment with limited resources, smolts lose the territorial behavior and form schools that represent an advantage against predation. Also, smolts change orientation in the river, exhibiting a negative rheotaxis, and they develop increased buoyancy (McCormick *et al.*, 1998).

A critical physiological change during smoltification is the development of hypo-osmoregulatory ability (Fig. 1.4). Parr have poor salinity tolerance, but during smoltification, fish develop, already in FW, the hypo-osmoregulatory capacities required for a successful SW entry. The peak of salinity tolerance is observed in mid-May, at the moment of the downstream migration (McCormick, 2013). The preparatory changes encompass the reorganization of the osmoregulatory mechanisms in all the osmoregulatory organs. However, along the years, most of the research has primarily focused on the gill and its function.

During smoltification, a preparatory reorganization of the gill, from an ion-absorbing to an ion-secreting capable epithelium, takes place already in FW. Number and size of ionocytes increase and also the structure of the multicellular complexes where larger apical crypts can be observed (Stefansson *et al.*, 2008). Transcript and

protein abundance of the key ion-transporter proteins in SW ionocytes (NKA, NKCC1 and CFTR) are all upregulated during smoltification. A specific expression pattern is observed for two NKA α -subunit isoforms. Gill mRNA levels and protein abundance of Nka α 1a decrease during smoltification and SW acclimation, whereas Nka α 1b increase (Christensen *et al.*, 2018; Nilsen *et al.*, 2007). Transcripts and proteins abundance in the gill increase also for the basolateral Nkcc1, as well as Cftr1 expression (Nilsen *et al.*, 2007; Pelis *et al.*, 2001). Furthermore, NKA activity in the gill significantly increases during smoltification, contributing to the enhanced ion secretory capacity and salinity tolerance of Atlantic salmon juveniles (McCormick and Saunders, 1987). Remodeling also takes place at the paracellular level to facilitate Na⁺ excretion, with changes in the expression pathway of the gill claudins. Specifically, increased mRNA levels were observed for *cldn10e* during smoltification and after SW exposure, while lower levels were observed for *cldn27a* and *cldn30* after SW transfer (Tipsmark *et al.*, 2008).

Changes take place also in the GIT of Atlantic salmon during smoltification. Drinking rates in wild brown trout increase during smoltification (Nielsen *et al.*, 1999), and likely in Atlantic salmon. As observed in other euryhaline teleosts, the drinking rates of Atlantic salmon smolts increase from ~0.2 mL/kg/h in FW to ~2.6 mL/kg/h and ~4.0 mL/kg/h after 24 h and 7 days in SW respectively (Fuentes and Eddy, 1997). Functional studies carried out in the intestine also showed an increased intestinal fluid transport (J_v) during smoltification, particularly in the posterior intestine (Sundell *et al.*, 2003; Veillette *et al.*, 1993). At the peak of smoltification, this increase has been accompanied by increased intestinal NKA activity and changes in paracellular permeability (Sundell *et al.*, 2003). More recent studies have focused on the putative molecular mechanisms underlying the functional changes observed in the intestine during smoltification. Like for the gill, during smoltification were observed in the intestine of Atlantic salmon changes in the expression of important transporters involved in the hypo-osmoregulatory mechanisms like Nka α 1c (the dominant NKA isoform present in the intestine of Atlantic salmon) and Nkcc2, (Sundh *et al.*, 2014). Transcripts upregulation and newly synthesized protein were observed during smoltification. Notably, the cellular distribution of these proteins changed in the enterocytes. Nka α 1c exhibited a more basal position during parr-smolt transformation in FW, while after transfer to SW, it redistributed to a more lateral position to optimize

its function in SW. Nkcc2, instead, moved from a sub-apical region during smoltification to a more apical localization after transfer to SW (Sundh *et al.*, 2014). During smoltification, increased transcriptional levels of *aqp8b* were observed in Atlantic salmon intestine, and after SW transfer, upregulation of *aqp1a*, *1b* and *8b* was also observed (Tipsmark *et al.*, 2010b). Similar to what observed in the gill, remodeling of the paracellular pathway also take place in the intestine depending on the environmental salinity. In the intestine, *cldn15* and *25b* mRNA levels increased after transfer to SW (Tipsmark *et al.*, 2010a).

Despite the important role of the kidney in osmoregulatory mechanisms in fish, very few studies have analyzed the physiological changes that occur in the kidney during smoltification. In spring, Eddy and Talbot, (1985), observed an increase in the urine production in smolt of Atlantic salmon. Later, NKA activity of the kidney was analyzed during smoltification, but no significant changes were observed (McCormick *et al.*, 1989). At the transcriptional level, increased levels of *aqp1a* and *10* were observed during smoltification, with peaks in April and March, respectively (Tipsmark *et al.*, 2010b). A recent study has focused on the expression profile of the Atlantic salmon kidney in FW and SW. The authors revealed a distinct distribution of transporters and tight junction components in the different segments of the kidney, and upregulation of key pathways associated with osmoregulatory mechanisms, such as aquaporins, claudins, NCC and CFTR following transfer to SW (Madsen *et al.*, 2020).

The period during which physiological changes reach their peak in conjunction with favorable environmental conditions for migration, is referred to as “smolt-window”. If fish are prevented from entering SW, they revert part of the smolting-related changes (“desmoltification”) (McCormick *et al.*, 1998).

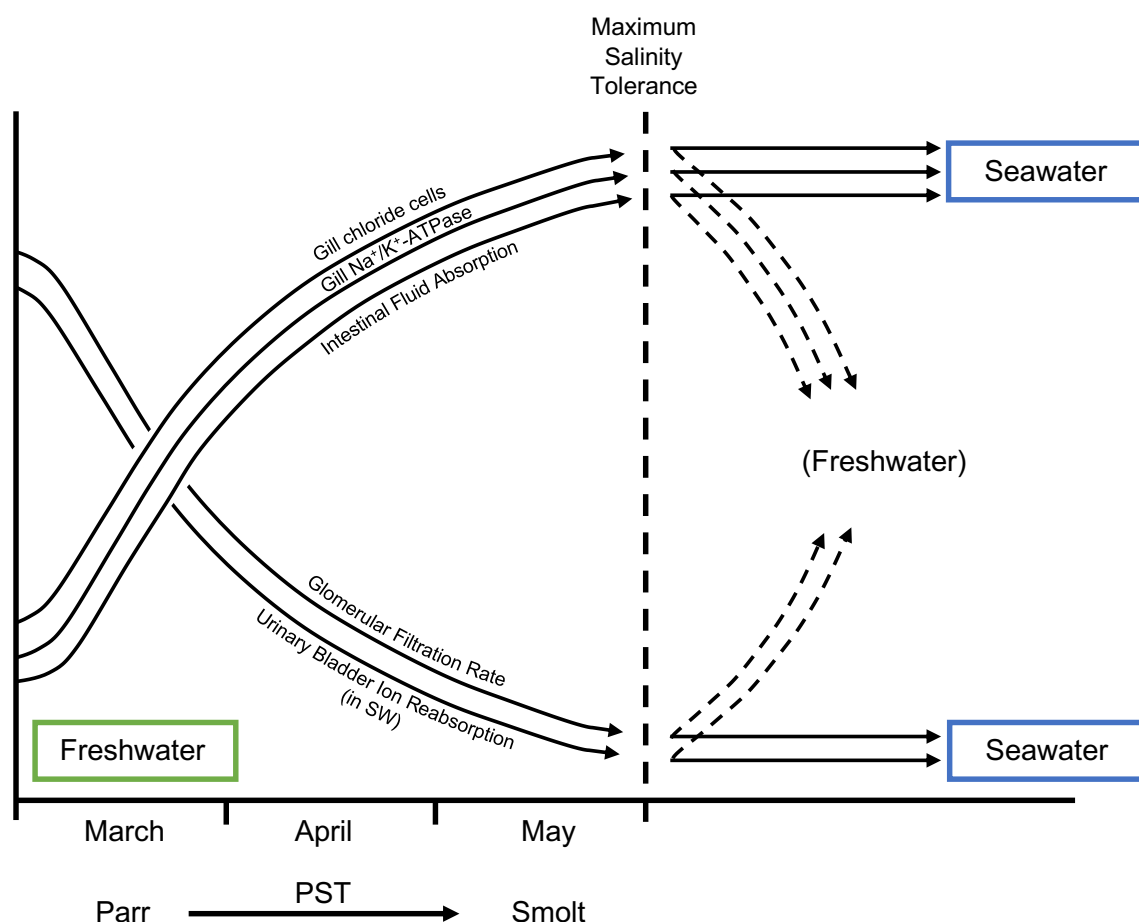


Figure 1.4. Functional changes associated with the parr-smolt transformation (PST) in Atlantic salmon (Modified from: McCormick and Saunders 1987).

1.3.4. Endocrine regulation of smoltification

Smolt development is a neuroendocrine-regulated event. Photoperiod is recognized as the primary environmental factor triggering smoltification. Photoperiod information is sent through the light-brain-pituitary axis to the endocrine system, initiating the cascade of mechanisms that regulate smolt development (Stefansson *et al.*, 2008).

The circulating levels of GH, cortisol, IGF-I and thyroid hormones (THs) increase during smoltification, while PRL levels reduce (McCormick, 2013). The synergically action of these hormones is responsible for the morphological and metabolic changes, the schooling behavior, and the development of hypo-osmoregulatory capacity observed during smoltification.

GH and IGF-I are important regulators of growth and metabolism in teleosts, and in salmon are likely involved in the reduction of K observed in smolts (Björnsson, 1997; Wood *et al.*, 2005). The action of these hormones also increases salinity

tolerance. Exogenous treatments with GH and IGF-I increased the expression of gill $\text{NKA}\alpha 1\text{b}$, Nkcc1 and Cftr (Pelis *et al.*, 2001; Tipsmark and Madsen, 2009).

Cortisol also stimulates salinity tolerance during smoltification, affecting many hypo-osmoregulatory mechanisms. Exogenous cortisol increases the ionocytes abundance, the expression of key ion transporters and tight junction components in gill and intestine, drinking rates, fluid absorption capacities of the intestine, and NKA activity in osmoregulatory organs (Cornell *et al.*, 1994; Fuentes *et al.*, 1996; Kiilerich *et al.*, 2007; Tipsmark *et al.*, 2009). Cortisol is involved in both hyper- and hypo-osmoregulatory mechanisms in teleosts (McCormick, 2001), and its action during smoltification seems linked with the GH-IGF-I axis, which acts as a switch to promote salt secretion (McCormick, 2013).

The thyroid hormones, comprising the prohormone thyroxine (T_4) and the active hormone triiodothyronine (T_3) are involved in many aspects of smoltification, such as silvering, olfactory imprinting, behavior, and visual sensitivity (Campinho, 2019; Stefansson *et al.*, 2008). Limited effects are reported on salinity tolerance (McCormick, 2001). However, these may have an indirect role through the GH-IGF-I and cortisol axes (McCormick, 2013).

PRL is generally considered to have an inhibitory action on smolt development in Atlantic salmon. It exerts an antagonistic action toward the SW-adaptative effect of cortisol and GH (Seidelin and Madsen, 1997), and treatments with PRL resulted in reduced NKA activity and $\text{NKA}\alpha 1\text{b}$ transcript levels in the gill of Atlantic salmon (Tipsmark and Madsen, 2009).

1.3.5. Salmon aquaculture

The high demand of proteins from aquatic resources and the considerable reduction of natural stocks have caused an exponential growth of the aquaculture industry in the last decades. Over half of fish in the markets comes from aquaculture recourses. Various species are produced globally but only some are clearly dominant. Of the finfish species produced in marine and coastal aquaculture, Atlantic salmon alone accounts for around 32.6% of the total production, with 2.7 million tons produced in 2021. The world's largest producer is Norway (FAO, 2022).

Besides the high technology applied throughout the production process, the success of Atlantic salmon industry is related with the high resistance of this species

and to its ability to adapt very well to various culture conditions. However, the industry still faces large losses due to high mortality (~15%) mainly after transfer to sea cages (Grefsrud *et al.*, 2023). Part of this mortality is caused by exceptional events such as sea lice, algae blooms, and infections. However, 4% of the mortality registered in the first weeks after sea transfer is due to suboptimal smolt quality caused by incomplete smoltification or delay in the transfer (loss of the smolt-window). In addition to direct mortality, poor osmoregulatory capacity increases the stress of the fish, leaving them more vulnerable to pathogens due to a compromised immune system (Oliveira *et al.*, 2021).

Considering the life cycle of this species, part of the production is carried out on land in FW, mainly in flow-through and once the smoltification is stimulated, the fish are transferred to sea cages located between the fjords or in coastal areas. However, alternative production systems are also in the developmental stage, such as recirculating aquaculture systems (RAS) (Dalsgaard *et al.*, 2013; Pino Martinez *et al.*, 2021).

A significant challenge for the industry is the proper stimulation of smoltification. Initially juvenile salmonids were exposed to natural photoperiods, but later to intensify production and produce out-season smolts with underyearling fish, temperature and photoperiod protocols were implemented to trigger smoltification at different times of the year (Clarke *et al.*, 1996; Duston and Saunders, 1995; Lysfjord *et al.*, 2004). Traditionally smoltification has been stimulated using a compressed square-wave summer-winter-summer photoperiod. Juveniles are reared under continuous light (24 h light) interrupted by 6 weeks of short (winter) photoperiod (≤ 12 h light) and a subsequent further period of continuous light (Handeland and Stefansson, 2001; Strand *et al.*, 2018). Under natural conditions, smoltification is size-dependent and salmonids hypo-osmoregulatory capacity increases with body size (McCormick and Saunders, 1987). Rearing conditions affect fish growth; hence, production protocols are also conceived to facilitate the production of larger smolts, more robust and potentially with better salinity tolerance.

To optimize production and improve fish welfare, great efforts have been put into the development of aquafeed for the modern aquaculture, enriched with functional additives (probiotics, enzymes, phytogenics, amino acids, etc.) to improve growth and health of the species cultivated (Encarnação, 2016; Olmos Soto *et al.*, 2015; Andersen *et al.*, 2016).

Possibly motivated by the enhancement in salinity tolerance resulting from the use of dietary salt in several studies (Perry *et al.*, 2006; Staurnes and Finstad, 2000; Striberny *et al.*, 2021), the salmon industry is increasingly adopting the use of specifically formulated feed enriched with salts (transition diets) during the final phase in FW. This trend is emerging as a potential alternative to the traditional standard photoperiodic protocols in smolt production.

1.3.6. Smoltification markers

Over the years, several parameters and indexes have been used to evaluate smolting development in farmed fish, aiming to select the optimal timing for SW transfer.

Smolt development is often determined based on the morphological changes characteristic of parr-smolt transformation. Commonly used is the smolt index, based on the visual assessment of parr marks, body silvering, and darkening of the fin margins, along with the reduction in K. Nevertheless, rearing conditions can differently affect fish morphology, and typical PST changes may not necessarily align with the development of hypo-osmoregulatory capacity, as observed in wild populations (Sundell *et al.*, 1998; Von Cramon-Taubadel *et al.*, 2005). For this reason, salmon hypo-osmoregulatory capacity is frequently evaluated through the SW challenge test to assess salinity tolerance. The test involves the direct transfer of fish to SW to determine survival rates, as well as plasma osmolality and ions concentration (Clarke *et al.*, 1996).

Smoltification is accompanied by a characteristic increase in gill NKA activity (McCormick, 2013). Consequently, a widely employed assay to assess ion secretory capacity in juveniles of Atlantic salmon involves measuring gill NKA activity (McCormick, 1993). Expression studies have further identified transcriptional and protein level changes during smoltification, with the switch in the expression of the NKA isoforms (Christensen *et al.*, 2018; Nilsen *et al.*, 2007).

The indexes developed to assess PST and predict a good performance upon SW transfer are linked to morphological changes or gill markers. However, none of the mentioned indexes is completely suitable to properly evaluate smolting development in the intense farming conditions. This work will expose the use of intestinal markers to assist in the characterization of PST related changes.

1.4. Objectives

This thesis aimed to study the role of the intestine in the ion and water homeostasis in Atlantic salmon (*Salmo salar*), with a particular focus on the development of the hypo-osmoregulatory capacities in different intestinal sections during artificial stimulation of smoltification and after transfer to SW. This main objective could be sub-divided in the following specific objectives:

1. Characterize at the functional and molecular levels the development of the hypo-osmoregulatory mechanisms in the intestine of Atlantic salmon following photoperiod manipulation and dietary treatment (salt mix and free tryptophan) to stimulate smoltification and salinity tolerance (**Chapter 2 and 3**).
2. Identify putative functional indexes and molecular markers in the intestine of Atlantic salmon indicative of a smolt ready to be transfer to SW (**Chapter 2 and 3**).
3. Characterize the regulatory action of cortisol on the development of the intestinal hypo-osmoregulatory capacity in Atlantic salmon juveniles outside the smolt-window and under natural on-growing conditions, with particular focus on the intestinal bicarbonate secretion cascade pathway (**Chapter 4**).

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CHAPTER

2

Photoperiod and dietary treatment in freshwater modulate the short-term intestinal response to seawater in Atlantic salmon (*Salmo salar*)

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Resumen

La estimulación y el momento de la esmoltificación son esenciales para la acuicultura del salmón del Atlántico (*Salmo salar*). En este capítulo se investigó la respuesta intestinal durante la manipulación de la dieta y del fotoperiodo durante la fase de agua dulce. Los juveniles de salmón del Atlántico clasificados como "pequeños" y "grandes" (~40 g y ~130 g respectivamente) recibieron tratamiento experimental en agua dulce (FW) durante 12 semanas, fueron posteriormente transferidos al agua de mar (SW) y seguidos durante siete días. Durante la fase de agua dulce, los peces experimentaron dos condiciones lumínicas diferentes, 24 L:0 D – 24 L ("grupos LL-LL") y 7 L:17 D – 24 L ("grupos SP-LL"), y fueron alimentados con una dieta control ("grupos LL-LL C" y "grupos SP-LL C") o con una dieta enriquecida con sal y triptófano libre ("grupos LL-LL + dieta" y "grupos SP-LL + dieta"). Analizamos la actividad de la Na^+/K^+ -ATPasa (NKA), las propiedades bioeléctricas del tejido intestinal en cámaras de Ussing y la composición del fluido intestinal. La actividad de la NKA mostró variaciones mínimas en relación con el tamaño de los peces, los tratamientos o la región intestinal estudiada (anterior o posterior). El fotoperiodo moduló las propiedades bioeléctricas epiteliales, corriente cortocircuito (I_{sc}) y la resistencia transepitelial (R_t) tanto del intestino anterior como del posterior, especialmente R_t . Adicionalmente, los experimentos farmacológicos dirigidos a los cotransportadores apicales $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ (NKCC2) y Na^+/Cl^- (NCC) revelaron efectos dependientes de la región intestinal y salinidad-dependientes. Asimismo, la estimulación de la producción de cAMP intracelular, generada in vitro con una combinación de forskolina e IBMX mostró respuestas dependientes de la región intestinal, la salinidad del agua y el tratamiento, con una clara especialización funcional del intestino anterior y posterior. Además, la composición del fluido intestinal reflejó la capacidad de procesamiento del agua de mar ingerida y mostró escasa variación en peces grandes. En resumen, nuestros datos sugieren una mejor pre-adaptación del intestino durante la esmoltificación en respuesta a la luz (grupos SP-LL), y muestran que la combinación de luz y dieta podrían ofrecer, en un contexto de acuicultura industrial, una ventaja para smolts más pequeños, pero no para los mayores. La composición del fluido intestinal en peces pequeños proporciona un índice de la función intestinal y puede considerarse un indicador a largo plazo del rendimiento en el salmón Atlántico en agua de mar.

Chapter 2

Palabras clave: salmón del Atlántico, esmoltificación, intestino, osmorregulación, cámara de Ussing, actividad de la Na^+/K^+ -ATPasa, fluido intestinal.

Abstract

Stimulation and timing of smoltification are essential for successful Atlantic salmon (*Salmo salar*) aquaculture. This study investigated intestinal responses during dietary and photoperiod manipulation in freshwater (FW) and after a subsequent seven days of residence in seawater (SW). “Small” and “large” Atlantic salmon parr (~40 g and ~ 130 g respectively) were treated in FW for 12 weeks and thereafter transferred to SW for seven days. During the FW phase, fish underwent two different light conditions, 24 L:0 D – 24 L (“LL-LL” groups) and 7 L:17 D – 24 L (“SP-LL” groups) or fed with either regular feed (“LL-LL C” and “SP-LL C” groups) or feed enriched with a salt mix plus free tryptophan (“LL-LL + diet” and “SP-LL + diet” groups). We analyzed Na^+/K^+ -ATPase (NKA) activity, tissue bioelectrical properties in Ussing chambers, and intestinal fluid composition. The NKA activity showed minor variations in relation to fish size, treatments, or intestinal region (anterior or posterior). Photoperiod modulated epithelial bioelectrical properties (I_{sc} and R_t) of the anterior and posterior intestine, particularly transepithelial resistance (R_t). Pharmacological experiments, targeting apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ (NKCC2) and Na^+/Cl^- (NCC) cotransporters revealed intestinal region- and water salinity-dependent effects. In addition, stimulation of the intracellular cAMP with forskolin and IBMX showed intestinal region-, water salinity-, and treatment-dependence responses with clear functional specialization of the anterior and posterior intestine. The intestinal fluid composition reflected the ability to process ingested SW and showed little variation in large fish. In summary, our data suggest a better preadaptation of the intestine during light-stimulated smoltification (SP-LL groups), and the combination of light and diet might give, in an industrial aquaculture setting, an advantage to smaller, but not larger smolts. Intestinal fluid composition in small fish can be used as an index of intestinal function and may act as a long-term performance proxy in SW Atlantic salmon.

Keywords: Atlantic salmon, smoltification, intestine, osmoregulation, Ussing chamber, Na^+/K^+ -ATPase activity, intestinal fluid.

2.1. Introduction

In nature, Atlantic salmon (*Salmo salar*) juveniles spend a variable period in freshwater (FW) before undergoing several preparatory changes for seawater (SW) entry. The set of morphological, physiological, endocrine, and behavioral changes that transform FW parr into a SW-ready smolts are known as parr-smolt transformation (PST) or simply smoltification (Hoar, 1988; Stefansson *et al.*, 2008). When fish have surpassed a minimum body size, increasing day length in spring activates the light-brain-pituitary axis to promote neuroendocrine mechanisms regulating the onset of smoltification (Ebbesson *et al.*, 2003; McCormick *et al.*, 1998). Other environmental factors, such as water temperature and flow, and endogenous mechanisms, also contribute to the stimulation and timing of PST (Eriksson and Lundqvist, 1982; McCormick, 2013).

In both FW and SW, the ionic and osmotic differences between smolts plasma and the surrounding medium cause an osmotic imbalance, opposite in the two environments. The gills, gastrointestinal tract, and kidney are the main organs that work in synergy to oppose the disequilibrium and maintain body homeostasis (Edwards and Marshall, 2013). A crucial change that allows smolts to survive and reach a new osmoregulatory steady state in SW is the development of hypo-osmoregulatory capacity while still in FW and the fast reorganization of the osmoregulatory mechanisms after SW transfer (McCormick, 2013).

Earlier studies on the development of the hypo-osmoregulatory mechanism during PST have focused mainly on the gill (McCormick, 2013). However, ion and osmotic homeostasis in SW is a highly integrated process where the intestine plays a fundamental role. Thus, upon transfer to SW, the first osmoregulatory response required is the onset of drinking, which increases about 30-folds compared to FW salmon smolts (Fuentes and Eddy, 1997). This increase of drinking causes an increased absorption of ions and water in the gastrointestinal tract (Smith, 1930) and the subsequent active secretion of ions by the chloride cells in the gills (Evans *et al.*, 2005).

Water ingested requires intestinal processing to become available to the plasma pool. This processing starts in the esophagus, where a relevant water-independent absorption of Na^+ and Cl^- take place (Hirano and Mayer Gostan, 1976; Parmelee and Renfro, 1983). The functional consequence is a substantial reduction

of osmolality of the imbibed fluid which constitutes a first step to promote water absorption in the intestine (Marshall and Grosell, 2006).

The main driving force for water absorption is NaCl movement. Several mechanisms are believed to be involved in this process. Thus, NaCl is or might be absorbed by the apical Na^+/Cl^- co-transporter (NCC) (Frizzell *et al.*, 1979; Watanabe *et al.*, 2011) and, together with K^+ , by the apical $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ co-transporter (NKCC2) (Musch *et al.*, 1982; Sundh *et al.*, 2014; Trischitta *et al.*, 1992). In addition, recent studies in SW species (Grosell *et al.*, 2005; Grosell and Genz, 2006; Kurita *et al.*, 2008) highlighted a putative important role for apical anion exchangers (Slc26 family, Ohana *et al.*, 2009) in facilitating Cl^- absorption and subsequent water movements. Thus, Cl^- enters the enterocyte by apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers and generates apical HCO_3^- secretion. As a result, there is an alkalization of the intestinal fluid that, combined with the high concentrations of Ca^{2+} and Mg^{2+} originated from the high drinking, creates the optimal chemical conditions for CaCO_3 and MgCO_3 precipitation in the form of aggregates (Walsh *et al.*, 1991; Wilson and Grosell, 2003). The precipitation in the form of carbonates removes ions from the solution, reduces the intestinal fluid osmolality, and favors net water absorption. This processing and the putative involvement of intestinal precipitation generates a unique ion profile in the intestinal fluid of SW fish. Na^+ and Cl^- concentration become lower than plasma levels, while divalent ions Ca^{2+} and Mg^{2+} , are several folds higher in the fluid than in the plasma (Alves *et al.*, 2019; Grosell *et al.*, 2007). How this process takes place in Atlantic salmon smolts remains still unknown. The electrochemical potential required to energize ion movement through channels and transporters is generated by the basolateral Na^+/K^+ -ATPase, whose activity in fish intestine is generally higher after SW adaptation (Barany *et al.*, 2020; Skou and Esmann, 1992).

The intestine of fish is a heterogeneous tissue at both morphological and functional levels. Using functional approaches, several studies described a region-specific role of the different tracts of the intestine in osmoregulatory mechanisms (Alves *et al.*, 2019; Gregório *et al.*, 2013; Grosell *et al.*, 2005; Ruhr *et al.*, 2014; Ruiz-Jarabo *et al.*, 2017b). In Atlantic salmon such studies are scarce but relevant. For instance, Sundell *et al.*, (2003) described intestinal region-dependent tissue resistance measured in Ussing chambers in FW and SW anterior and posterior intestine. Which are also evident in rainbow trout in FW and SW (Sundell and Sundh, 2012). Usher *et*

al., (1991b) showed changes in water absorption *in vitro* in the intestine of Atlantic salmon during the PST and after transfer to SW, and Veillette *et al.* (1993) show a functional intestinal regionalization of fluid transport during the PST.

In the industrial production of Atlantic salmon, several methods are used to stimulate smoltification, *e.g.*, osmoregulatory maturation. A compressed natural photoperiod is commonly adopted, where the fish is exposed to a period of short-day length (“winter”) followed by an increase in day length (“spring”) during the last part of the FW phase (Duston and Saunders, 1990; Sigholt *et al.*, 1995). This treatment is hereafter termed “light treatment”. However, to enhance the growth rate in FW, many farmers skip the short-day treatment to achieve bigger smolt size and a shorter growth period in SW (Pino Martinez *et al.*, 2021; Ytrestøyl *et al.*, 2020). In the absence of light treatment, so-called “smoltification feeds” may be used to stimulate PST. Generally, these feeds are added a salt/ion mixture known to stimulate SW tolerance in juvenile salmonids (Basulto, 1976; Staurnes and Finstad, 2000). The feed used in the present experiment was in addition added free tryptophan, which, like ions, is known to activate G-protein coupled calcium-sensitive receptors (CaSR) to promote SW tolerance (Loretz, 2008). This method, hereafter termed a “dietary stimulation”, is adopted in the weeks preceding SW transfer.

The transfer from FW to SW still represents a critical period with significant loss of animals in salmon farming due to suboptimal smolt quality and high level of infections (Aunsmo *et al.*, 2008; Hjeltne *et al.*, 2019). Therefore, the aim of the present study was to understand if the intestinal function provides valuable information about the timing of SW transfer of Atlantic salmon undergoing different strategies of smolting stimulation. To achieve this objective, we investigated the intestinal responses in FW and after a short-term (seven days) SW challenge to 1) characterize the region-specific osmoregulatory mechanisms evoked during smoltification in the anterior and posterior intestine by mean of a functional approach in Ussing chamber; 2) focusing on the end result of the intestinal function (fluid composition), investigate if dietary stimulation, with a mix of salt and amino acid tryptophan, in combination with photoperiod manipulation, can improve the intestinal physiological machinery required for the FW-SW transition, in small (~40 g) and large (~130 g) Atlantic salmon pre-smolts.

2.2. Materials and methods

2.2.1. Chemicals

Forskolin (FK) and 3-isobutyl-1-methylxanthine (IBMX) were from Cayman Chemical (MI, USA). All other chemicals were from Sigma-Aldrich (Madrid, Spain). In pharmacology experiments, final concentrations of the chemicals in Ussing chambers were as follows: bumetanide (BUM) 200 μ M, hydrochlorothiazide (HCTZ) 1000 μ M, forskolin (FK) 10 μ M and 3-isobutyl-1-methylxanthine (IBMX) 500 μ M, prepared as concentrated stocks in DMSO. The amount of DMSO in the solution never exceeded 0.2% of the total volume of the Ussing chamber.

2.2.2. Animals and experimental design

The experimental design of the present study has been described in detail by Striberny *et al.*, (2021). In brief, fertilized Atlantic salmon eggs (*Salmo salar*) were obtained from AquaGen strain (AquaGen, Trondheim, Norway) and hatched and raised to parr at the Aquaculture Research Station in Tromsø (Norway). In mid-March 2017, at start-feeding, juveniles were divided into two groups. A group of fish was kept in FW at 4 °C till two weeks before the start of the experiment (6th February 2018). During the two weeks, the temperature was increased by 0.5 °C/day till 10 °C. This group at the beginning of the experiment had a body mass mean ~ 40 g and it is referred to them as “small” fish. The other group was kept in FW at 10 °C until September 2017, after which the temperature was decreased and kept on 4 °C till November 2017 and increased again to 10 °C until the start of the experiment (6th February 2018). At the beginning of the experiment, these fish had a body mass average ~ 130 g and termed “large” fish. Until this point, both size groups had been kept at continuous light (24 L:0 D).

On 6th February 2018, 1400 small fish and 1000 large fish were divided into eight circular tanks (300 L/tank) for each size group and kept in flow-through FW at 10 °C. Four tanks per size were subjected to six weeks of short photoperiod with 7 h of light, and 17 h of darkness (7 L:17 D, “SP” groups) and the others remained under 24 h of light (24 L:0 D, “LL” groups). At the end of the six weeks under short photoperiod for SP groups (21st March), all fish were brought back to continuous light (24 L:0 D). At this time-point, the water temperature was increased to 12 °C, and two tanks, for each light condition and size, were fed with pellet supplemented with salt and the

amino acid tryptophan ("LL-LL + diet" and "SP-LL + diet" groups). The rest of the 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 SW transfer (11th May). The composition of the two used feeds is shown in Table 2.1. On 11th May, 50 fish from each treatment and size were transfer to 33‰ SW at 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 for SP-LLs. Feeding was withheld in the 24 h before each sampling. However, food was present in the intestine of FW fish. SW fish intestine were devoid of food.

The experimental design and the sampling time-points used in the present work are schematically represented in Fig. 2.1. For each time-point, size, and experimental condition, ten fish were sampled, five from each duplicate tank. Animals were anesthetized with an overdose of benzocaine (160 ppm), blood collected, and fish sacrificed by decapitation. The intestine was isolated, washed in salmon saline (see below), and samples of the anterior intestine (2–3 cm caudal of the point of insertion of the last pyloric caeca) and posterior intestine (2–3 cm posterior to the ileorectal sphincter) were collected for electrophysiological characterization and intestinal Na^+/K^+ -ATPase activity measurements. Also, during the sampling of fish in SW at day seven after transfer, intestinal fluid from individual animals was collected.

The experiment was conducted following guidelines provided in Norwegian and European legislation related to animal research. Formal approval of the experimental protocol was given by the Norwegian Food Safety Authority, FOTS ID 13891.

Table 2.1. 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

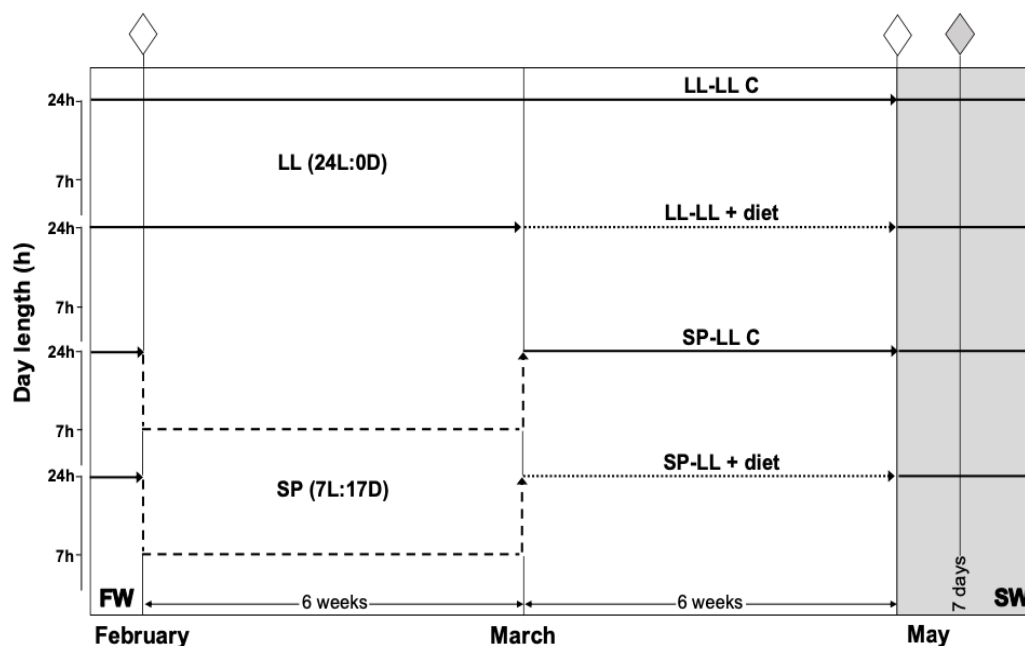


Figure 2.1. Graphic representation of the experimental design showing the four experimental conditions to which small (~40 g) and large (~130 g) Atlantic salmon (*Salmo salar*) were subjected from February to May. The white background represents the freshwater phase (FW) while the grey the seawater phase (SW). Solid arrows indicate the use of continuous light (24 L:0 D, LL), dash arrows the use of short photoperiod (7 L:17 D, SP), and round dot arrows the use of salt and tryptophan diet. White diamonds represent the FW sampling-points while grey diamond the SW sampling-point.

2.2.3. Intestinal fluid

FW fish lacked intestinal fluid; therefore, the fluid collection took place only after seven days in SW. After anesthesia, the abdominal cavity was cut open, and the intestinal tract clamped with two hemostatic forceps (from the end of pyloric caeca to the anal sphincter), the contents were then emptied into 1.5 mL tubes, centrifuged (12,000 rpm, 6 min, RT) and fluid stored at -20°C for later analysis. Osmolality was measured using a Vapor Pressure Osmometer (Model 5600 Wescor, Logan, UT, USA). Sodium ion concentration was determined using a flame photometer (BWB-XP, BWB Technologies, UK), and chloride ion was measured by coulometric titration with a Chloride Analyzer (Model 925 Corning, Medfield, MA, USA). The difference in osmolality (ΔOsm) and chloride ion (ΔCl^{-}) were calculated between plasma (PL) and intestinal fluid (IF) per individual fish (plasma values from Striберny *et al.*, (2021)) following the formula $\Delta\text{value} = \text{Plasma level} - \text{Intestinal fluid level}$; in mOsm kg^{-1} for osmolality or mmol L^{-1} for chloride ion.

2.2.4. Intestinal Na⁺/K⁺-ATPase activity

Mucosal biopsies of the anterior and posterior intestine were placed in 200 μ L of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3), rested on ice for 2–3 min and immediately frozen at – 80 °C to avoid tissue degradation by proteases. Intestinal Na⁺/K⁺-ATPase (NKA) activity was determined using a previously developed method (McCormick, 1993).

Samples were randomly analyzed and NKA activity was measured within 25 min of samples thawing. The specific activity (μ mol ADP mg protein⁻¹ h⁻¹) was determined by linking ATP hydrolysis to the oxidation of nicotinamide adenine dinucleotide (NADH) measured at 340 nm for 10 min at 25 °C in the presence or absence of 0.5 mM ouabain. Immediately after each NKA assay, protein content of each sample was adjusted to 1 mg mL⁻¹. Protein content was determined by the Bradford Protein Assay (BioRad Laboratories, Hercules, CA, USA) using bovine serum albumin as standard (BSA, BioRad Laboratories, Hercules, CA, USA).

2.2.5. Ussing chamber experiments

Intestinal sections from the anterior and posterior intestine were washed with chilled fresh saline to remove undigested food and mounted within five minutes of sacrifice on tissue holders of 0.25 cm² or 0.5 cm² (P2404 and P2305, Physiological Instruments, San Diego, CA, USA) between two half-Ussing chambers (P2400 or P2300, Physiological Instruments, San Diego, CA, USA) holding 2 mL of saline kept at 10 °C and gassed bilaterally with humidified air. All intestinal sections of a single tank (five fish, ten intestinal preparations, five anterior and five posterior intestines) were run simultaneously. Saline composition for FW experiments was: 7.5 mM NaHCO₃, 2.5 mM KCl, 130 mM NaCl, 1 mM NaH₂PO₄, 1 mM MgSO₄, 2.5 mM CaCl₂, 5 mM Na-HEPES. Apical saline received 10 mM mannitol to equilibrate 10 mM glucose in the basolateral saline. Saline pH was adjusted at 7.80 using 4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), and osmolality set to 320 mOsm kg⁻¹ with mannitol (Vapor Pressure Osmometer Model 5600 Wescor, Logan, UT, USA). For SW experiments, saline received an additional 20 mM NaCl, pH was adjusted to 7.80, and osmolality to 350 mOsm kg⁻¹ with mannitol.

In our experimental setup the transepithelial potential (TEP, mV) was referenced (grounded) to the apical side (mucosa). Short-circuit current (I_{sc} , μ A cm⁻²)

was monitored by clamping the epithelia to 0 mV. For clarity, I_{sc} was expressed as negative for anion absorption, and positive for the secretion of anions. Transepithelial resistance (R_t , $\Omega \text{ cm}^2$) was calculated by Ohm's law using the current deflections induced by 2 mV bipolar pulses of 3 s every minute. When the tissue achieved steady state (usually 30–40 min after mounting), basal values were recorded.

The response to pharmacological treatments was tested in the last period in FW (FW-May) and after seven days in SW (SW 7d-May). Bumetanide (BUM, 200 μM , apical) or hydrochlorothiazide (HCTZ, 1000 μM , apical) were used to inhibit respectively $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ (NKCC) and Na^+/Cl^- (NCC) co-transporters. A cocktail of forskolin and 3-isobutyl-1-methylxanthine (FK, 10 μM + IBMX, 500 μM) applied bilaterally was used to raise intracellular cAMP. After monitoring effects for 30 min, individual responses were recorded and shown as short-circuit current differences between the treatment values and the basal values of individual preparations (ΔI_{sc} , $\mu\text{A cm}^{-2}$).

Voltage clamping and current injections used VCC MC8 Multi-channel voltage/current clamp or VCC600 amplifiers (with automatic fluid resistance compensation) (Physiological Instruments, San Diego, CA, USA), connected with Ag/AgCl electrodes (with tip asymmetry $<1 \text{ mV}$) to either side of the Ussing chamber with 3-mm-bore agar bridges (1 M KCl in 3% agar). Bioelectrical parameters for each tissue were recorded onto a computer through Lab-Trax-4/16 acquisition systems (World Precision Instruments, Sarasota, FL, USA) using LabScribe3 (iWorx Systems Inc., CB Sciences, NH, USA).

2.2.6. Statistical analysis

All results are shown as mean $\pm$ SEM. Homogeneity of variances and normality was assessed before statistical analysis. When necessary, data were transformed to comply with ANOVA assumptions. In each sampling point and for each fish size and intestinal region, differences between experimental groups were assessed by two-way ANOVA, considering light (LL-LL and SP-LL) and diet (control and dietary treatment) as main factors. The ANOVA analysis were followed by the Bonferroni *post-hoc* test when significant interaction between the two factors was observed. In the specific case of pharmacological experiments with bumetanide, when an experimental group was missing one-way ANOVA was carried out, followed by the Bonferroni *post-hoc* test. All

statistical analyses were performed using Prism 6.0 (GraphPad Software). Statistical significance was accepted at $p < 0.05$.

2.3. Results

2.3.1. Animals and mortality

At the beginning of the experiment small fish body mass averaged 42.2 ± 2.0 g while large fish 131.8 ± 5.9 g. From February to May small and large fish increased their body mass reaching respectively the weight of 108.4 ± 3.1 g and 296.6 ± 9.9 g at the time of SW transfer in May.

During the experimental period, low mortality was observed in the SW stage. For small fish, a total of 8 animals died out of 130. Two fish died in the LL-LL C group and 3 in the SP-LL C during the SW challenge test in May (Striberny *et al.*, 2021), 1 fish died in the LL-LL + salt and 2 in the SP-LL C during the first seven days in SW.

2.3.2. Intestinal fluid analysis

2.3.2.1. Carbonate aggregates

Regardless of their treatment, carbonate aggregates were present in the intestinal fluid of all fish sacrificed after seven days in SW. However, due to the intensive sampling and *ex-vivo* experimentation, quantification was not possible.

2.3.2.2. Osmolality

Intestinal fluid osmolality ($\text{Osm}_{(\text{IF})}$, mOsm Kg^{-1}) lower than plasma osmolality in individual fish. $\text{Osm}_{(\text{IF})}$ in small fish averaged between 276 and 317 mOsm Kg^{-1} and was significantly affected by diet ($p = 0.0021$, two-way ANOVA, Fig. 2.2A) with the lowest $\text{Osm}_{(\text{IF})}$ shown by SP-LL + diet group (276 mOsm Kg^{-1} , Fig. 2.2A). In small fish, ΔOsm between plasma and intestinal fluid was significantly affected by diet ($p = 0.0157$, two-way ANOVA, Fig. 2.2B). The dietary treated groups presented an ΔOsm ~70% higher than control groups (Fig. 2.2B). Large fish had $\text{Osm}_{(\text{IF})}$ averages between 290 and 313 mOsm Kg^{-1} . There was a significant interaction between light and diet ($p = 0.0071$, two-way ANOVA, Fig. 2.2C). The LL-LL + diet group had a significantly higher osmolality than SP-LL + diet (Bonferroni *post-hoc* test, Fig. 2.2C). In large fish, no significant differences in ΔOsm between plasma and intestinal fluid existed in response to treatments at seven days in SW (two-way ANOVA, Fig. 2.2D).

2.3.2.3. Chloride ion

Chloride ion concentration in the intestinal fluid ($\text{Cl}_{(\text{IF})}^-$, mmol L^{-1}) was consistently lower than plasma concentration in individual fish. In small fish, $\text{Cl}_{(\text{IF})}^-$ concentration was between 38 mmol L^{-1} and 55 mmol L^{-1} (Fig. 2.3A). Diet significantly affected $\text{Cl}_{(\text{IF})}^-$ levels ($p = 0.0020$), and there was a significant interaction between light and diet ($p = 0.0149$, two-way ANOVA, Fig. 2.3A). In the group LL-LL + diet, $\text{Cl}_{(\text{IF})}^-$ concentration was ~40% lower than the group LL-LL C (Fig. 2.3A). In small fish, ΔCl^- between plasma and intestinal fluid did not differ significantly between treatments (two-way ANOVA, Fig. 2.3B). However, LL-LL C presented the lowest ΔCl^- (98 mmol L^{-1}). In large fish, $\text{Cl}_{(\text{IF})}^-$ ranged between 49 and 58 mmol L^{-1} and was significantly affected by light ($p = 0.0376$, two-way ANOVA, Fig. 2.3C). ΔCl^- between plasma and intestinal fluid in large fish were significantly affected by light ($p = 0.0017$) and diet ($p = 0.0030$, two-way ANOVA, Fig. 2.3D).

2.3.2.4. Sodium ion

In small fish, sodium ion concentrations in the intestinal fluid ($\text{Na}_{(\text{IF})}^+$, mmol L^{-1}) were affected by light ($p = 0.0345$) and diet ($p < 0.0001$) and showed a significant interaction of the two factors ($p = 0.0207$, two-way ANOVA, Fig. 2.4A). $\text{Na}_{(\text{IF})}^+$ ranged from 41 mmol L^{-1} to 59 mmol L^{-1} with significantly higher levels in LL-LL C group than the other groups (Bonferroni *post-hoc* test, Fig. 2.4A). $\text{Na}_{(\text{IF})}^+$ in large fish was significantly affected by light ($p = 0.0041$) and presented significant interaction of light and diet ($p = 0.0312$, two-way ANOVA, Fig. 2.4B). Averages were between 45 and 69 mmol L^{-1} , and LL-LL groups had $\text{Na}_{(\text{IF})}^+$ levels significantly higher ($\sim 65\text{--}69 \text{ mmol L}^{-1}$) than SP-LL + diet (45 mmol L^{-1}) (Bonferroni *post-hoc* test, Fig. 2.4B).

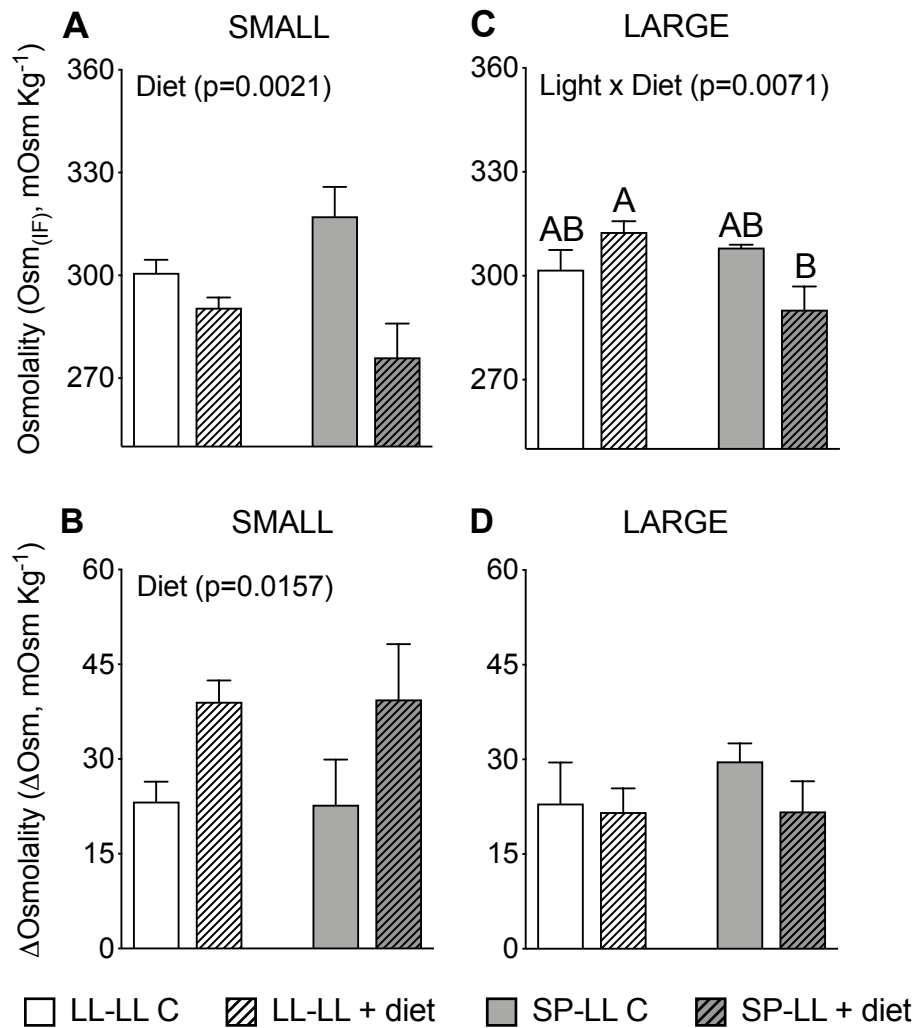


Figure 2.2. Intestinal fluid osmolality ($Osm_{(IF)}$, mOsm Kg^{-1}) and difference in osmolality between plasma and intestinal fluid (ΔOsm , mOsm Kg^{-1}) in small (~40 g, A, B) and large (~130 g, C, D) Atlantic salmon (*Salmo salar*), after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 8-11$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA). When significant interaction between light and diet was observed, Bonferroni *post-hoc* test was carried out and significant differences among experimental groups indicated with different uppercase letters.

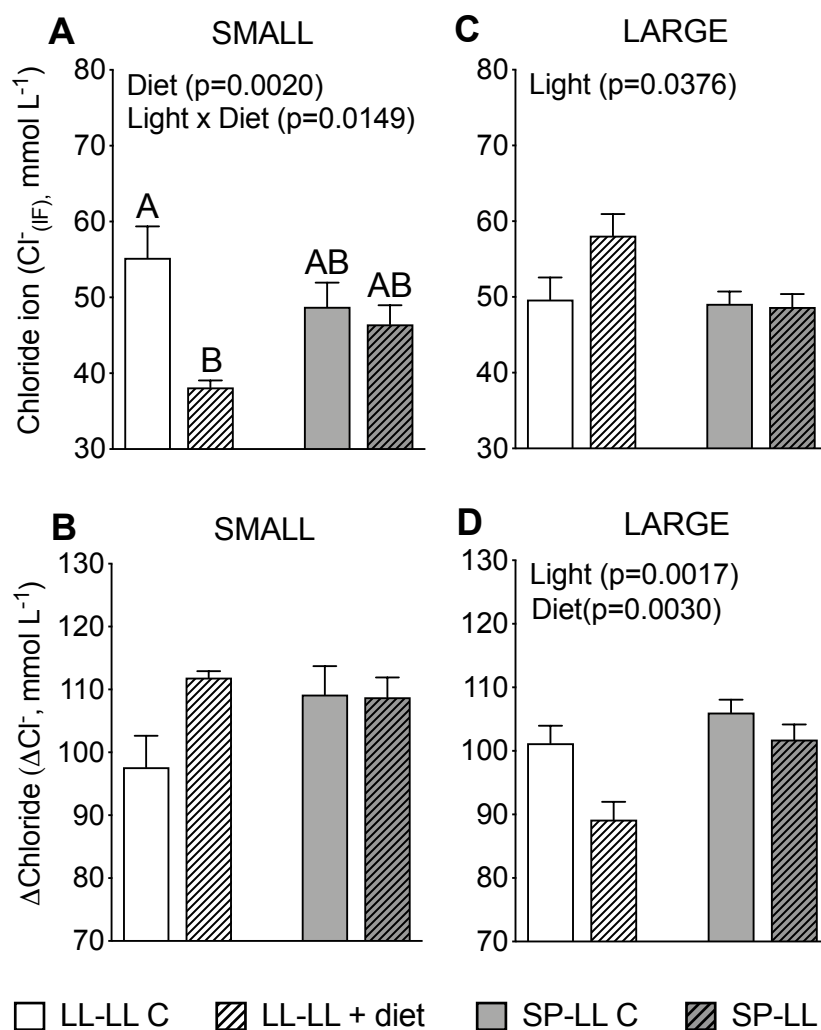


Figure 2.3. Chloride ion concentrations in the intestinal fluid ($\text{Cl}^-_{(\text{IF})}$, mmol L^{-1}) and difference in chloride ion concentrations between plasma and intestinal fluid (ΔCl^- , mmol L^{-1}) in small (~ 40 g, A, B) and large (~ 130 g, C, D) Atlantic salmon (*Salmo salar*), after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 7-11$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA). When significant interaction between light and diet was observed, Bonferroni *post-hoc* test was carried out and significant differences among experimental groups indicated with different uppercase letters.

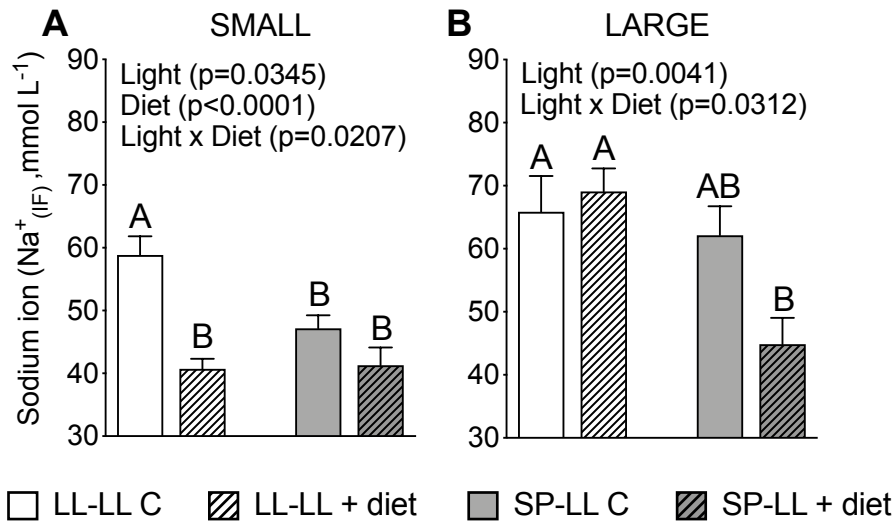


Figure 2.4. Sodium ion concentrations in the intestinal fluid ($\text{Na}^+_{(\text{IF})}$, mmol L^{-1}) in small (~ 40 g, A) and large (~ 130 g, B) Atlantic salmon (*Salmo salar*), after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 8-11$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA). When significant interaction between light and diet was observed, Bonferroni *post-hoc* test was carried out and significant differences among experimental groups indicated with different uppercase letters.

2.3.3. Intestinal Na^+/K^+ -ATPase activity

This study has no data on gill ATPase activity; however, a cohort of a parallel experiment was shown to perform well long-term and grow in SW (Striberny *et al.*, 2021). Due to the small size of the intestine at the beginning of the experiment in February, the Na^+/K^+ -ATPase (NKA) activity assay could not be successfully performed. Therefore, the NKA activity results are reported only for FW and SW sampling points in May.

In small fish, NKA activities averaged between 3.2 and 4.6 $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$ in the anterior intestine. The activity did not change significantly in response to light or diet in either FW or SW (two-way ANOVA, Fig. 2.5A). In contrast, the NKA activity in the posterior intestine was affected by the light in FW ($p = 0.0012$, two-way ANOVA, Fig. 2.5B), presenting values between 2.5 and 4.8 $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$.

After seven days in SW, all groups had similar NKA activities (Fig. 2.5B). In large fish, NKA activity in the anterior intestine averaged between 2.8 and 4.9 $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$ (Fig. 2.5C). NKA activity was affected by light in FW ($p = 0.0121$, two-way ANOVA, Fig. 2.5C). Upon transfer to SW, there were no significant differences among treatments (two-way ANOVA, Fig. 2.5C). NKA activity was stable

in the posterior intestine in both FW and SW, averaging between 2.5 and 3.6 $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$ (two-way ANOVA, Fig. 2.5D).

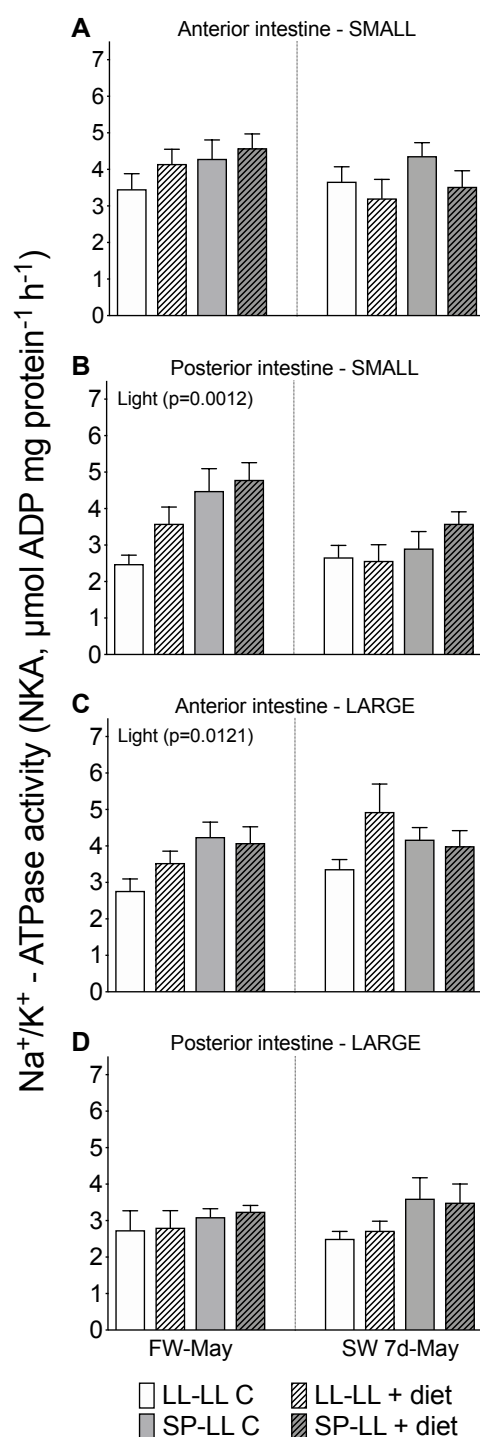


Figure 2.5. Na⁺ K⁺-ATPase activity (NKA, $\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$) of the anterior and posterior intestine in small (~40 g, A, B) and large (~130 g, C, D) Atlantic salmon (*Salmo salar*). NKA values were measured before SW transfer (FW - May) and after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 7-10$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA).

2.3.4. Ussing chamber experiments: basal short-circuit current (I_{sc})

In the open circuit mode, intestinal preparations showed small but consistent transepithelial voltages, either positive or negative depending on the region, experimental group or salinity. The experiments were subsequently performed under voltage clamp and in our setup, negative currents indicate net absorption of anions, while positive values indicate secretory currents. Current values (I_{sc}) are shown as $\mu A\ cm^{-2}$.

The anterior intestine of small fish presented absorptive I_{sc} (negative current) in both FW and SW, except for the SP-LL C group that showed a positive/secretory I_{sc} at seven days in SW (Fig. 2.6A). I_{sc} in the anterior intestine averaged between +2.15 and $-18.8\ \mu A\ cm^{-2}$ (Fig. 2.6A). After seven days in SW, there was a significant effect of light ($p = 0.0045$, two-way ANOVA, Fig. 2.6A). The posterior intestine of small fish presented negative I_{sc} in FW and SW, with average values between -4.78 and $-11.5\ \mu A\ cm^{-2}$ (Fig. 2.6B). In FW in May, light significantly affected I_{sc} ($p = 0.0202$, two-way ANOVA, Fig. 2.6B) with lower values for fish subjected to SP-LL than groups under LL-LL conditions. After seven days in SW, I_{sc} was not significantly affected by light or diet or by the combination of both (two-way ANOVA, Fig. 2.6B).

In large fish, the anterior and the posterior intestine presented, in all the experimental groups, negative/absorptive I_{sc} (Fig. 2.6C, D). In the anterior intestine, I_{sc} averaged between -5.16 and $-12.32\ \mu A\ cm^{-2}$ (Fig. 2.6C), while in the posterior intestine, averages were between -3.78 and $-9.56\ \mu A\ cm^{-2}$ (Fig. 2.6D). For the two considered regions, I_{sc} was not significantly affected by light or diet or by the interaction of the two factors (two-way ANOVA, Fig. 2.6C, D).

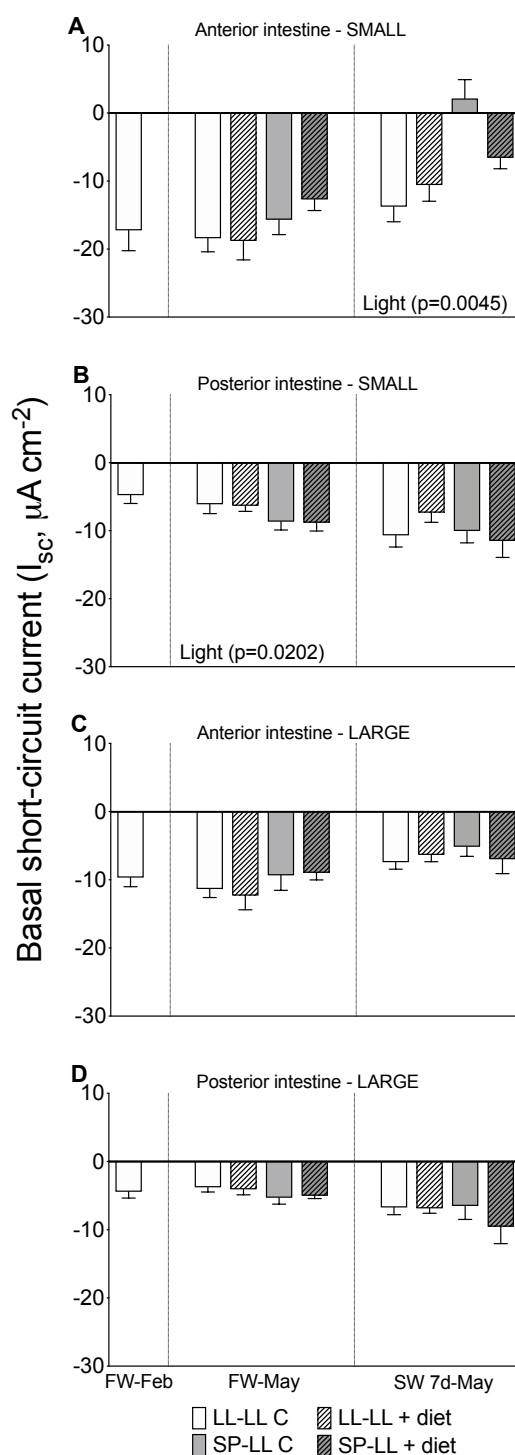


Figure 2.6. Basal short-circuit current (I_{sc} , $\mu A\ cm^{-2}$) of the anterior and posterior intestine in small (~40 g, A, B) and large (~130 g, C, D) Atlantic salmon (*Salmo salar*). I_{sc} values were measured in Ussing chamber using the voltage-clamp technique, under symmetric osmotic conditions, at the beginning of the experiment (FW - Feb), before SW transfer (FW - May) and after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 7-10$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA).

2.3.5. Pharmacology treatments

In pharmacology experiments carried out in Ussing chamber in the last period in FW (May) and after seven days in SW, short-circuit current differences (ΔI_{sc} , $\mu A\ cm^{-2}$) with positive values indicate inhibition of the prevailing absorptive current (I_{sc}), and negative values show stimulation of absorption. Experiments with levels of DMSO equivalent to those used in the pharmacological characterizations were performed, and were without effect on tissue bioelectrical parameters, ruling out effects of the vehicle alone (Fig. 2.7A).

2.3.5.1. Bumetanide effect on basal I_{sc}

In both fish sizes, the effect of apical bumetanide (BUM, 200 μM), a specific inhibitor of $Na^+/K^+/2Cl^-$ cotransporter (NKCC), showed positive ΔI_{sc} for all the considered groups in the anterior and posterior intestine (Fig. 2.7), therefore inhibition of the absorptive I_{sc} (Fig. 2.7B). Due to technical problems during the experiments in the posterior intestine of the SP-LL C in FW in May and in the anterior and posterior intestine of large fish SP-LL + diet in SW, the bumetanide effect was not determined (ND, Fig. 2.7E, F).

Bumetanide effect in the anterior intestine of small fish had ΔI_{sc} between +1.80 and +4.91 $\mu A\ cm^{-2}$ (Fig. 2.7C), while in the posterior intestine it had ΔI_{sc} between +0.30 and +0.91 $\mu A\ cm^{-2}$ (Fig. 2.7D). The response to bumetanide was not significantly affected by light or diet in the two intestinal region (two-way ANOVA, Fig. 2.7C, D).

In the anterior intestine of large fish, the ΔI_{sc} response to apical bumetanide, ranged from +0.90 to +2.75 $\mu A\ cm^{-2}$ (Fig. 2.7E), while it ranged from +0.20 to +1.26 $\mu A\ cm^{-2}$ in posterior intestine (Fig. 2.7F).

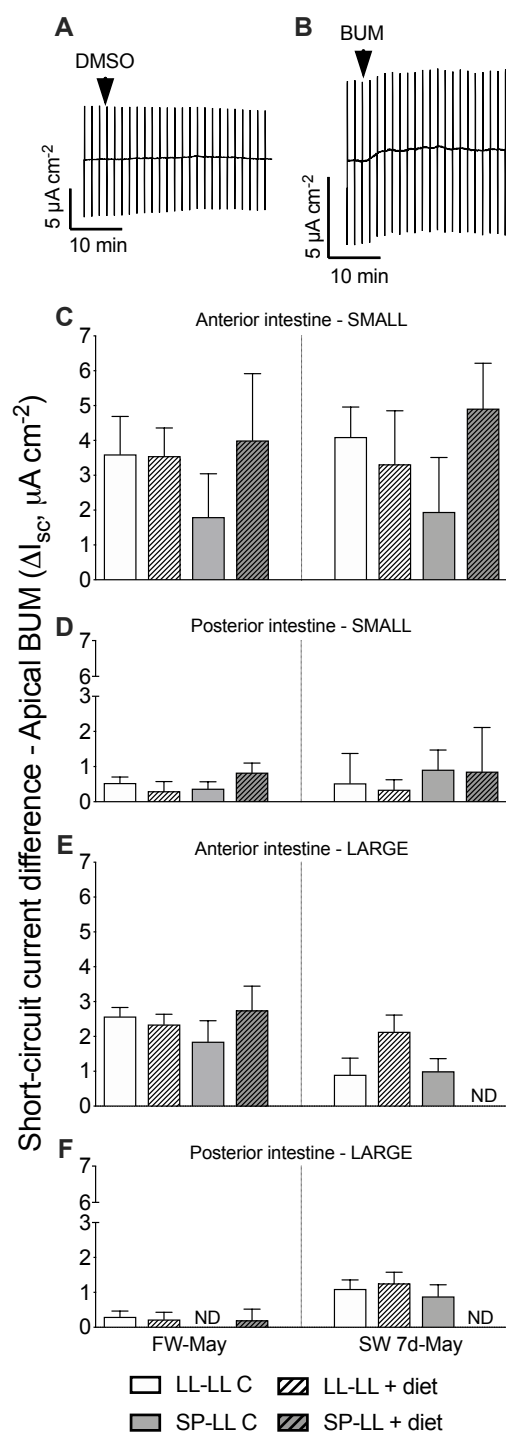


Figure 2.7. Response to DMSO (0.2%, original trace, A) and difference in short-circuit current (ΔI_{sc} , $\mu A\ cm^{-2}$) in response to apical bumetanide (200 μM , original trace, B) in the anterior and posterior intestine of small (~40 g, C, D) and large (~130 g, E, F) Atlantic salmon (*Salmo salar*). Pharmacological treatments were carried out in Ussing chamber, using the voltage-clamp technique, under symmetric osmotic conditions, before SW transfer (FW - May) and after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 6-8$). In the analysis, light and dietary treatment are the two considered factors; significant differences were not detected among treatments ($p < 0.05$, two-way ANOVA).

2.3.5.2. Hydrochlorothiazide effect on basal I_{sc}

The response to the apical application of hydrochlorothiazide (HCTZ, 1000 μM), a specific inhibitor of Na^+/Cl^- cotransporter (NCC), showed positive ΔI_{sc} for all the experimental groups (Fig. 2.8), with inhibition of the absorptive I_{sc} (Fig. 2.8A).

The response to apical HCTZ was not significantly affected by light or diet in either intestinal region of small fish (two-way ANOVA, Fig. 2.8B, C). In the anterior intestine, average ΔI_{sc} ranged from +1.68 to +8.8 $\mu\text{A cm}^{-2}$, while in the posterior intestine, ΔI_{sc} ranged from +0.56 to +2.8 $\mu\text{A cm}^{-2}$.

In the anterior intestine of large fish, the response to apical HCTZ (Fig. 2.8D) was not significantly affected by light or diet (two-way ANOVA) and ΔI_{sc} ranged from +0.27 to +3.20 $\mu\text{A cm}^{-2}$. The effect of apical HCTZ in the posterior intestine of large fish had ΔI_{sc} between 0 and +1.80 $\mu\text{A cm}^{-2}$ (Fig. 2.8E). In FW, the posterior intestine ΔI_{sc} was significantly affected by light ($p = 0.0055$) and presented a significant interaction between light and diet ($p = 0.0472$, two-way ANOVA, Fig. 2.8E). Fish from the SP-LL + diet group had significantly higher ΔI_{sc} than those from the LL-LL + diet treatment (Bonferroni *post-hoc* test, Fig. 2.8E).

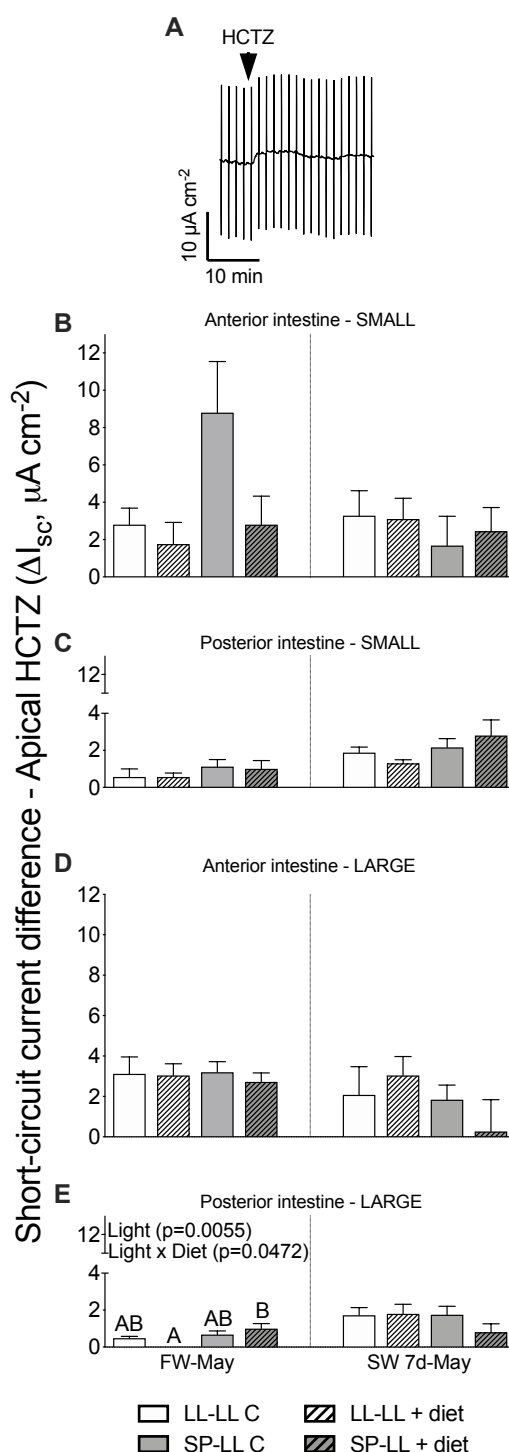


Figure 2.8. Difference in short-circuit current (ΔI_{sc} , $\mu\text{A cm}^{-2}$) in response to apical hydrochlorothiazide (HCTZ, 1000 μM , original trace, A) in the anterior and posterior intestine of small (~40 g, B, C) and large (~130 g, D, E) Atlantic salmon (*Salmo salar*). Pharmacological treatment was carried out in Ussing chamber, using the voltage-clamp technique, under symmetric osmotic conditions, before SW transfer (FW - May) and after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 3-8$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA). When significant interaction between light and diet was observed, Bonferroni *post-hoc* test was carried out and significant differences among experimental groups indicated with different uppercase letters.

2.3.5.3. *Forskolin and 3-isobutyl-1-methylxanthine effect on basal I_{sc}*

The response to bilateral forskolin (FK, 10 μ M) + 3-isobutyl-1-methylxanthine (IBMX, 500 μ M), used to raise intracellular cAMP, resulted in higher (Fig. 2.9A), less (Fig. 2.9B) or unchanged absorptive I_{sc} in both intestinal regions of small and large fish. In the anterior intestine of small fish, ΔI_{sc} ranged between -4.50 and $+2.00$ μ A cm^{-2} (Fig. 2.9C). In both FW and SW, LL-LL groups and SP-LL groups presented opposite responses, with positive ΔI_{sc} for LL-LLs and negative ΔI_{sc} in the case of SP-LLs. After seven days in SW, light significantly affected the response to FK + IBMX ($p < 0.0001$, two-way ANOVA, Fig. 2.9C). The posterior intestine of small fish presented values between 0 and $+0.75$ μ A cm^{-2} and light significantly affected ΔI_{sc} in FW ($p = 0.0016$, two-way ANOVA, Fig. 2.9D).

ΔI_{sc} in the anterior intestine of large fish, in response to bilateral FK + IBMX, averaged between -0.12 and $+1.54$ μ A cm^{-2} with a decrease of absorptive I_{sc} for all the experimental groups except SP-LL + diet after seven days in SW (Fig. 2.9E). The response to bilateral FK + IBMX in the anterior intestine was not affected by light or diet, neither in FW nor in SW (two-way ANOVA, Fig. 2.9E). The posterior intestine of large fish showed ΔI_{sc} between -1.6 and 0.36 μ A cm^{-2} (Fig. 2.9F). In FW, all the experimental groups responded to bilateral FK + IBMX with a higher absorptive I_{sc} . ΔI_{sc} was significantly affected by light ($p = 0.0007$) and presented a significant interaction between light and diet ($p = 0.0469$, two-way ANOVA, Fig. 2.9F). After seven days in SW, dietary treatment significantly affected ΔI_{sc} ($p = 0.0001$, two-way ANOVA, Fig. 2.9F); while control groups showed an increase in the absorptive I_{sc} after bilateral FK + IBMX, fish treated with the enriched diet, showed reduction of it.

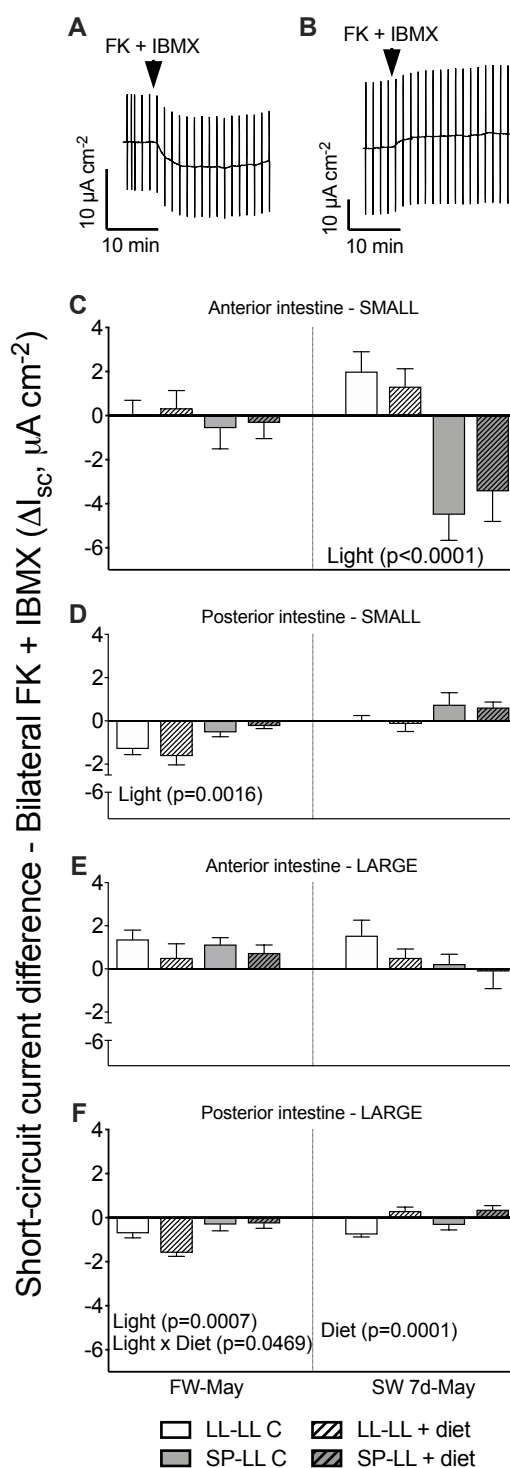


Figure 2.9. Difference in short-circuit current (ΔI_{sc} , $\mu\text{A cm}^{-2}$) in response to bilateral forskolin (FK, 10 μM) + 3-isobutyl-1-methylxanthine (IBMX, 500 μM) (original traces, A-B) in the anterior and posterior intestine of small (~40 g, C, D) and large (~130 g, E, F) Atlantic salmon (*Salmo salar*). Pharmacological treatment was carried out in Ussing chamber using the voltage-clamp technique, under symmetric osmotic conditions, before SW transfer (FW-May) and after seven days in SW (SW 7d - May). Each bar represents the mean $\pm$ SEM ($n = 5-10$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two-way ANOVA). When significant interaction between light and diet was observed, Bonferroni *post-hoc* test was carried.

2.3.6. Tissue resistance (R_t)

In small fish, the anterior intestine had a tissue resistance (R_t , $\Omega \text{ cm}^2$) between 45 and 85 $\Omega \text{ cm}^2$ (Fig. 2.10A) and it was significantly affected by light in both FW and SW in May ($p = 0.0394$ and $p = 0.0006$ respectively, two-way ANOVA, Fig. 2.10A). In FW, the posterior intestine R_t averaged between 147 and 209 $\Omega \text{ cm}^2$ and, in May, R_t was significantly affected by the light ($p = 0.0204$, two-way ANOVA, Fig. 2.10B). After seven days in SW, R_t averaged between 75 and 188 $\Omega \text{ cm}^2$ and it was significantly affected by both light and diet ($p = 0.0003$ and $p = 0.0278$ respectively, two-way ANOVA, Fig. 2.10B).

In large fish, the R_t of the anterior intestine was between 89 and 138 $\Omega \text{ cm}^2$, without significant effects of treatments not in FW nor in SW (two-way ANOVA, Fig. 2.10C). In the posterior intestine of FW animals, R_t averages were between 152 and 293 $\Omega \text{ cm}^2$. In FW (May), light significantly affected R_t of the posterior intestine ($p = 0.0040$) and it was observed, also, a significant interaction between the two experimental factors ($p = 0.0218$, two-way ANOVA, Fig. 2.10D). SP-LL C group had the lowest R_t , significantly different from the LL-LL groups (Bonferroni *post-hoc* test, Fig. 2.10D). After seven days in SW, R_t averages in the posterior intestine were between 128 and 227 $\Omega \text{ cm}^2$ (Fig. 2.10D). A significant effect of light was observed ($p = 0.0012$, two-way ANOVA, Fig. 2.10D) with LL-LL groups showing higher R_t than SP-LL groups.

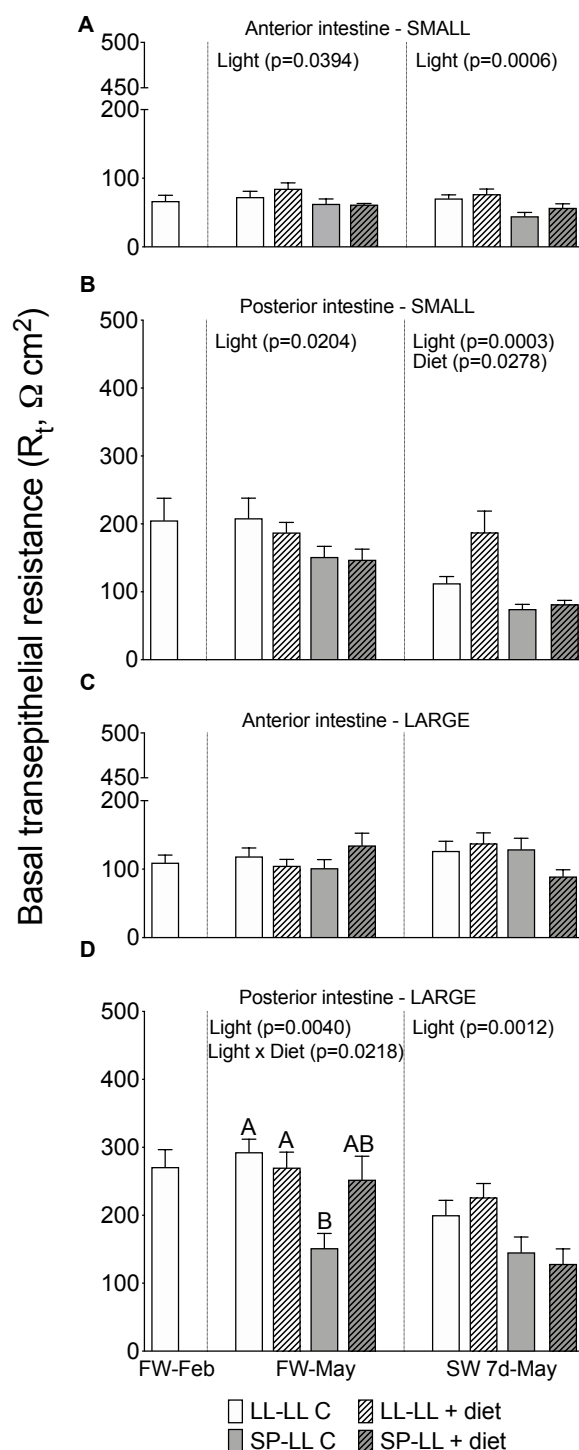


Figure 2.10. Basal transepithelial resistance (R_t , $\Omega \text{ cm}^2$) of the anterior and posterior intestine in small (~40 g, A, B) and large (~130 g, C, D) Atlantic salmon (*Salmo salar*). R_t was manually calculated (Ohm's law), during short-circuit current experiments, using current deflections induced by a 2 mV bipolar pulse, at the beginning of the experiment (FW - Feb), before SW transfer (FW- May) and after seven days in SW (SW 7d-May). Each bar represents the mean $\pm$ SEM ($n = 7-10$). In the analysis, light and dietary treatment are the two considered factors; significant p values are reported in the graph ($p < 0.05$, two- way ANOVA). When significant interaction between light and diet was observed, Bonferroni *post-hoc* test was carried out and significant differences among experimental groups indicated with different uppercase letters.

2.4. Discussion

The present study functionally characterized the effects of light vs. dietary stimulated PST on intestinal osmoregulatory responses before and after SW transfer in Atlantic salmon of two different size classes, representative of 0+ and 1+ smolts in the farming industry. All experimental conditions allowed small and large fish to develop some intestinal hypo-osmoregulatory competence required for SW acclimation, albeit with an improved ability found in fish subjected to short photoperiods and/or feeding with salt and tryptophan enriched feed compared to untreated fish.

Previous studies in Atlantic salmon demonstrate the capacity of smolts to completely re-equilibrate their osmoregulation after 4–8 days in SW (McCormick *et al.*, 1989; Prunet and Boeuf, 1985). Therefore, we focused on the intestinal physiological changes in FW and after the first seven days in SW.

Atlantic salmon smolts need to increase drinking to compensate for the dehydrating effect of SW (Fuentes and Eddy, 1997). However, an efficient processing of the ingested water is required to achieve an optimal osmoregulatory balance. Intestinal fluid processing in marine and euryhaline teleosts causes a decrease in osmolality from ~1100 mOsm (of the ingested SW) to values close to plasma osmolality (Alves *et al.*, 2019; Ruiz-Jarabo *et al.*, 2017a). In such conditions, NaCl absorption across the enterocytes generates an osmotic gradient between plasma and intestinal fluid to favor water absorption (Veillette *et al.*, 1993). Here, the osmotic gradient (ΔOsm , Fig. 2.2B, D) was positive in all experimental groups with lower osmolality in the intestinal fluid than the plasma. This observation probably reflects differences in drinking and intestinal processing of ingested SW, or likely a combination of both. However, the rationale applied to this combined processing should be as follows: the higher the difference between plasma and intestinal fluid osmolality, the better the processing of ingested SW. Therefore, ΔOsm could be considered a suitable index of the fish capacity to process the ingested SW and their consequent ability to regulate water absorption. In this study, dietary treated small fish had a ~70% higher ΔOsm (Fig. 2.2B) than the control groups indicating positive effects of diet on intestinal osmoregulatory mechanisms. Interestingly, ΔOsm in large fish was unaffected by dietary or light treatment (Fig. 2.2D), thus arguing that the benefits of

dietary and photoperiod manipulation are better suited for smaller smolts to enhance performance upon SW challenge.

In the intestine, water movement follows Cl^- and Na^+ absorption (Marshall and Grosell, 2006); therefore, the concentration of Cl^- and Na^+ in the intestinal fluid are also important indicators of how fish cope with osmotic stress in SW. Similarly to the results on osmolality, lower Cl^- and Na^+ concentration in the intestinal fluid in the groups treated with the combination of short photoperiod and dietary salt indicate beneficial effects of light and dietary stimulated PST, particularly in small fish (Fig. 2.3A and Fig. 2.4A).

The presence of carbonate precipitates in the intestine of all fish in SW, and the results of the intestinal fluid analysis confirm the acquired capacity of SW processing, regardless of fish size and experimental treatment. However, the results suggest that for small fish, but not for large fish, the combination of light and dietary treatments allows better SW processing in the intestine and better performance at the osmoregulatory level.

The intestine of fish has a dual function; it is involved in nutrient absorption and osmoregulatory mechanisms, being particularly challenging in SW teleost (Collie and Ferraris, 1995). Nutrient uptake occurs predominantly in the anterior intestine, while osmoregulatory processes are present along the whole intestine (Loretz, 1995). In our experimental conditions, comparisons between FW and SW animals must be interpreted with caution, considering the different feeding statuses of the fish in the two environmental salinities. One of the objectives of the present study was to recreate the conditions usually adopted in the aquaculture systems where fish receive daily meals. Still, feeding is withheld in the initial period in SW, due to the lack of appetite in the fish after the transfer (Usher *et al.*, 1991a). In FW fish, undigested food was still present in the intestine on sampling days, while it was absent in SW fish. Therefore, the results may be due to a cumulative effect of feeding mechanisms and pre-adaptative changes in FW animals. At the same time, they are exclusively due to osmoregulation processes in SW. Despite this subtle dissimilarity in conditions, the present study results allow us to describe changes in the intestinal response in FW and SW that occur differently in relation to the group considered.

Ion movement across the epithelium and the intestinal fluid absorption relies on the activity of the basolateral Na^+/K^+ -ATPase (NKA). After SW adaptation, salmonids species generally show a higher intestinal NKA activity in SW than in FW. However,

in the present study, NKA activity in the intestine of small and large fish did not significantly differ between the last period in FW and the initial SW phase (Fig. 2.5). The dissimilarity in results could be due to the different residence times in SW, lower in this study than the previous, and experimental conditions (Colin *et al.*, 1985; Fuentes *et al.*, 1997). A further deviation is represented by the similar NKA values observed between anterior and posterior intestine, for most of the groups investigated (Fig. 2.5), unlike previous studies in trout (Colin *et al.*, 1985; Nielsen *et al.*, 1999). This may be associated with the increase in the posterior intestine functionality after smoltification, with levels of activity similar to those measured in the anterior intestine. The activity of NKA was affected by the light treatment. In the last period in FW, in fact, the posterior intestine of small fish and the anterior intestine of large fish (Fig. 2.5B, C) showed higher NKA activity in groups that went through short photoperiod, indicating a potentially higher readiness of these groups for SW entry.

Active absorptive processes in the intestine of Atlantic salmon were marginally affected by the experimental conditions used in the present study (Fig. 2.6), however, the transepithelial resistance (R_t) showed photoperiod dependence with lower values for the short photoperiod groups (SP-LLs) (Fig. 2.10). Short-day experience could represent a signal for the increased need of intestinal absorption by the reduction of selectivity in the intestine.

The functional differences between the anterior and posterior intestine were reflected in the basal bioelectrical properties of the two regions. Under symmetric conditions in the Ussing chamber, the small and large fish showed prevailing absorbing processes, both in FW and SW, but with different magnitude for the anterior and posterior intestine (Fig. 2.6). Also, the selectivity of the two regions showed differences as previously described (Loretz, 1995). The anterior intestine (Fig. 2.10A and Fig. 2.10C) did not show differences in the resistance between FW and SW as observed in eel (Ando *et al.*, 1975), while the resistance in the posterior intestine (Fig. 2.10B and Fig. 2.10D) tended to be lower after seven days in SW than before SW exposure. The reduction of tissue resistance after transfer to SW was previously observed in Coho salmon (Collie, 1985). However, later studies in Atlantic salmon and rainbow trout showed an increase in the transepithelial resistance in SW fish (Sundell *et al.*, 2003; Sundell and Sundh, 2012). Differences between our data and these studies are likely related to fish size and time in SW.

The putative effects of light and dietary treatments in intestinal ion transport

were tested using pharmacological experiments in the Ussing chamber, focusing on Cl^- and Na^+ movements. The coupled flow of both ions in the enterocytes was analyzed using bumetanide as the specific inhibitor of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) (Fig. 2.7), of which NKCC2 represents the apical isoform (Gamba, 2005), and HCTZ, a specific inhibitor of the apical Na^+/Cl^- cotransporter (NCC) (Fig. 2.8) (Bazzini *et al.*, 2005). Our results confirm the presence of functional NKCC2 and NCC in the apical side of the enterocytes of the Atlantic salmon intestine. However, the inhibitory effect of bumetanide and HCTZ on the absorptive current was poorly affected by the light and diet, showing an independent functioning of NKCC2 and NCC pathways from the treatments used. The pharmacological experiment with bumetanide (Fig. 2.7) also showed a regional distribution of NKCC2 with more functional protein (larger bumetanide-sensitive effects) in the anterior intestine, particularly in FW animals, and in line with observations in other marine teleosts (Ando *et al.*, 2014; Fuentes *et al.*, 2018).

Even if the immediate need for SW teleosts is Cl^- absorption to take up water from the ingested SW, Cl^- efflux and fluid secretion can be observed in teleost as demonstrated in killifish (Marshall *et al.*, 2002a), Japanese eel (Ando *et al.*, 2014) and toadfish (Ruhr *et al.*, 2014). The capacity to move Cl^- in both directions could be related to a different localization of the cystic fibrosis transmembrane conductance regulator channels (CFTR) present in the apical and/or basolateral membrane of the enterocytes or in cytosolic vesicles established in several species (Bodinier *et al.*, 2009; Gregório *et al.*, 2013; Marshall *et al.*, 2002; Marshall and Singer, 2002; Wong *et al.*, 2016). Studies in killifish and sea bream highlight the importance of CFTR during SW acclimation (Gregório *et al.*, 2013; Marshall and Singer, 2002) while gene expression studies in eel, showed higher expression of *Cftr* in FW animals suggesting an important role also in FW, to maintain hydration, decreasing fluid viscosity, and avoiding obstruction during the passage of the intestinal content (Mekuchi *et al.*, 2013; Wong *et al.*, 2016). In Atlantic salmon gills, two different isoforms of *Cftr* were identified for the first time in an organism, *CftrI* and *CftrII* (Chen *et al.*, 2001), and both isoforms are also expressed in the intestine (Sundh *et al.*, 2014). In gills, Singer *et al.*, (2002) suggested a temporal response of the two isoforms during SW acclimation of Atlantic salmon, with *cftrII* involved in the early response to SW and *cftrI* in more extended periods. In the present study, the pharmacological manipulation with FK + IBMX was carried out to increase intracellular cAMP, thus stimulating CFTR activity by the cAMP-

PKA signaling pathway (Moon *et al.*, 2015). Surprisingly, the responses to cAMP stimulation were heterogeneous, *i.e.* in some groups I_{sc} increased to show secretory currents, and in other groups current levels were more absorptive after cAMP stimulation (Fig. 2.9). The only likely explanation for these observations is the distribution of the Cftr protein to the apical or basolateral membrane of the enterocytes, to drive the chloride current to secretion or absorption respectively. The basolateral located channels could facilitate the mobilization of Cl^- into the bloodstream for subsequent elimination at the gill level (Evans *et al.*, 2005), while the apical CFTR could be involved in Cl^- recycling to ensure the functioning of the Cl^-/HCO_3^- exchangers for carbonate aggregate formation (Bodinier *et al.*, 2009; Grosell *et al.*, 2005). In our study, stimulation of the cAMP showed differences in response to the treatments received and the environmental salinity, suggesting an essential function of CFTR in the modulation of intestinal osmoregulatory processes in Atlantic salmon. Immunohistochemistry studies could clarify CFTR distribution in the enterocytes and help to better understand the functional role of this channel during SW acclimation in Atlantic salmon, but despite several attempts, also by us, a specific antibody for Cftr in Atlantic salmon has not yet been optimized.

In conclusion, the present study showed a functional specialization of the anterior and posterior intestine in FW and upon transfer to SW in Atlantic salmon. While the anterior intestine is strongly involved in absorptive processes already in FW, the posterior intestine undergoes physiological maturation to optimize its function when in SW. The intestinal fluid composition upon transfer to SW may become an advantageous index to predict SW performance in juvenile Atlantic salmon. Low osmolality, combined with low levels of chloride and sodium ions (much lower than plasma levels), indicate, in fact, proper processing of the ingested SW, as expected in well-acclimated SW fish.

Experimental manipulation of parr with photoperiod and diet does not have a significant impact in larger Atlantic salmon parr. However, smaller fish respond to the combination of both experimental factors with a better performance at the intestinal level after SW transfer, possibly contributing to improved growth (Striberny *et al.*, 2021). Thus, our data indicate that, for smaller smolts, the combination of light “as winter signal” and dietary treatment with salt mix plus free tryptophan can enhance adaptation and performance in SW of Atlantic salmon in the aquaculture system.

2.5. References

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CHAPTER

3

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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Resumen

La transferencia al agua de mar (SW) representa una etapa crítica en la producción del salmón del Atlántico. El éxito de la transferencia se relaciona con el desarrollo óptimo de las capacidades hipo-osmorreguladoras durante el proceso de esmoltificación. Aunque se han adoptado diversas estrategias en acuicultura para estimular la esmoltificación, aún se produce una considerable pérdida de peces después de la transferencia a jaulas marinas. Por lo tanto, en este estudio investigamos las respuestas moleculares en los intestinos anterior y posterior del salmón Atlántico, después de 1) un tratamiento de fotoperiodo (24 horas de luz (L): 0 horas de oscuridad (D) → 24L:0D vs. 7L:17D → 24L:0D) y 2) tratamiento con dieta funcional (alimentación regular o alimentación enriquecida con una mezcla de sal/triptófano), combinado con o sin un tratamiento fotoperiódico en agua dulce (FW). El objetivo fue evaluar cómo se modulan los mecanismos hipo-osmorreguladores intestinales con estos tratamientos, e identificar posibles marcadores intestinales indicativos de un smolt preparado para SW. Utilizando PCR cuantitativa en tiempo real (qPCR), investigamos los niveles de transcripción de transportadores y canales involucrados en los movimientos iónicos a través de los enterocitos, componentes de las uniones estrechas y receptores celulares. Las dos regiones intestinales mostraron perfiles genéticos y respuestas diferentes ante los tratamientos experimentales. En el intestino anterior, la exposición a un fotoperiodo corto (7L:17D) aumentó la expresión de *nkaa1c*, *nkcc1*, *nkcc2*, *slc26a6* y *cftr1*, tanto en FW como en SW. Además, *nkaa1b*, *ocln* y *prlr* se expresaron al alza en FW, y *cldn15* en grupos de SW expuestos a este fotoperiodo. El intestino posterior fue menos sensible a los tratamientos experimentales, aunque se observó una regulación al alza de *nkcc1*, *nkcc2*, *slc26a6* y *cftr1* en FW en los grupos de fotoperiodo corto. Por lo tanto, nuestros resultados muestran que la exposición a una señal invernal en FW activa de manera más efectiva los mecanismos hipo-osmorreguladores en el intestino del salmón del Atlántico, donde un papel coordinado y complementario de los intestinos anterior y posterior garantizan un procesamiento óptimo del agua de mar ingerida. El tratamiento con dietas funcionales tuvo un efecto positivo, pero más marginal, en la regulación de los genes investigados, mejorando principalmente el impacto del fotoperiodo corto cuando se combinaron ambos tratamientos. En general, proponemos el cotransportador apical de $\text{Na}^+/\text{K}^+/\text{2Cl}^-$, *nkcc2*, y el intercambiador apical de $\text{Cl}^-/\text{HCO}_3^-$,

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slc26a6, como posibles marcadores moleculares de agua dulce en el intestino anterior para evaluar la "preparación para SW" en los smolts de salmón del Atlántico.

Palabras clave: salmón del Atlántico, fotoperiodo, tratamiento dietético, intestino, marcador molecular, hipo-osmorregulación.

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, here we investigated the molecular responses in the anterior and posterior intestines of Atlantic salmon, following 1) a photoperiod treatment (24h light (L):0h dark (D) → 24L:0D vs. 7L:17D → 24L: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 cellular receptors. The two intestinal regions showed different gene profiles and responsiveness towards the experimental treatments. In the anterior intestine, the exposure to short photoperiod (7L:17D) upregulated *nkaα1c*, *nkcc1*, *nkcc2*, *slc26a6*, and *cftr1*, in FW and SW. Also, *nkaα1b*, *ocln*, and *prlr* were upregulated in FW and *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 *cftr1* 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 intestines 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 $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, *nkcc2*, and the apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger, *slc26a6*, as potential FW molecular markers in the anterior intestine to assess “SW-readiness” in Atlantic salmon smolts.

Keywords: Atlantic salmon, photoperiod, dietary treatment, intestine, molecular marker, hypo-osmoregulation.

3.1. 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 (≤ 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 that foresee 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 (GIT) 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⁻/HCO₃⁻ exchangers (AEs) belonging to the solute carrier family 26 (Slc26) (Grosell *et al.*, 2009; Kurita *et al.*, 2008). The functioning of the Cl⁻/HCO₃⁻ exchangers promotes Cl⁻ absorption but also leads to high luminal HCO₃⁻ concentrations and alkalinization of the intestinal fluid, which cause CaCO₃ and MgCO₃ 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), Atlantic salmon included (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 $\alpha 1a$ and $\alpha 1b$ 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 *nka α 1a*, *nka α 1b*, *nka α 1c*, *nkcc1*, *nkcc2*, *slc26a3*, *slc26a6*, *cftrI*, *cftrII*, *ocln*, *cldn15*, *casr*, *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.

3.2. Materials and methods

3.2.1. 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 (24L:0D) and in FW at 4 °C till two weeks before the start of the experiment (6th February, 2018). The temperature was increased by 0.5 °C /day to 10°C for two weeks.

On 6th February, 2018, 1400 fish (body mass of ~40 g) 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 7h of light and 17h of darkness (7L:17D, “SP” groups), and the others remained under 24h of light (24L:0D, “LL” groups). At the end of the six weeks under a short photoperiod for SP groups (21st March), all fish were brought back to continuous light (24L: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 11th May, 50 fish from each treatment were transferred to 33‰ SW at 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 7h 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 (body mass of ~105 g). 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.

Table 3.1. 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

3.2.2. 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.

3.2.3. 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*, *ef1 α* , and *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 a single reference gene for anterior and posterior intestines comparisons. Therefore, transcriptomic data available for the anterior and posterior intestines 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 3.2 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 3.2. 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>).

3.2.4. Quantitative real-time PCR (qPCR)

Real-time PCR amplifications were performed in duplicate for all the samples in a final volume of 6 μ l with 1X SensiFAST SYBR Hi-ROX (Bioline), optimal concentrations of forward and reverse primers (from 200 to 300 nM, Table 3.2), and 1 μ L of 1:6 diluted cDNA. For *18S*, *ef1 α* , 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

Table 3.2. 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)	T_a (°C)	E (%)	R^2	NCBI acc. n./Refer.
<i>18S rRNA</i>	18S ribosomal RNA	F: CCTGCGGCTTAATTTGACTC R: AACCAGACAAATCGCTCCAC	139	200	58	94.2	0.994	AJ427629.1
<i>actb</i>	Beta actin	F: ACACCTTCTACAACGAGCTGAG R: TGATCTGGGTCATCTTCTCCCT	105	200	60	96.6	0.980	AF012125.1
<i>arpc1b</i>	Actin-related protein 2/3 complex subunit 1b	F: CGCGTATGCTGGTCTCTGAA R: GCAGATGGATATGAGGCGTGA	145	200	60	95.5	0.995	XM_045690514.1
<i>ef1a</i>	Elongation factor 1 alpha	F: AGGCATTGACAAGAGAACCATT R: TGATACCACGCTCCCTCTC	119	200	60	100.7	0.986	Striberny and Jørgensen, 2017
<i>b2m</i>	Beta-2-microglobulin	F: CAAATGGCACAGCTTCTGATG R: GTGTTCAAATCTAATGGTACCA	125	200	60	95.5	0.999	NM_001123699.1
<i>ubap2</i>	Ubiquitin-associated protein 2	F: GGAATGAGATGTTTCGAGCAGG R: GCTAACAAGTCTGATTTGGGAAGG	130	200	60	94.2	0.998	XM_014142130.2
<i>nkaa1a</i>	Na ⁺ /K ⁺ - ATPase subunit alpha 1a	F: CCAGGATCACTCAATGTCACTCT R: TCTATCAAAGGCAATGAGTTTAATATCATTGTAAAA	93	250	60	100.0	0.997	Striberny <i>et al.</i> , 2021
<i>nkaa1b</i>	Na ⁺ /K ⁺ - ATPase subunit alpha 1b	F: GAGGTTGGGTGGAACAGGAG R: AGCTGAGTGCACCATCGCAG	162	250	60	102.1	0.999	Striberny <i>et al.</i> , 2021
<i>nkaa1c</i>	Na ⁺ /K ⁺ - ATPase subunit alpha 1c	F: GGTGTCTTGCTGCTCGGAATT R: GGAGCTGTGCTTGCTTCATCA	113	250	60	102.1	0.995	AY319389.1
<i>nkcc1</i>	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter 1	F: ACGTGTCCCACATCTCAG R: GAGGGCTTGGATGAGTCT	121	200	60	95.5	0.991	Kiilerich <i>et al.</i> , 2007b
<i>nkcc2</i>	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter 2	F: AAAGACCAACCTCCAGGTCA R: AACCAGGCCATGTAGAGGTG	129	200	60	106.1	0.991	XM_014127389.1
<i>cftr1</i>	Cystic fibrosis transmembrane conductance regulator I	F: ACGACCCCTGTCCAAAGATAG R: TGTAAACGCAGGTAGGTGTTCC	131	250	60	93.6	0.997	AF155237.1
<i>cftrII</i>	Cystic fibrosis transmembrane conductance regulator II	F: TGCTTAAGGTTAGTGCCCTCAGG R: AAGGCTACTTCAGGTTAATCAC	111	250	60	97.4	0.999	NM_001123534.1
<i>slc26a3</i>	Cl ⁻ /HCO ₃ ⁻ exchanger	F: CTTCTTGGCACTTCCAGAC R: ACTCTCTGCTCATCGCTGGT	150	250	60	91.2	0.992	XM_021577256.1
<i>slc26a6</i>	Cl ⁻ /HCO ₃ ⁻ exchanger	F: GCTGAGCACTTTTCCTGTCC R: GAGCATAGGCCATACCCTGA	125	250	60	91.9	0.993	XM_014134693.1
<i>ocln</i>	Occludin	F: AAGGCAGAGATGTGAAGGACTG R: GATGCTATTGGCTGAGGCATTG	119	300	60	99.9	0.995	XM_014123589.2
<i>cldn15</i>	Claudin 15	F: GAGGCAACCGGATTCTTCATGT R: GTTCTCATACTGTCGGGTGGAG	126	300	60	99.3	0.998	XM_014139145.2
<i>casr</i>	Calcium sensing receptor	F: AATGGGAGCACCAAGTTTCAG R: AATGTCCTGTAGGGCCTGTG	147	200	60	98.3	0.999	NM_001123571.1
<i>prlr</i>	Prolactin receptor	F: CCAGATTCATCAGATGGGAGGG R: GGATCTGCTGAGATGGTACTCG	135	200	60	99.6	0.997	DQ508436.1

primer pair specific annealing temperature (T_a , Table 3.2) 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 3.2). The range of acceptance for the amplification efficiency was set between 90% and 110%.

3.2.5. 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 intergroup 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 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 intestines, was used to normalize the target genes investigated.

3.2.6. Statistical analysis

All results are shown as mean $\pm$ 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 intestines. All statistical analyses were performed using GraphPad Prism 8.4.0 for macOS (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at $p < 0.05$.

3.3. Results

3.3.1. 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 intestines (Duarte *et al.*, 2023; Gaetano *et al.*, 2023).

3.3.2. 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 intestines 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. Tab. 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. Tab. 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 intestines was *18S* - *actb* with a stability value of 0.182 (Suppl. Tab. 1).

3.3.3. mRNA abundance of target genes

3.3.3.1. Light and diet effect

In the anterior intestine of Atlantic salmon, light treatment significantly regulated in FW the mRNA levels of *nkaa1b* (Fig. 3.1C), *nkaa1c* (Fig. 3.1E), *nkcc1* (Fig. 3.2A), *nkcc2* (Fig. 3.2C), *slc26a6* (Fig. 3.3C), *cftrl* (Fig. 3.4A), *ocln* (Fig. 3.5A), and *prlr* (Fig. 3.6C), and in SW the levels of *nkaa1a* (Fig. 3.1A), *nkaa1c* (Fig. 3.1E), *nkcc1* (Fig. 3.2A), *nkcc2* (Fig. 3.2C), *slc26a6* (Fig. 3.3C), *cftrl* (Fig. 3.4A), and *cldn15* (Fig. 3.5C) (two-way ANOVA). For these genes, higher mRNA levels were observed in the groups exposed to the SP-LL photoperiod, except for *nkaa1a*, which showed lower transcript levels in the SP-LL groups than in the LL-LL groups (Fig. 3.1A). In the posterior intestine, *nkcc1* (Fig. 3.2B), *nkcc2* (Fig. 3.2D), *slc26a6* (Fig. 3.3D), and *cftrl* (Fig. 3.4B) 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. 3.3B).

Dietary treatment significantly affected the mRNA levels of the genes investigated only in FW fish. In the anterior intestine, dietary treatment affected *nkaa1b* (Fig. 3.1C), *nkaa1c* (Fig. 3.1E), *nkcc2* (Fig. 3.2C), and *ocln* (Fig. 3.5A) (two-way ANOVA), with higher mRNA levels observed for the groups treated with the experimental diet. Increased mRNA levels were also observed for *nkcc1* in the posterior intestine of FW fish (two-way ANOVA; Fig. 3.2B). mRNA levels of *cftrl* 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. 3.4C, 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. 3.3C). 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. 3.2A). In the posterior intestine, the interaction of light and diet was observed in FW for *nka α 1a* (Fig. 3.1B), *cftrI* (Fig. 3.4B), *cftrII* (Fig. 3.4D), and *casr* (Fig. 3.6B) (two-way ANOVA followed by Bonferroni *post-hoc* test). The Bonferroni *post-hoc* test revealed significantly higher *nka α 1a* mRNA levels in the LL-LL C group than in the SP-LL C (Fig. 3.1B), higher *cftrI* levels in the SP-LL C than in the LL-LL C group (Fig. 3.4B), higher *cftrII* levels in the SP-LL C group than in the SP-LL + diet (Fig. 3.4D), and higher *casr* levels in the LL-LL C group than in LL-LL + diet and SP-LL C groups (Fig. 3.6B). In SW, significant interaction between light and diet was observed in the posterior intestine for *nka α 1a* (Fig. 3.1B), *nka α 1b* (Fig. 3.1D), *nkcc1* (Fig. 3.2B) and *casr* (Fig. 3.6B) (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. 3.6B).

The regulation dynamics of the genes investigated in this study are summarized in a heatmap in Fig. 3.7.

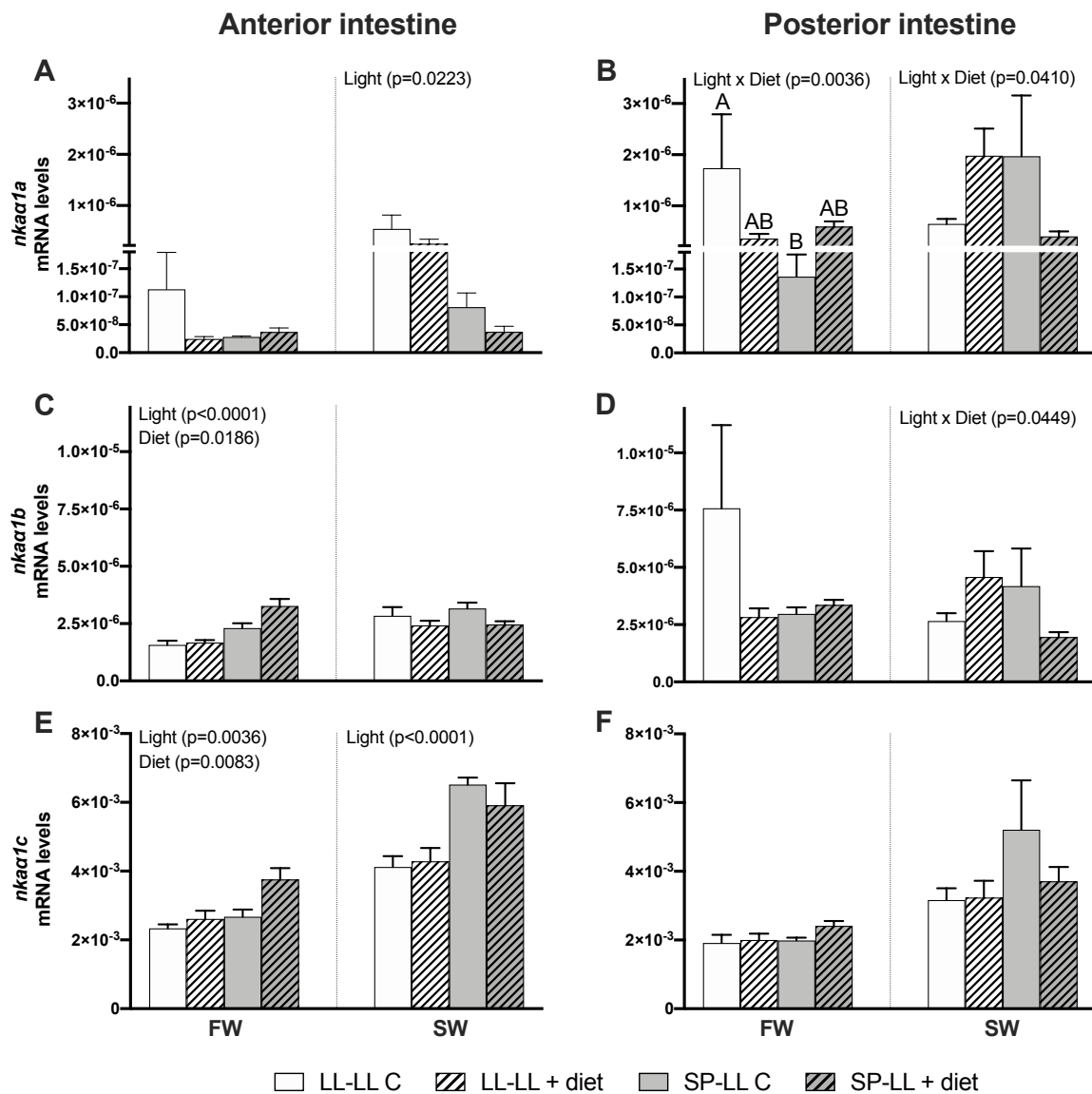


Figure 3.1. Relative *nkaa1a* (A, B), *nkaa1b* (C, D), and *nkaa1c* (E, F) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean $\pm$ 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.

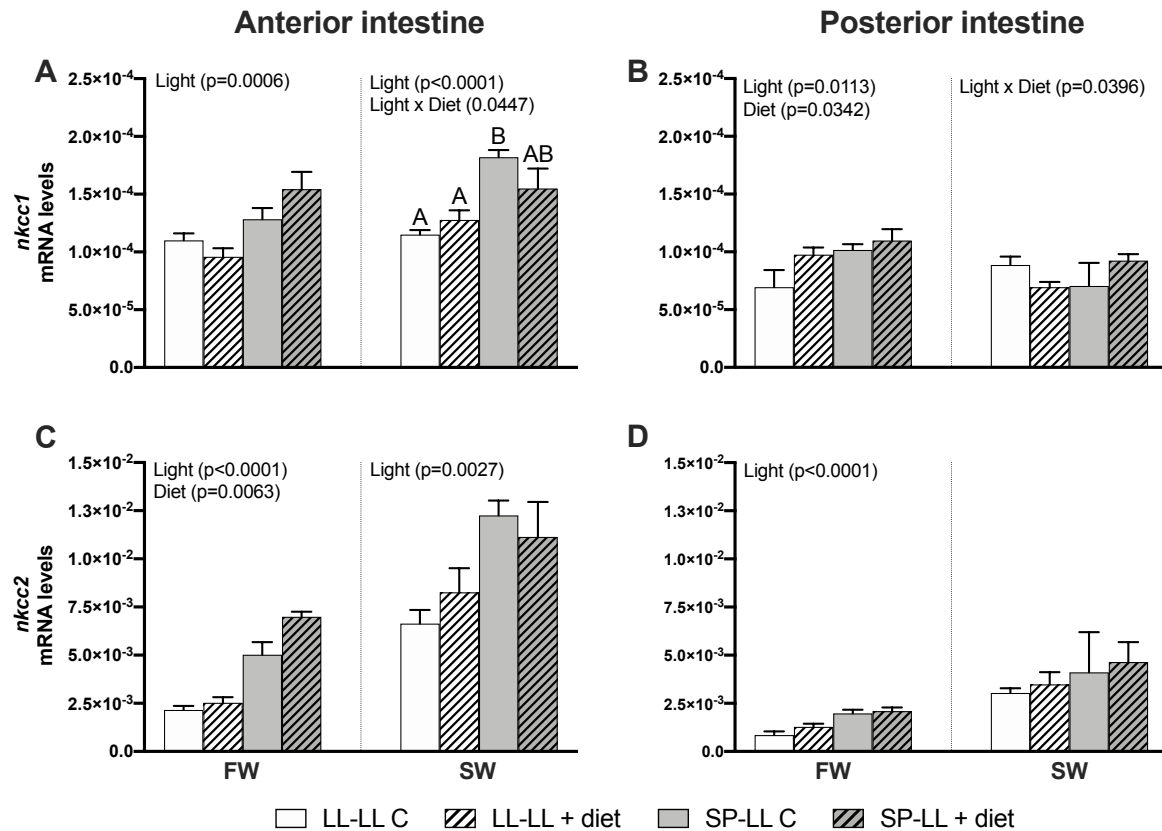


Figure 3.2. Relative *nkcc1* (A, B) and *nkcc2* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean $\pm$ 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.

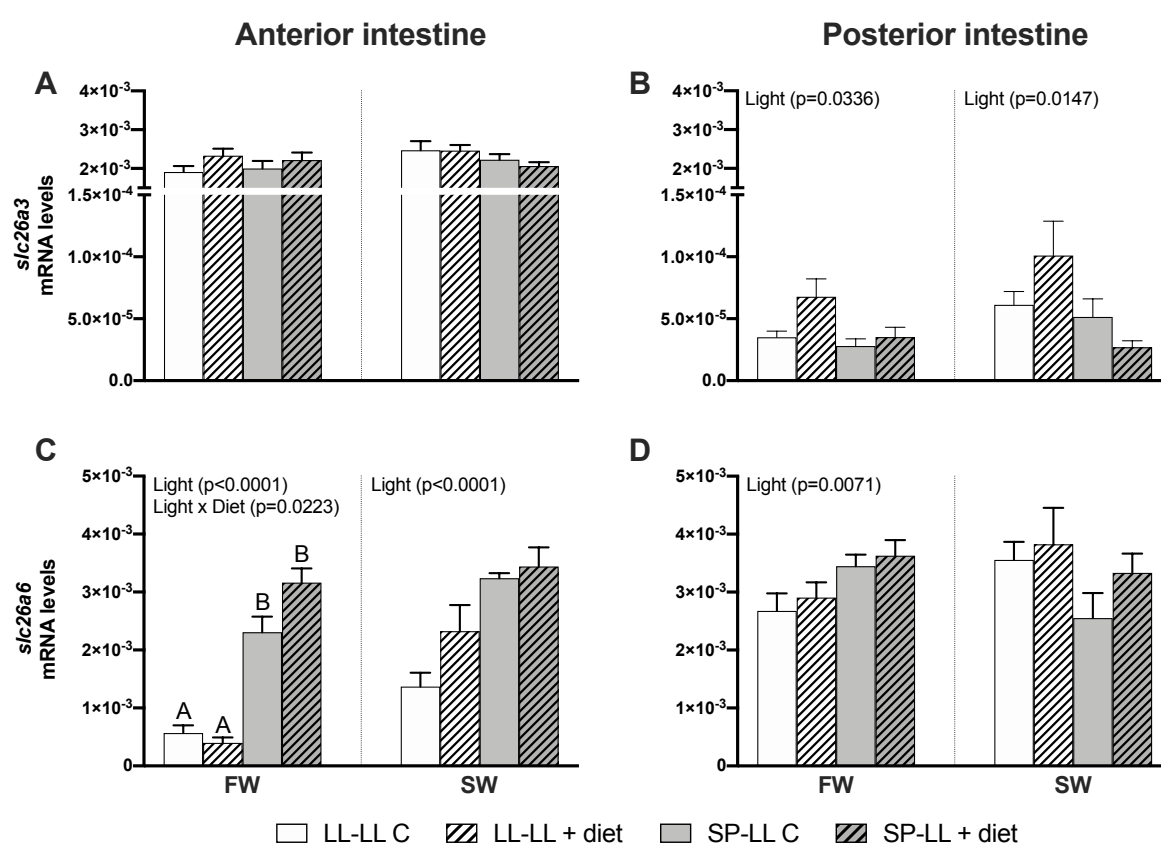


Figure 3.3. Relative *slc26a3* (A, B) and *slc26a6* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean $\pm$ 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.

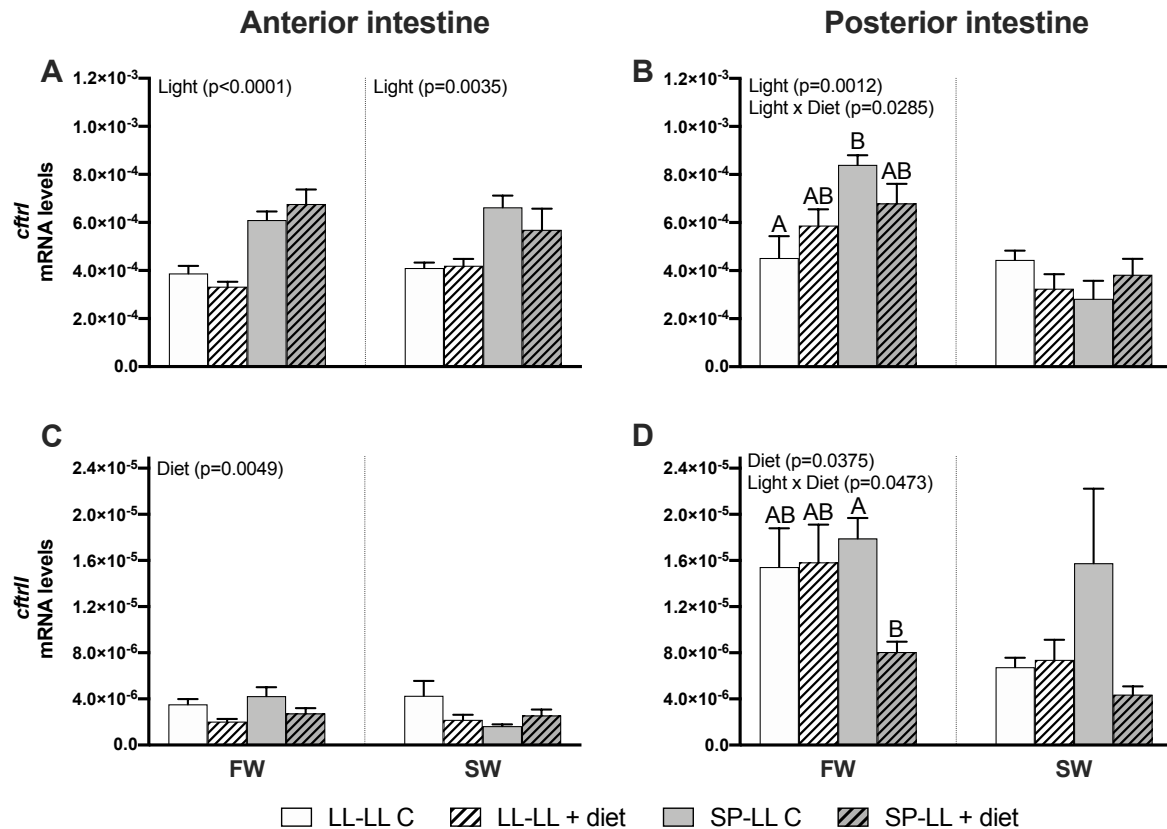


Figure 3.4. Relative *cftrI* (A, B) and *cftrII* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean $\pm$ 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.

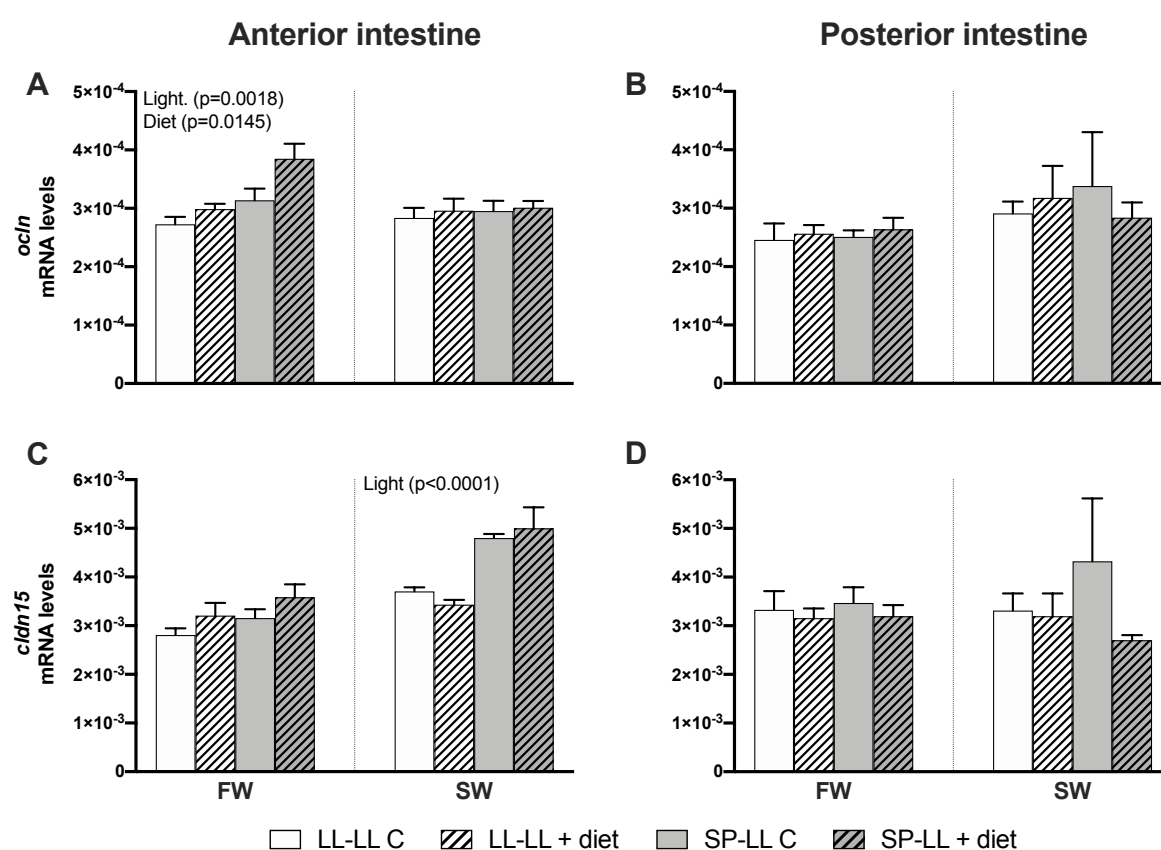


Figure 3.5. Relative *ocln* (A, B) and *cldn15* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean $\pm$ 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.

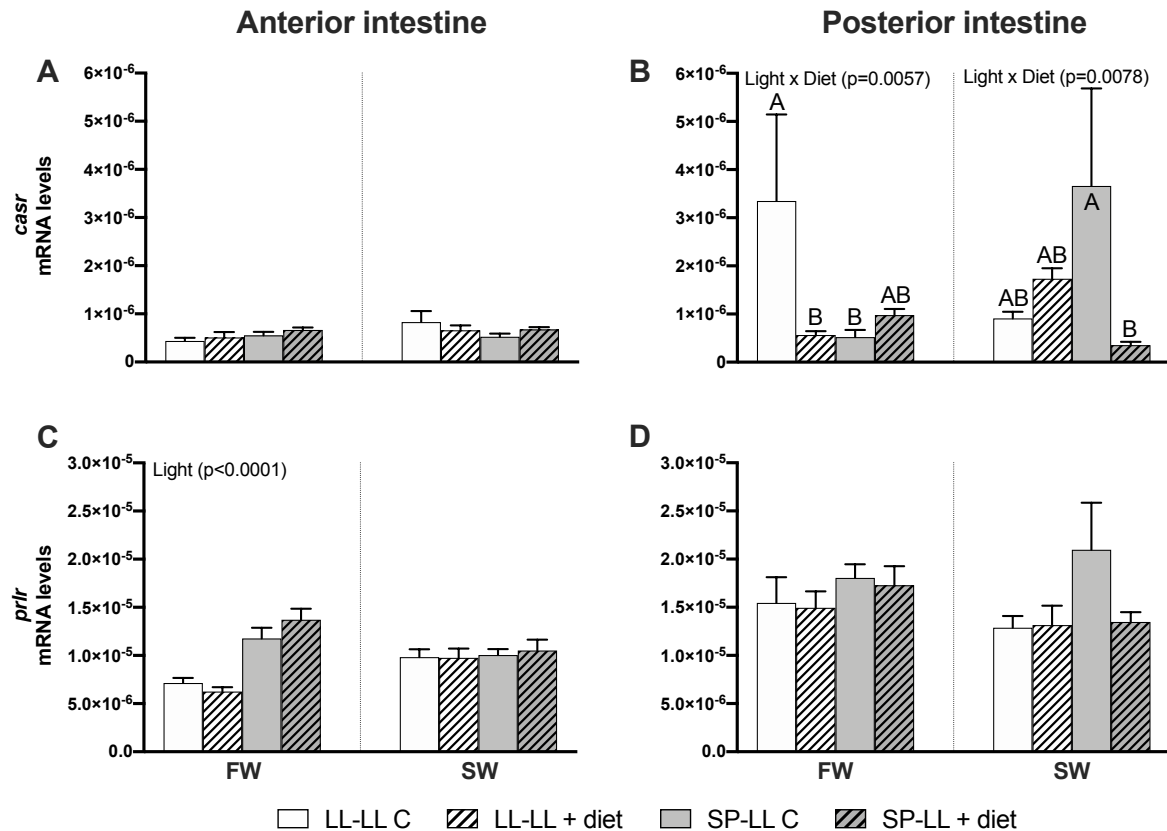


Figure 3.6. Relative *casr* (A, B) and *prlr* (C, D) mRNA levels in the anterior intestine (left) and posterior intestine (right) of Atlantic salmon (*Salmo salar*) in FW and after seven days in SW. Target genes are normalized using the geometric mean between *18S* and *actb*. Data are shown as mean $\pm$ 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.

3.3.3.2. Anterior intestine vs. posterior intestine

In FW, the posterior intestine of Atlantic salmon showed significantly higher *nkaa1a* and *cftrll* mRNA levels than the anterior intestine in all the experimental groups (unpaired t-test; Table 3.3). Moreover, higher mRNA levels in the posterior intestine were observed for *nkaa1b* in the LL-LL + diet group, *slc26a6* in the LL-LL groups and SP-LL C, *cftrl* 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 3.3). *nkcc2* and *slc26a3* levels were significantly higher in the anterior intestine of all FW groups (unpaired t-test; Table 3.3). Higher mRNA levels in the anterior intestine were also observed for *nkaa1c* 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 3.3).

When comparing the two intestinal regions in SW, significantly higher mRNA levels were observed in the posterior intestine for *nkaa1a* in the groups LL-LL + diet and SP-LL + diet, *slc26a6* in the LL-LL C groups, *cftrll* in the LL-LL + diet and SP-LL groups, and for *casr* in the group LL-LL + diet (unpaired t-test; Table 3.3). 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 3.3). Also, higher levels in the anterior intestine were detected for *nkaa1c* in SP-LL + diet group, *cftrl* in SP-LL C, *cldn15* in SP-LL + diet group, and for *casr* in the group SP-LL + diet (unpaired t-test; Table 3.3).

Table 3.3. 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 intestines. 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
<i>nkaa1a</i>	0.0362	0.0007	0.0239	<0.0001
<i>nkaa1b</i>	0.0789	0.0111	0.0734	0.8073
<i>nkaa1c</i>	0.0806	0.0540	0.0098	0.0017
<i>nkcc1</i>	0.0103	0.8298	0.0333	0.0196
<i>nkcc2</i>	0.0005	0.0015	0.0003	<0.0001
<i>slc26a3</i>	<0.0001	<0.0001	<0.0001	<0.0001
<i>slc26a6</i>	<0.0001	<0.0001	0.0031	0.2354
<i>cftrl</i>	0.5840	0.0017	0.0005	0.9556
<i>cftrlI</i>	0.0002	<0.0001	<0.0001	<0.0001
<i>ocln</i>	0.3384	0.0375	0.0167	0.0014
<i>cldn15</i>	0.1529	0.8857	0.4132	0.2857
<i>casr</i>	0.0363	0.5879	0.6024	0.0378
<i>prlr</i>	0.0010	<0.0001	0.0022	0.1518
Seawater (SW)				
GENE	LL-LL C	LL-LL + diet	SP-LL C	SP-LL + diet
<i>nkaa1a</i>	0.2328	0.0035	0.0548	0.0004
<i>nkaa1b</i>	0.7223	0.0776	0.5195	0.0653
<i>nkaa1c</i>	0.0512	0.0876	0.1996	0.0095
<i>nkcc1</i>	0.0077	<0.0001	0.0003	0.0028
<i>nkcc2</i>	0.0005	0.0036	0.0056	0.0068
<i>slc26a3</i>	<0.0001	<0.0001	<0.0001	<0.0001
<i>slc26a6</i>	<0.0001	0.0544	0.0756	0.8078
<i>cftrl</i>	0.5053	0.1299	0.0021	0.1861
<i>cftrlI</i>	0.0604	0.0116	0.0137	0.0447
<i>ocln</i>	0.7745	0.7033	0.6562	0.5703
<i>cldn15</i>	0.3233	0.6083	0.7592	0.0003
<i>casr</i>	0.5582	0.0002	0.0880	0.0041
<i>prlr</i>	0.0512	0.1665	0.0574	0.0645
AI > PI		PI > AI		

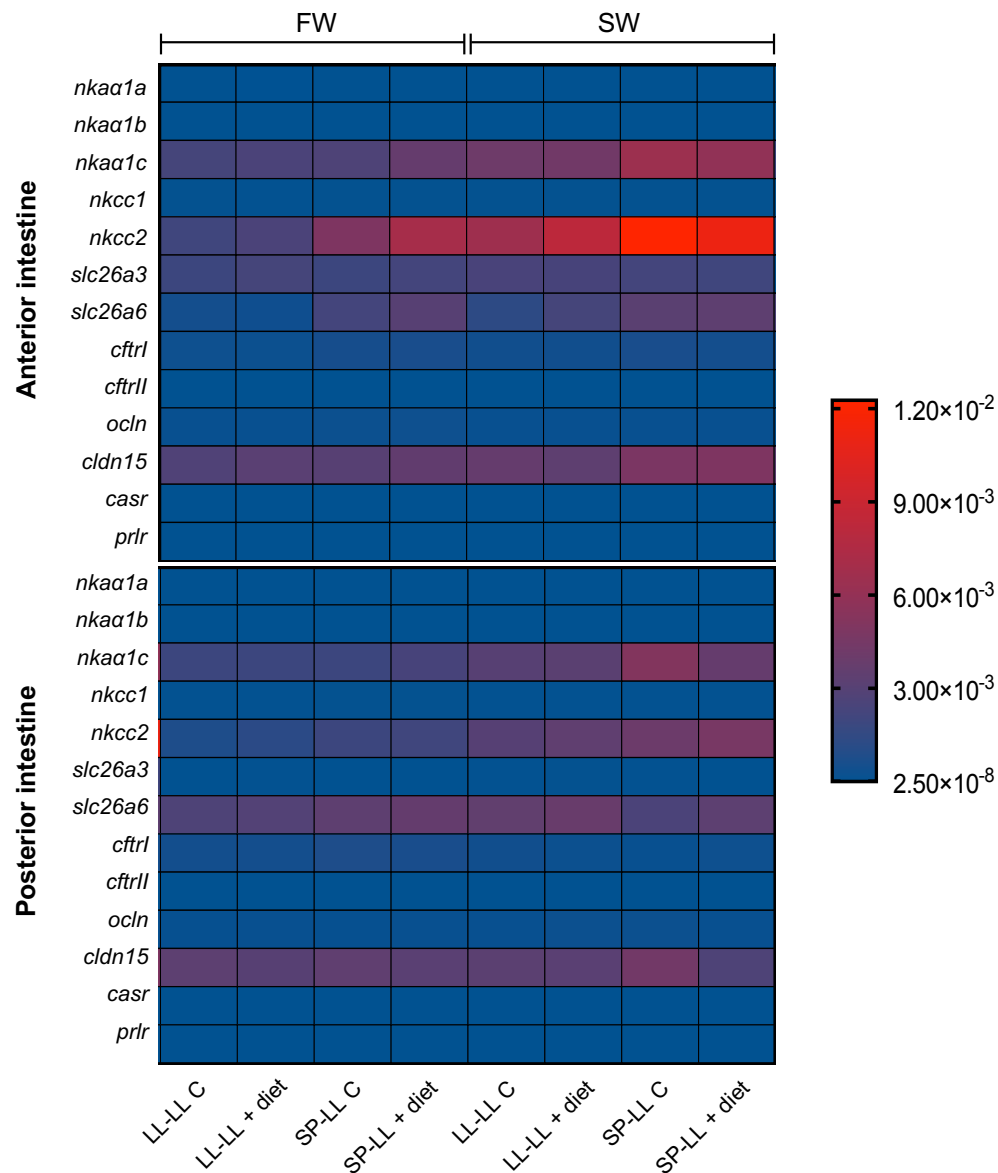


Figure 3.7. Heatmap summarizing the relative mRNA levels of the genes investigated in the anterior and posterior intestines of Atlantic salmon (*Salmo salar*) after exposure to light and dietary treatment in FW and following seven days of residence in SW.

3.4. Discussion

The present study used quantitative real-time PCR (qPCR) to investigate 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.

Discrete regions of the intestine display morphological, molecular, and functional differences in teleosts (Alves *et al.*, 2019; Gregório *et al.*, 2013; Ruhr *et al.*, 2014; Ruiz-Jarabo *et al.*, 2017), salmonids included (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 intestines' 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 intestines (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 intestines 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 intestines 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 3.3), the two regions were differently affected by the photoperiod and dietary treatments used in this study. The putative SW-enhancing treatments strongly modulate the anterior intestine, while the posterior intestine is 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 endocrine factors, like growth hormone (GH) and cortisol, are directly connected with regulating hypo-osmoregulatory mechanisms 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), Arctic 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). 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*). Although the effect of the diet on the regulation of the molecular pathway investigated was marginal compared to the upregulation caused by the short photoperiod, a reorganization of the enterocytes at the molecular level occurred in response to the dietary treatment. 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. 3.6A, 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). For the latter, NKA creates the electrochemical gradient required to energize ion movements across fish enterocytes, and its activity is often higher after adaptation to SW (Barany *et al.*, 2020; Sundell *et al.*, 2003), but not always (Gaetano *et al.*, 2023). This study analyzed three isoforms of the α catalytic subunit of the NKA, *nkaα1a*, *nkaα1b*, *nkaα1c*. Contrary to the observations in salmonid gills (Nilsen *et al.*, 2007; Richards *et al.*, 2003), 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. 3.1A-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. 3.1E), as previously observed (Sundh *et al.*, 2014; Tipsmark *et al.*, 2010a), reaffirms the

crucial role of the isoform $\alpha 1c$ 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). In SW fish, NKCC2 removes 1Na^+ , 1K^+ , and 2Cl^- from the intestinal lumen, contributing to a sensible reduction of the osmotic pressure, thus favoring water absorption from the ingested SW. 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. 3.2C), 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). Furthermore, these results further confirm higher pan-NKCC immunodetection on the apical enterocyte membrane due to the accumulation of Nkcc2 (Duarte *et al.*, 2023). 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. 3.4A-D), and both isoforms exhibited different mRNA abundance between intestinal regions (Table 3.3); 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. These results suggest 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 intestines suggested the presence of CFTR 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, apically 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 $\text{Cl}^-/\text{HCO}_3^-$ 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. 3.2A, B) may support the involvement of this secretory pathway in the short photoperiod groups occurring with a similar pattern observed for *cftrl*, particularly in the anterior intestine (Fig. 3.2A and 3.4A). 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. Only immunohistochemistry studies could define the distribution of the two isoforms in the enterocytes and 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 the hypo-osmoregulatory mechanisms of fish as it promotes water absorption from the intestinal fluid reducing its osmolality in a double way: it enables Cl^- entry in the enterocytes in exchange for HCO_3^- , and the latter sustains Ca^{2+} and Mg^{2+} precipitation as intestinal precipitates in the lumen (Grosell *et al.*, 2005, 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 intestines (Fig. 3.3A-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. 3.3C), reaching levels matching those of SW fish and the posterior intestine (Fig. 3.3C, D). Given the relevant role of $\text{Cl}^-/\text{HCO}_3^-$ 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. 3.5A-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. 3.5A, 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. 3.5B, 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-translation 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; PRL, instead, is considered to have an inhibitory effect (McCormick, 2013). PRL plasma levels decrease during smoltification in Atlantic salmon (Prunet *et al.*, 1989), as well as the transcript levels of PRL receptors (*prlr*) in the gill (Kiillerich *et al.*, 2007a). But the dynamics of the *prlr* in the intestine are still unknown. Considering this and the inhibitory action of PRL on the intestinal bicarbonate secretion demonstrated in the sea bream (Ferlazzo *et al.*, 2012), we investigated *prlr* in the anterior and posterior intestines. The mRNA levels of *prlr* were marginally regulated by the treatments used in this study (Fig. 3.6C, 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. 3.6C). 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 24h SW challenge through the smoltification period (Kiillerich *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. Four main findings yielded from our work:

1. The exposure to a winter signal, followed by an increase in day length, plays a critical role in the efficient activation of the intestinal molecular mechanisms required for the processing of the ingested SW in Atlantic salmon.
2. The dietary treatment, involving a combination of salt mix and free tryptophan, enhances at the molecular levels some of the pathways associated with the hypo-osmoregulatory capacity in the intestine and synergizes with the short photoperiod, improving its action. However, it cannot be the sole stimulating factor.
3. Atlantic salmon’s anterior and posterior intestines exhibit distinct responses towards photoperiod and dietary treatment, and their characteristic

transcriptional profile reflects the coordinated and complementary role of these two regions in SW processing.

4. The apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, *nkcc2*, and the apical $\text{Cl}^-/\text{HCO}_3^-$ 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.

3.5. References

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CHAPTER

4

Cortisol regulates the osmoregulatory functions of the intestine in Atlantic salmon (*Salmo salar*).

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Resumen

La adquisición de la capacidad hipo-osmorreguladora, necesaria para la exitosa transición al agua de mar (SW) en el salmón del Atlántico anádromo (*Salmo salar*), es regulada por factores endocrinos. Aunque se ha demostrado que el cortisol afecta la capacidad secretora de cloruro en las branquias, su papel en la función intestinal es menos claro. Este estudio examinó la influencia del cortisol exógeno y la salinidad en la capacidad hipo-osmorreguladora intestinal y en la regulación de transportadores iónicos. Parr y post-smolts de salmón Atlántico en agua dulce (FW) recibieron implantes intraperitoneales con 0 $\mu\text{g g}^{-1}$ o 40 $\mu\text{g g}^{-1}$ de cortisol. Doce días después de la inyección, la mitad de los peces fueron expuestos a 25 ppt (parr) o 30 ppt (post-smolts) de SW durante 48 h. El tratamiento con cortisol resultó en una disminución significativa en la osmolalidad del plasma y las concentraciones de cloruro en los parr después de 48 h en SW en comparación con los controles. El cortisol aumentó la actividad de la NKA en las branquias y el intestino anterior en parr y post-smolts en FW y SW. En SW, los peces tratados con cortisol mostraron un volumen intestinal reducido y una menor masa de precipitados de carbonato. Los niveles de ARNm de *nka α 1c* y *nkcc2* fueron regulados al alza por el cortisol y la exposición a SW. Además, el cortisol también reguló positivamente los niveles de ARNm de genes involucrados en la vía de secreción intestinal de HCO_3^- (*slc26a3*, *slc26a6*, *nbc1*, *atp6v1a* y *gn*), de manera región y salinidad dependiente. Por otro lado, se observaron incrementos en los niveles de ARNm en respuesta al cortisol también para los receptores *gr1*, *gr2* y *mr*, especialmente en el intestino posterior de los parr. Nuestras observaciones indican que el cortisol mejora la tolerancia a la salinidad en parr y postsmolts del salmón del Atlántico e identifican los transportadores de iones clave regulados por el cortisol, incluyendo las vías involucradas en la secreción de HCO_3^- y la precipitación de carbonato.

Palabras clave: parr, post-smolts, salmón del Atlántico, intestino, hipo-osmorregulación, cortisol, precipitados intestinales.

Abstract

The acquisition of hypo-osmoregulatory capacity, necessary for the successful transition to seawater (SW) in anadromous Atlantic salmon (*Salmo salar*), is regulated by endocrine factors. Although cortisol has been shown to affect the salt secretory capacity of the gill, its role in intestinal function is less clear. This study examined the influence of exogenous cortisol and salinity on intestinal osmoregulatory capacity and ion transporter regulation. Atlantic salmon parr and post-smolts in freshwater (FW) were given intraperitoneal implants with 0 $\mu\text{g g}^{-1}$ or 40 $\mu\text{g g}^{-1}$ cortisol. Twelve days after injection, half of the fish were exposed to 25 ppt (parr) or 30 ppt (post-smolts) SW for 48 h. Cortisol treatment resulted in a significant decrease in plasma osmolality and chloride concentrations in parr after 48 h in SW compared to controls. Cortisol increased NKA activity in the gill and anterior intestine in parr and post-smolts in FW and SW. In SW, cortisol-treated fish showed reduced intestinal fluid volume and a lower mass of carbonate precipitates. The mRNA levels of *nka α 1c* and *nkcc2* were upregulated by cortisol and SW exposure. Cortisol also elevated mRNA levels of genes involved in the intestinal HCO_3^- secretion pathway (*slc26a3*, *slc26a6*, *nbc1*, *atp6v1a*, and *gn*), in a salinity- and region-dependent manner. Increased mRNA levels in response to cortisol were also observed for the receptors *gr1*, *gr2*, and *mr*, particularly in the posterior intestine of parr. Our findings indicate that cortisol enhances salinity tolerance in parr and post-smolts of Atlantic salmon and identify key ion transporters regulated by cortisol, including the pathways involved in the HCO_3^- secretion and carbonate precipitation.

Keywords: parr, post-smolts, Atlantic salmon, intestine, hypo-osmoregulation, cortisol, intestinal precipitates.

4.1. Introduction

Hydromineral imbalance is a universal challenge among teleost species. Due to the osmotic differences between their extracellular body fluids (approximately 300-350 mOsm kg⁻¹) and the surrounding environment, fish passively lose ions and gain water in freshwater (FW), and face ion gain and water loss in seawater (SW). Complex physiological mechanisms have evolved to counteract these imbalances and maintain ionic and osmotic homeostasis, primarily involving the gill, gastrointestinal tract (GIT), and kidney. In FW, fish absorb ions mainly through the gill and produce large amounts of dilute urine, whereas, in SW, fish drink the external medium to absorb water and eliminate the excess of ions primarily across the gill (Marshall and Grosell, 2006).

In teleosts, the GIT is a multifunctional organ central to osmoregulatory mechanisms. In FW, ion absorption associated with nutrient uptake complements the hyper-osmoregulatory action of the gill and kidney (Wood and Bucking, 2010). However, in SW, the GIT assumes a more central role in maintaining ionic and water balance. Marine teleosts drink SW to compensate for the passive water loss into the surrounding hyperosmotic environment (Fuentes and Eddy, 1997; Perrott *et al.*, 1992), and along the GIT SW is processed to promote water absorption (Grosell, 2014). The esophagus selectively absorbs Na⁺ and Cl⁻, reducing the NaCl content of the ingested SW by approximately 50% (Hirano and Mayer Gostan, 1976; Parmelee and Renfro, 1983; Takei *et al.*, 2017). In the intestine, ion concentration is further reduced to allow water absorption. The ions gained along the GIT are subsequently excreted through the gill and kidney, ensuring overall ionic and osmotic balance (Larsen *et al.*, 2014).

The inward cotransport of Na⁺ and Cl⁻ from the intestinal lumen takes place via NKCC2 and NCC cotransporters (Trischitta *et al.*, 1992; Watanabe *et al.*, 2011), energized by the electrochemical gradient generated by the activity of the basolateral Na⁺/K⁺-ATPase (NKA) (Skou and Esmann, 1992). A significant influx of Cl⁻ also occurs in exchange for HCO₃⁻ mediated by anion exchanges (AEs). This pathway, whose contribution is accountable for ~50% of Cl⁻ uptake in the lemon sole (*Parophrys vetulus*) (Grosell *et al.*, 1999), relies on apical DIDS-sensitive anion exchangers belonging to the Slc26 family (Grosell *et al.*, 2001). Studies in different species identified Slc26a3 and particularly Slc26a6 as the family members involved in the apical Cl⁻/HCO₃⁻ exchange in the intestine of SW-acclimated species (Alves *et al.*, 2019; Gregório *et al.*, 2013; Grosell *et al.*, 2009b; Kurita *et al.*, 2008; Ruiz-Jarabo *et*

al., 2017b; Takei *et al.*, 2019). HCO_3^- originates in part from the hydration reaction of metabolic CO_2 , mediated by the cytosolic carbonic anhydrase (CAc) (Grosell *et al.*, 2009a, 2007; Grosell and Genz, 2006), and in part enters the enterocyte along with Na^+ through the basolateral cotransporter $\text{Na}^+/\text{HCO}_3^-$ (NBC1, Slc4a4) (Kurita *et al.*, 2008; Taylor *et al.*, 2010). In addition to contributing to the net Cl^- uptake, AEs lead to the luminal precipitation of carbonates (ichthyocarbonates). The high HCO_3^- concentrations and the alkaline pH of the lumen cause the precipitation of Ca^{2+} and, to a lesser extent, of Mg^{2+} , both present in the ingested SW and concentrated in the intestinal lumen, as CaCO_3 and MgCO_3 ($\text{Ca}(\text{Mg})\text{CO}_3$) precipitates, causing a further reduction of the intestinal fluid osmolality which favors water absorption (Grosell and Oehlert, 2023; Walsh *et al.*, 1991; Wilson *et al.*, 2002).

Euryhaline teleosts exhibit the capacity to thrive in a wide range of salinity, owing to their ability to regulate the osmoregulatory mechanisms in response to the demands of the surrounding environment. The transition of euryhaline species from FW to SW requires physiological adjustments within their osmoregulatory organs, which are governed by the endocrine system (Takei *et al.*, 2014). Various hormones regulate osmoregulatory processes in teleost fishes, and cortisol is one of the primary hormones involved. Under the regulatory control of the hypothalamic-pituitary-interrenal axis (HPI axis), the interrenal cells of teleosts synthesize and release cortisol that regulates several physiological functions including metabolism, growth, osmoregulation, stress response, and immune function (Aluru and Vijayan, 2009; Gorissen and Flik, 2016; Mommsen *et al.*, 1999). While it was initially identified as a hormone primarily associated with SW adaptation, growing evidence demonstrates its involvement in osmoregulatory mechanisms in FW and brackish water (BW) as well (Chasiotis and Kelly, 2011; Cruz *et al.*, 2013; Mancera *et al.*, 2002, 1994; Takagi *et al.*, 2011), confirming the dual osmoregulatory function of this hormone (McCormick, 2001). While the effect of cortisol on the gill of SW fish is well-established (Chang *et al.*, 2023; Kiillerich *et al.*, 2007a, 2011b; Ojima *et al.*, 2009; Pelis and McCormick, 2001), its effect on the intestine is less clear. Early studies on Atlantic salmon and rainbow trout showed that cortisol treatment in FW increases the drinking response upon transfer to SW (Fuentes *et al.*, 1996). In eels, cortisol enhances ion and water absorption in the intestine (Hirano and Utida, 1968; Utida *et al.*, 1972), and similar results were also observed in salmonids (Sundell and Sundh, 2012; Veillette *et al.*,

1995). Cortisol also increases the expression of aquaporins in the intestine of European eel (*Anguilla anguilla*) (Martinez *et al.*, 2005) and silver sea bream (*Sparus sarba*) (Deane *et al.*, 2011). However, the molecular (ion transporter) targets of cortisol and its role in the intestine alkalization have not been established.

Anadromous Atlantic salmon migrate from FW to SW as part of its life cycle (Thorstad *et al.*, 2011). The downstream migration of juveniles is preceded by smoltification (also smolting or parr-smolt transformation), a complex endocrine-regulated set of morphological, physiological, and behavioral changes preparatory for migration and SW entry (Hoar, 1988; McCormick, 2013). During smoltification, a fundamental change is the development of salinity tolerance, which is driven in part by increases in plasma cortisol which occur in spring (McCormick, 2013; McCormick *et al.*, 2019; Shrimpton and McCormick, 1998). Changes driven by cortisol in the gill have received more attention than in other osmoregulatory organs and include enhancement of the abundance of key proteins required for ion secretion in SW, including Nka, Nkcc, and Cftr (Kiillerich *et al.*, 2007a; McCormick *et al.*, 2008; Pelis and McCormick, 2001), and regulation of the mRNA levels of tight junction components genes, like *cldn10e*, associated with the formation of the leaky epithelia characteristic of SW gill (Tipsmark *et al.*, 2009). Studies employing exogenous cortisol demonstrated that in the intestine, cortisol mediates the increased fluid absorption capacity of the posterior intestine observed during smoltification (Cornell *et al.*, 1994; Veillette *et al.*, 1995) and regulates transcriptional levels of tight junction components genes (Tipsmark *et al.*, 2010). However, several mechanisms underlying the signaling action mediated by cortisol in the intestine of Atlantic salmon are still unknown. By employing an integrative approach, this study aimed to characterize the action of cortisol on the development of hypo-osmoregulatory capacity in the intestine of parr and post-smolt Atlantic salmon. Special emphasis was given to the molecular mechanisms linked to the HCO_3^- secretion cascade. While the physiological relevance of this pathway in maintaining water balance in SW fish is well-established, to our knowledge, no studies have explored its role in Atlantic salmon physiology or investigated the potential regulatory role of cortisol on HCO_3^- secretion and carbonate precipitation in teleosts.

4.2. Materials and methods

4.2.1. Animals and holding conditions

Atlantic salmon, 0+ parr, were obtained from the U.S. Department of Agriculture Hatchery in Franklin, Maine, and reared at the USGS Conte Anadromous Fish Laboratory (Turners Falls, MA, USA) for 3 months until they reached ~18 g. Atlantic salmon post-smolts were obtained from the Kensington State Fish Hatchery (Kensington, CT, USA) as parr and reared at the USGS Conte Anadromous Fish Laboratory for one year until they reached ~85 g. Each group was kept in several 1.5 m diameter tanks supplied with flow-through dechlorinated tap water with a seasonal temperature profile (8-15 °C) under a natural photoperiod. Fish were fed *ad libitum* twice daily with commercial dry pellets (Bio-Oregon, Longview, WA, USA). Feed was withheld for 24 hours before injection. Experiments were carried out between July and November 2021.

All rearing and experiments were conducted in accordance with U.S. Geological Survey institutional guidelines and an approved IACUC protocol (LSC-9070).

4.2.2. Experimental design and samples generation

On the day of injection, fish were randomly netted, anesthetized with MS-222 (100 mg L⁻¹; pH 7.0), and injected intraperitoneally with 5 µL g⁻¹ body mass (M_b) of the vehicle (1:1 shortening and vegetable oil) (Specker *et al.*, 1994) to reach doses of 0 µg g⁻¹ (control) or 40 µg g⁻¹ cortisol (hydrocortisone, Pfaltz & Bauer, Waterbury, CT, USA). To identify control and treated fish, animals were paint tagged with different colors between the anal fin rays. After 30 min of recovery, injected fish were transferred to 1 m diameter tanks supplied with 15 °C flow-through dechlorinated tap water (FW). Twelve days after injection, half of the fish were transferred to artificial seawater (SW) (Crystal Sea, Marine Enterprises International, Baltimore, MD, USA) in 1 m diameter tanks with recirculated system and temperature control (15 °C). The water salinity used for parr experiments was 25 ppt (maximum salinity that allows for direct transfer of this life stage), while for post-smolts, it was 30 ppt. The remaining fish were subjected to a FW-FW transfer. Both FW and SW tanks were fitted with a particle, charcoal, and biological recirculating system, and the temperature was maintained at 15 °C. Forty-eight hours after transfer fish were anesthetized with MS-222 (200 mg L⁻¹; pH 7.0), the morphometric parameters were measured, and blood

was collected from the caudal peduncle into heparinized syringes within 5 min of netting. Body mass (M_b , g), fork length (L_f , cm), and total length (L_t , cm) were assessed for each fish. The intestine was isolated from the end of the pyloric caeca to the anal sphincter, and the intestinal content was emptied in pre-weighted tubes for successive analysis. Gill, anterior intestine (posterior to the pyloric caeca till the ileorectal sphincter), and posterior intestine (posterior to the ileorectal sphincter) were collected and placed in either 100 μL ice-cold SEI buffer (250 mmol L^{-1} sucrose, 10 mmol L^{-1} EDTA, 50 mmol L^{-1} imidazole, pH 7.3) and immediately frozen and stored at -80°C or in RNALater (Invitrogen, Waltham, MA, USA) at 4°C for 24 h, and frozen at -20°C until RNA extraction. Due to the small size of parr, measurement of bioelectrical properties was carried out only for the anterior intestine of post-smolts.

After injection, feed was limited. Parr were fed to satiation on day 4 and 8 post-injection, while post-smolts on day 6. Since cortisol is known to influence food intake, this limited ration was used to minimize the confounding influence of food intake without starving fish.

4.2.3. Plasma and intestinal fluid analysis

Plasma cortisol levels were measured using a validated direct competitive enzyme immunoassay as described by (Carey and McCormick, 1998). The standard curve ranged from 1 to 320 ng mL^{-1} . Sensitivity as defined by the standard curve was determined to be 0.3 ng mL^{-1} . The average inter-assay variation was 6.22% and intra-assay variation was 3.23%.

Plasma and intestinal fluid samples, collected from parr and post-smolts, were analyzed to determine osmolality and ionic composition in individual fish. Plasma osmolality was measured in duplicate using a Vapor Pressure Osmometer (Wescor, Logan, UT, USA) using a sample volume of 8-10 μL . For the intestinal fluid, a single measurement was carried out due to the limited volume available, but when possible, the measurement was done in duplicate. Plasma chloride concentrations were measured by the Ag:Cl titration method using a Buchler-Cotlove chloridometer (Labconco, Kansas City, MO, USA). Due to the lower concentrations and volume in the intestine, chloride intestinal fluid concentrations were determined with a commercial colorimetric kit (BioAssay Systems, Hayward, CA, USA). Calcium and magnesium concentrations in plasma and intestinal fluid were also measured using

commercial colorimetric kits (BioAssay Systems, Hayward, CA, USA). Colorimetric assays were run in duplicate in 96-well microplates following the manufacturer instructions, on a BioTek Synergy 2 spectrophotometer (BioTek Instruments, Winooski, VT, USA).

4.2.4. Na^+/K^+ - ATPase activity

NKA activity assays were carried out in gill and anterior intestine samples as described by McCormick (1993). The specific activity ($\mu\text{mol ADP mg protein}^{-1} \text{ h}^{-1}$) was determined by linking ATP hydrolysis to the nicotinamide adenine dinucleotide (NADH) oxidation. Ten microliters of each sample were run in duplicate in 96-well microplates at 340 nm for 10 min at 25°C on a BioTek Synergy 2 spectrophotometer (BioTek Instruments, Winooski, VT, USA). The NKA activity was calculated by the difference between total ATP hydrolysis in the absence and presence of 0.5 mmol L⁻¹ ouabain. Protein content was determined on the homogenate using a BCA protein assay (Thermo Fisher Scientific, Rockford, IL, USA).

4.2.5. Intestinal content and titratable alkalinity

Due to the almost complete absence of intestinal fluid in FW fish, the intestinal content collection occurred only for SW fish. The intestinal content was weighed within 30 min of collection and centrifuged (12,000 g, 6 min, RT) to separate the fluid from the precipitates. The intestinal fluid was transferred to clean pre-weighted tubes, and the volume was measured to the nearest 0.1 μL (0.1 mg assuming a density of 1). The aggregates were weighed to the nearest 0.1 mg. An aliquot of intestinal fluid was stored at 4 °C to measure HCO_3^- within 24h. The remaining intestinal fluid volume was frozen at 4 °C for further analysis.

Titratable alkalinity was manually measured in the intestinal fluid using the double titration method. Forty microliters of the intestinal fluid samples (20 μL for parr) were diluted in 10 mL NaCl (40 mmol L⁻¹), gassed with 100% O₂ for 30 min to remove the CO₂, and titrated to pH 3.8 with 5 mmol L⁻¹ HCl; an additional gassing period of 20 min was applied to remove any remaining CO₂, then the sample was back titrated to its original pH with 5 mmol L⁻¹ NaOH. Intestinal fluid HCO_3^- concentration was calculated from the volume difference between acid and base used during the titration and the molarity of the titrants (Gregório *et al.*, 2014).

4.2.6. Bioelectrical properties

On the sampling day, epithelial electrophysiology was performed in the freshly excised post-smolt anterior intestine (for FW and SW fish) in Ussing chambers to characterize intestinal bioelectrical properties under asymmetric conditions (Fuentes *et al.*, 2010). Tissues were washed with chilled fresh saline and mounted on tissue holders (0.25 cm^2) within five minutes of sacrifice. The holders were positioned between two half-Ussing chambers containing 5.5 mL of basolateral or apical saline, and kept at $15\text{ }^{\circ}\text{C}$. Before use and during the experiment, basolateral saline was gassed with $0.5\%\text{ CO}_2 + 99.5\%\text{ O}_2$, while apical saline was gassed with $100\%\text{ O}_2$. The composition of the two salines is reported in Table 4.1 After 30 min of tissue stabilization, the saline was replaced in both half chambers, and the preparations were left undisturbed for 2 h. During the experiment, transepithelial electrical potential (TEP, mV) was monitored every 30 min by clamping the epithelia to $0\text{ }\mu\text{A cm}^{-2}$ by mean Ag/AgCl electrodes (with tip asymmetry $< 1\text{ mV}$) to either side of the Ussing chamber with 3-mm-bore agar bridges (1 M KCl in $3\%\text{ agar}$) connected to a VCC MC6 Multichannel voltage/current clamp amplifier (with automatic fluid resistance compensation) (Physiological Instruments, San Diego, CA, USA). The TEP was referenced (grounded) to the apical side (mucosa). Transepithelial resistance (R_t , $\Omega\text{ cm}^2$) was manually calculated (Ohm's law) using the voltage deflections induced by $10\text{ }\mu\text{A cm}^{-2}$ bipolar pulses of 3 s every 57 s.

Table 4.1. Composition of salines used during current-clamp experiments in Ussing chamber.

	Basolateral saline (mmol L ⁻¹)	Apical saline (mmol L ⁻¹)
NaCl	135.0	40.0
KCl	2.5	2.6
MgSO ₄ · 7H ₂ O	1.0	126.0
HEPES - Na	5.0	-
NaHCO ₃	7.5	-
NaH ₂ PO ₄	1.0	-
Na ₂ HPO ₄	-	1,0
CaCl ₂ · 2H ₂ O	2.5	3,0
Glucose	10.0	-
pH	7.8	7.8
Osmolality (mmol/kg)	~ 310	~ 310

4.2.7. RNA extraction, cDNA synthesis, and quantitative real-time PCR

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. Assessment of RNA quantity and quality was performed using the NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was also 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 1.1 µg of total RNA in a reaction volume of 20 µL. Samples were stored at -20°C for further use. Primer pairs were extracted from previous publications, or they were designed using the web interface Primer3Plus (Untergasser *et al.*, 2012) as indicated in Table 4.2. Real-time PCR amplifications were performed in duplicate for all the samples in a final volume of 6 µL with 3 µL PerfeCTa SYBR Green SuperMix, ROX (Quanta BioSciences, MA, USA), optimal concentrations of forward and reverse primers (250 nM), 1 µL of 10x diluted cDNA (100x dilution for *ef1a* and *actb*; 1000x dilution for *18S*), and sterile nuclease-free water up to the final volume. 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 2 min, followed by 44 cycles of 95°C for 10 s and primer pair specific annealing temperature (T_a , Table 4.2) 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. For normalization of cDNA loading, all samples were run in parallel with several potential reference genes (Table 4.2). The stability of the reference genes was verified by the online tool RefFinder (Xie *et al.*, 2012). After analysis, *18S* and *arpc1b* were selected as reference genes, and their geometric mean was used for target gene normalization. To estimate efficiencies, a standard curve was generated for each primer pair from 10-fold serial dilutions of DNA cloned into pGEM-T Easy Vector. All calibration curves exhibited correlation coefficients $R^2 > 0.990$, and the corresponding real-time PCR efficiencies were between 90% and 110% (Table 4.2).

Table 4.2. 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), annealing temperature (T_a), qPCR efficiency (E), correlation coefficient (R^2), and NCBI accession number or reference.

Gene symbol	Gene name	Primer sequences (5' to 3')	Size (bp)	T_a (°C)	E (%)	R^2	NCBI acc. n./ Refer.
<i>18S rRNA</i>	18S ribosomal RNA	F: CCTGCGGCTTAATTTGACTC R: AACCAGACAAATCGCTCCAC	139	58	104.5	0.999	Chapter 3
<i>actb</i>	Beta actin	F: ACACCTTCTACAACGAGCTGAG R: TGATCTGGGTCATCTTCTCCCT	105	60	104.5	0.998	Chapter 3
<i>arpc1b</i>	Actin-related protein 2/3 complex subunit 1b	F: CGCGTATGTCTGGTCTCTGAA R: GCAGATGGATATGAGGCGTGA	145	60	104.4	0.999	Chapter 3
<i>ef1a</i>	Elongation factor 1 alpha	F: AGGCATTGACAAGAGAACCATT R: TGATACCACGCTCCCTCTC	119	60	101.4	0.996	Striberny and Jørgensen, 2017
<i>nkaa1c</i>	Na ⁺ /K ⁺ - ATPase subunit alpha 1c	F: GGTTGTCTTGCTGCTCGGAATT R: GGAGCTGTGTCTTGGTTCATCA	113	60	103.7	0.999	Chapter 3
<i>nkcc2</i>	Na ⁺ /K ⁺ /2Cl ⁻ cotransporter 2	F: AAAGACCAACCTCCAGGTCA R: AACCAGGCCATGTAGAGGTG	129	60	101.5	0.993	Chapter 3
<i>slc26a3</i>	Cl ⁻ /HCO ₃ ⁻ exchanger	F: CTTCTTGCCACTTCCAGAC R: ACTCTCTGCTCATCGCTGGT	150	60	103.6	0.997	Chapter 3
<i>slc26a6</i>	Cl ⁻ /HCO ₃ ⁻ exchanger	F: GCTGAGCACTTTTCTGTCC R: GAGCATAGGCCATACCCTGA	125	60	92.0	0.997	Chapter 3
<i>nbc1</i>	Na ⁺ /HCO ₃ ⁻ cotransporter	F: TGGACCTGTTCTGGGTAGCAA R: AGCACTGGGTCTCCATCTTCAG	123	60	97.1	0.996	NM_001124325.1
<i>atp6v1a</i>	vacuolar H ⁺ -ATPase subunit a	F: CTCTTCCCGTGTGTCCAGGGTG R: GACATCTCATTTCACGCTCTCC	146	60	100.6	0.996	XM_014129312.2
<i>gr1</i>	Glucocorticoid receptor 1	F: CCAGCGGTCCAACCTCATAAGAT R: GTGTTTTGTCGTGCTCTCCATC	136	60	98.7	0.998	XM_045717356.1
<i>gr2</i>	Glucocorticoid receptor 2	F: CATGGCAGACCAGTGTGAAC R: AGCAGCAGCAGAACCTTCAT	102	60	106.0	0.997	McCormick <i>et al.</i> , 2008
<i>mr</i>	Mineralocorticoid receptor	F: TATGGGTCTCTTCCAGTCAGGT R: GGCTTCGTAACCTGCCATTTCATC	127	60	105.8	0.999	XM_045722750.1
<i>gn</i>	Guanylin	F: CTAACCCTTCCCTGCCACAG R: GCAAAGGCACAGATCTCACA	115	60	99.7	0.998	XM_014133644.1

4.2.8. Calculations and statistical analysis

Body mass change (ΔM_b), Fulton's condition factor (K), and specific growth rate (SGR) were calculated as follows: $\Delta M_b = M_{b \text{ day14}} - M_{b \text{ day0}}$; $K = (M_{b \text{ day14}} \times L_{f \text{ day14}}^{-3}) \times 100$; $SGR = ((\ln M_{b \text{ day14}} - \ln M_{b \text{ day0}})/14) \times 100$. Due to the impossibility to identify individual fish in parr experiments, these parameters were calculated at the tank level per treatment.

All results are shown as mean $\pm$ SEM. Outliers were identified by the ROUT method, and homogeneity of variances and normality was assessed before statistical analysis. When necessary, data were transformed using the Log function for the qPCR data and the square root function for the other datasets to comply with ANOVA assumptions. For both parr and post-smolt, differences between experimental groups for plasma analysis (cortisol, osmolality, Cl^- , Ca^{2+} , and Mg^{2+}), NKA activity, transepithelial potential (TEP), transepithelial resistance (R_t), and mRNA levels were assessed by two-way ANOVA, considering cortisol dose (control and cortisol) and salinity (FW and SW) as main factors. Two-way ANOVA was followed by the Bonferroni *post-hoc* test when interaction between factors was detected. Biometric analysis (ΔM_b , K, and SGR), intestinal fluid analysis (osmolality, Cl^- , Ca^{2+} , Mg^{2+} , total volume, and HCO_3^-) and intestinal precipitates were analyzed with the unpaired t-test. All statistical analyses were performed using GraphPad Prism 8.4.0 for macOS (GraphPad Software, San Diego, CA, USA). Statistical significance was accepted at $p < 0.05$.

4.3. RESULTS

4.3.1. Mortality

During the 14 days of experiments, tanks were inspected at least once daily to check for mortality and fish welfare. No mortality was observed for post-smolts. Control parr did not show mortality in FW but had 31.5% mortality in SW, whereas cortisol-treated parr had a mortality of 8.3 % in FW.

4.3.2. Biometric analysis

On the sampling days, parr average M_b ranged between 16.2 g and 18.2 g, while in post-smolts ranged between 82.4 g and 91.1 g (Table 4.3). Body mass reduction was observed in the fish during the experimental period. The reduction was

higher for cortisol-injected fish but not significantly for either parr or post-smolts (Table 4.3). Also, K and SGR did not significantly differ between control and cortisol groups in either parr or post-smolts (Table 4.3).

Table 4.3. Fish body mass sampling day (M_b), body mass variation (ΔM_b), Fulton's condition factor (K), and specific growth rate (SGR) in parr and post-smolts of Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data presented as mean $\pm$ SEM. No significant differences observed between control and cortisol groups (unpaired t-test, $p < 0.05$).

		M_b (g)		ΔM_b (g)	K	SGR (%)
PARR	Control FW	18.2 $\pm$ 0.7	Control	-1.1 $\pm$ 0.04	0.98 $\pm$ 0.02	-0.54 $\pm$ 0.08
	Cortisol FW	16.2 $\pm$ 0.6				
	Control SW	16.6 $\pm$ 0.5	Cortisol	-1.7 $\pm$ 0.2	0.97 $\pm$ 0.01	-0.68 $\pm$ 0.08
	Cortisol SW	16.8 $\pm$ 0.6				
POST-SMOLT	Control FW	91.1 $\pm$ 6.2	Control	-8.6 $\pm$ 1.5	0.88 $\pm$ 0.02	-0.64 $\pm$ 0.1
	Cortisol FW	87.7 $\pm$ 6.2				
	Control SW	88.1 $\pm$ 4.9	Cortisol	-10.9 $\pm$ 1.0	0.85 $\pm$ 0.01	-0.87 $\pm$ 0.06
	Cortisol SW	82.4 $\pm$ 5.3				

4.3.3. Plasma cortisol, osmolality, and ion concentrations

Plasma cortisol levels were significantly affected by treatment in parr and post-smolts, with elevation of the levels in the cortisol groups in both FW and SW (Fig. 4.1A and 4.1B). In post-smolts, there was also a significant interaction between treatment and salinity, with cortisol plasma levels significantly higher in the cortisol-treated groups than in the control group in FW, but not in SW (Fig. 4.1B).

No difference in plasma osmolality or chloride was observed between control and cortisol-treated fish in FW (Table 4.4). SW significantly increased the plasma osmolality and chloride levels in both parr and post-smolts. In parr, cortisol treatment significantly reduced plasma osmolality and chloride levels in SW, and a similar trend was seen in post-smolts.

Plasma calcium concentrations in parr were affected by salinity, and interaction between treatment and salinity was observed (Table 4.4). Cortisol elevated plasma calcium levels in FW but not in SW (Table 4.4). SW exposure caused a significant increase in calcium concentration only in the control fish (Table 4.4). In post-smolts,

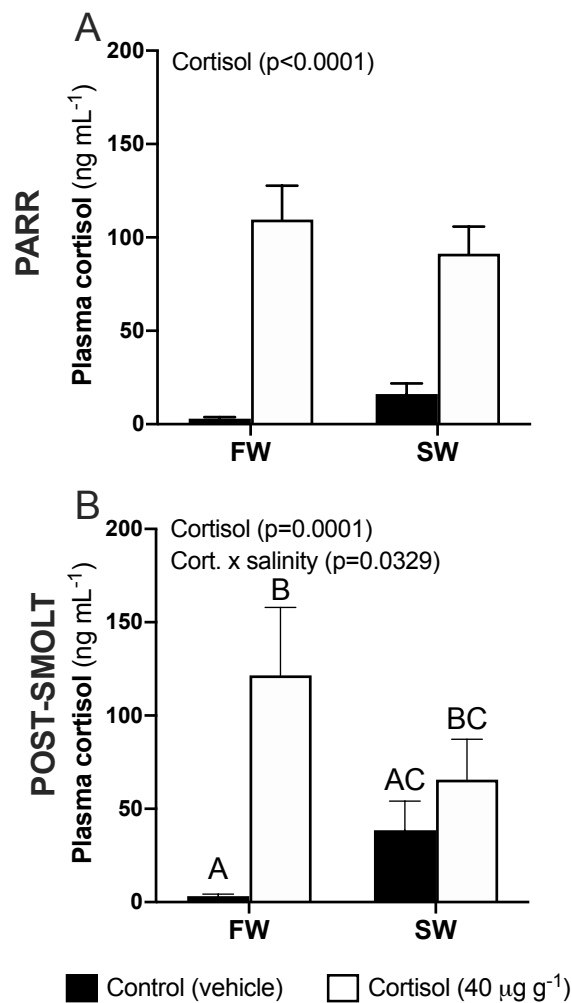


Figure 4.1. Plasma cortisol in parr (A) and post-smolt (B) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Each bar represents the mean $\pm$ SEM ($n=14-19$ for parr; $n=8-10$ for post-smolt). When a significant interaction between cortisol dose and salinity was observed with two-way ANOVA, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

plasma calcium concentrations were not affected by cortisol treatment or salinity (Table 4.4).

SW exposure caused a significant increase in plasma magnesium levels, particularly in the control group, in both parr and post-smolts (Table 4.4). In FW, plasma magnesium levels were similar in control and cortisol groups, but in SW lower levels were observed in the cortisol groups (Table 4.4).

4.3.4. Intestinal content analysis

Levels of intestinal fluid in FW fish were too low to be collected. In SW parr, the intestinal fluid osmolality of the control group was significantly higher than the cortisol-treated group, while in SW post-smolts, no significant difference was observed (Table 4.4). Despite a decreasing trend in cortisol groups, the concentration of intestinal fluid ions (Cl^- , Ca^{2+} , and Mg^{2+}) did not significantly change between the control and cortisol groups in either parr or in post-smolts (Table 4.4).

The volume of intestinal fluid collected in the SW control parr was significantly higher than the fluid collected in the cortisol-treated fish (Fig. 4.2A). Reduction of the intestinal fluid in the cortisol group was also observed in SW post-smolts; however, no significant difference was observed (Fig. 4.2D). Similarly, the mass of precipitates accumulated in the intestine was significantly lower in the cortisol-treated groups than in controls, for both SW parr and post-smolts (Fig. 4.2B, E). The HCO_3^- concentration measured in the intestinal fluid were in the range observed for marine teleosts and did not significantly change due to salinity or cortisol treatment in either life stages (Fig. 4.2C, F).

Table 4.4. Plasma and intestinal fluid osmolality and ion concentrations (Cl^- , Ca^{2+} and Mg^{2+}) in parr and post-smolt Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data are reported as mean $\pm$ SEM (n=11-20 for parr; n=9-10 for post-smolt). In the analysis of plasma, cortisol dose (F) and salinity (S) are the two considered factors (two-way ANOVA); when a significant interaction between the two factors (FxS) was observed, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Intestinal fluid data were analyzed by unpaired t-test. Statistical significance accepted at $p < 0.05$. Abbreviation “ns” indicates not significant statistical differences.

			PLASMA		INTESTINAL FLUID
			FW	SW	SW
PARR	Osmolality (mOsm Kg ⁻¹)	Control	300 $\pm$ 1 ^A	393 $\pm$ 10 ^B	382 $\pm$ 9
		Cortisol	307 $\pm$ 3 ^A	313 $\pm$ 2 ^A	310 $\pm$ 9
			F (p<0.0001); S (p<0.0001); FxS (p<0.0001)		(p<0.0001)
	Chloride (mmol L ⁻¹)	Control	133.8 $\pm$ 1.4 ^A	177.1 $\pm$ 4.4 ^B	36.84 $\pm$ 1.96
		Cortisol	135.5 $\pm$ 1.5 ^A	139.0 $\pm$ 1.6 ^A	33.60 $\pm$ 2.23
			F (p<0.0001); S (p<0.0001); FxS (p<0.0001)		(ns)
	Calcium (mmol L ⁻¹)	Control	2.35 $\pm$ 0.05 ^A	2.79 $\pm$ 0.07 ^B	14.19 $\pm$ 0.85
		Cortisol	2.59 $\pm$ 0.03 ^B	2.60 $\pm$ 0.06 ^B	12.26 $\pm$ 1.10
			F (ns); S (p=0.0002); FxS (p=0.0002)		(ns)
POST-SMOLT	Osmolality (mOsm Kg ⁻¹)	Control	0.76 $\pm$ 0.02	0.99 $\pm$ 0.05	156.4 $\pm$ 8.55
		Cortisol	0.75 $\pm$ 0.02	0.83 $\pm$ 0.04	155.8 $\pm$ 8.03
			F (p=0.0332); S (p=0.0002); FxS (ns)		(ns)
	Chloride (mmol L ⁻¹)	Control	303 $\pm$ 2	356 $\pm$ 16	349 $\pm$ 15
		Cortisol	310 $\pm$ 1	331 $\pm$ 10	327 $\pm$ 9
			F (ns); S (p=0.0005); FxS (ns)		(ns)
	Calcium (mmol L ⁻¹)	Control	148.6 $\pm$ 0.7	177.0 $\pm$ 8.4	32.16 $\pm$ 2.42
		Cortisol	150.5 $\pm$ 1.0	163.0 $\pm$ 4.7	31.23 $\pm$ 1.38
			F (ns); S (p=0.0002); FxS (ns)		(ns)
POST-SMOLT	Calcium (mmol L ⁻¹)	Control	2.73 $\pm$ 0.07	2.89 $\pm$ 0.05	14.30 $\pm$ 1.34
		Cortisol	2.80 $\pm$ 0.07	2.84 $\pm$ 0.07	12.82 $\pm$ 1.06
			F (ns); S (ns); FxS (ns)		(ns)
	Magnesium (mmol L ⁻¹)	Control	0.73 $\pm$ 0.03	0.93 $\pm$ 0.07	167.50 $\pm$ 9.05
		Cortisol	0.70 $\pm$ 0.02	0.76 $\pm$ 0.02	153.50 $\pm$ 7.43
			F (p=0.0212); S (p=0.0044); FxS (ns)		(ns)

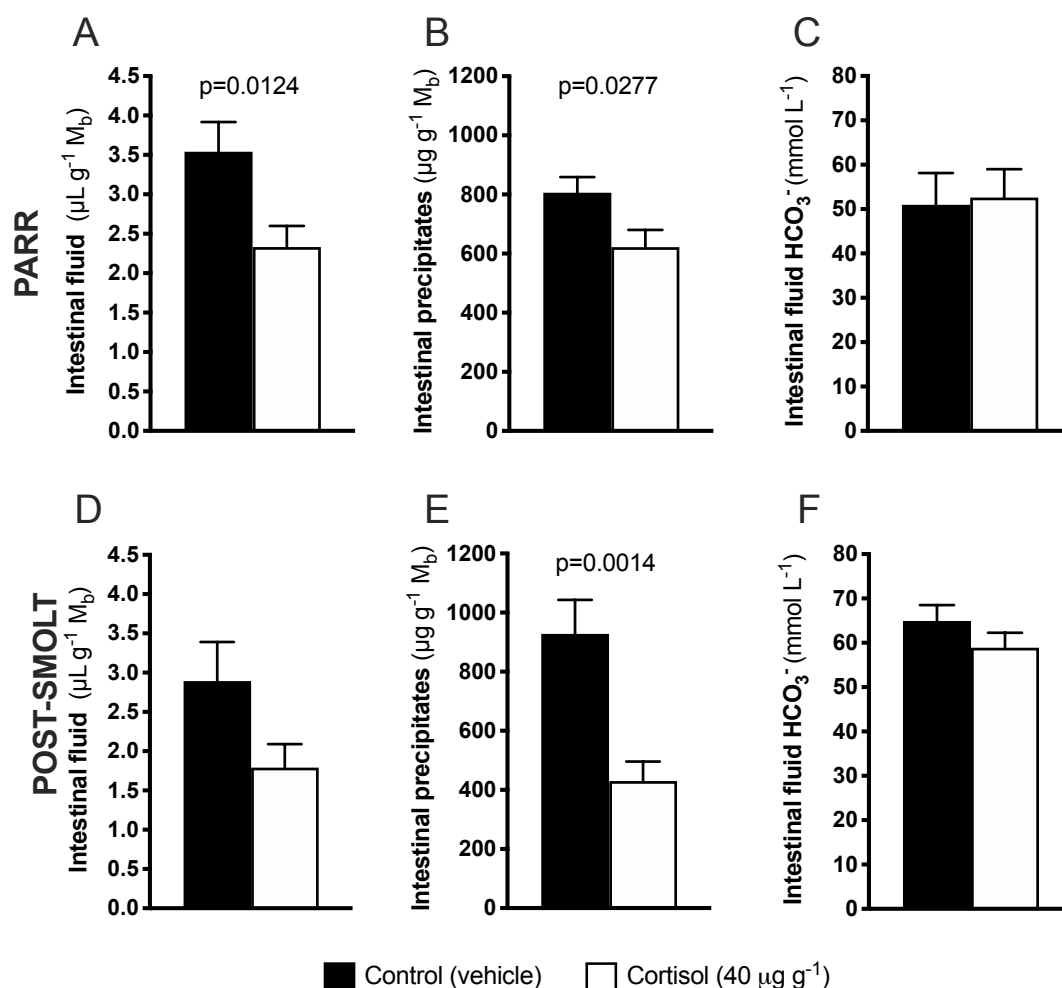


Figure 4.2. Intestinal fluid volume, intestinal precipitates, and intestinal fluid HCO_3^- concentration in parr (A to C) and post-smolt (D to F) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. The intestinal fluid volume and intestinal aggregates were normalized to the fish body mass (M_b). Each bar represents the mean $\pm$ SEM ($n=11-19$ for parr; $n=9-10$ for post-smolt). Statistical significance accepted at $p<0.05$ (unpaired t-test). Only significant effects are reported.

4.3.5. Bioelectrical properties

Transepithelial potential (TEP) and transepithelial resistance (R_t) were monitored for 2 h in the anterior intestine of post-smolts. TEP showed negative values in the control group in FW and in both experimental groups in SW (Fig. 4.3A), whereas the cortisol group in FW values close to zero (Fig. 4.3A). TEP was significantly affected by salinity, showing more negative values after SW transfer. A significant interaction was also observed between cortisol treatment and salinity, with the FW groups having TEP significantly different than the SW groups (Fig. 4.3A). R_t was significantly affected by the cortisol treatment and interaction between treatment and salinity was observed (Fig. 4.3B). The FW cortisol group showed a significantly lower R_t than the FW control group. After transfer to SW, no effect of cortisol treatment was observed. (Fig. 4.3B).

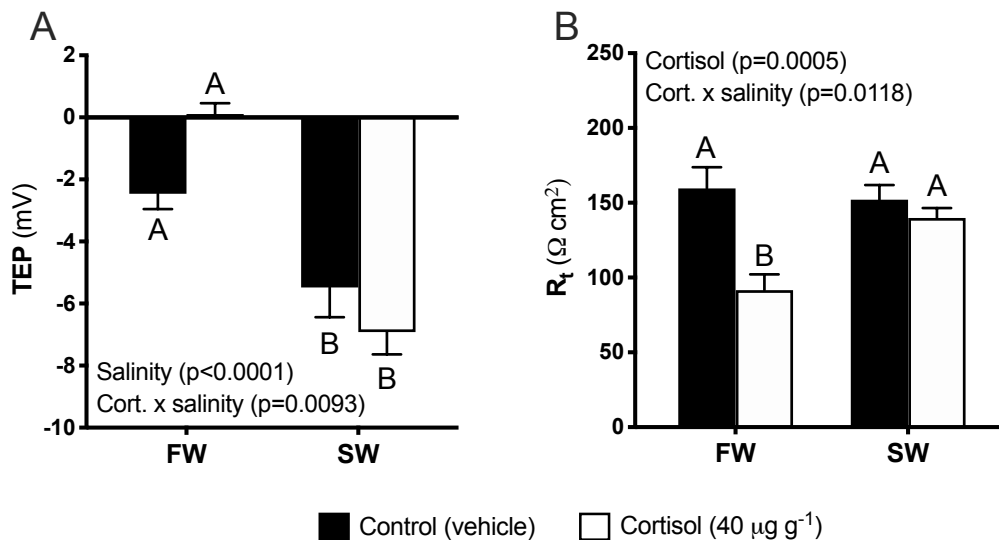


Figure 4.3. Transepithelial potential (TEP, mV, A) and transepithelial resistance (R_t , $\Omega \text{ cm}^2$, B) measured in the anterior intestine of post-smolt Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. TEP was measured in Ussing chamber using the current-clamp technique, under asymmetric conditions. TEP and R_t per fish were calculated as average of the values recorded every 30 min for 2 h. Each bar represents the mean $\pm$ SEM ($n=8-10$). When a significant interaction between cortisol dose and salinity was observed with two-way ANOVA, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

4.3.6. NKA activity

In both parr and post-smolts, cortisol significantly increased gill (Fig. 4.4A, C) and anterior intestine NKA activity (Fig. 4.4B, D) in FW and SW. In parr, gill NKA activity was also affected by salinity, showing higher levels in SW (Fig. 4.4A).

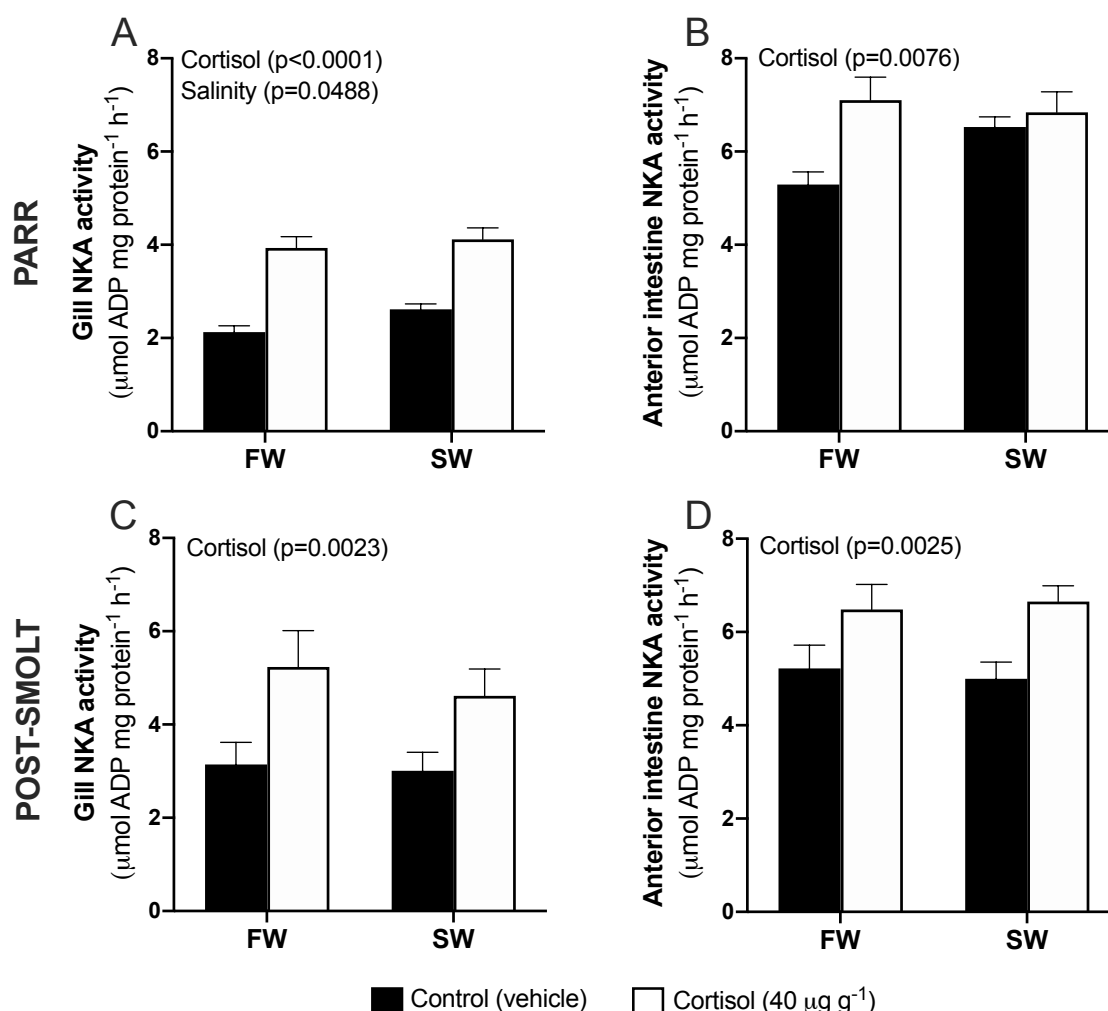


Figure 4.4. Na^+/K^+ -ATPase (NKA) activity in the gill and anterior intestine of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or $40 \mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Each bar represents the mean $\pm$ SEM ($n=13-19$ for parr; $n=9-10$ for post-smolt). In the analysis, cortisol dose and salinity are the two considered factors (two-way ANOVA). Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

4.3.7. Transcriptional response

In parr and post-smolts, *nka1c* mRNA levels were significantly affected by cortisol and salinity. In both anterior and posterior intestines, cortisol treatment and SW exposure caused an increase in *nka1c* mRNA levels (Fig. 4.5).

In the anterior and posterior intestines of parr, *nkcc2* mRNA levels were significantly affected by salinity, with an increase in mRNA abundance in SW. (Fig. 4.6A, B). There was an interaction of cortisol and salinity, with cortisol increasing *nkcc2* mRNA levels in FW, whereas they were slightly lower in SW (Fig. 4.6A, B). In post-smolts, *nkcc2* mRNA levels were significantly increased by both cortisol treatment and SW exposure (Fig. 4.6C, D).

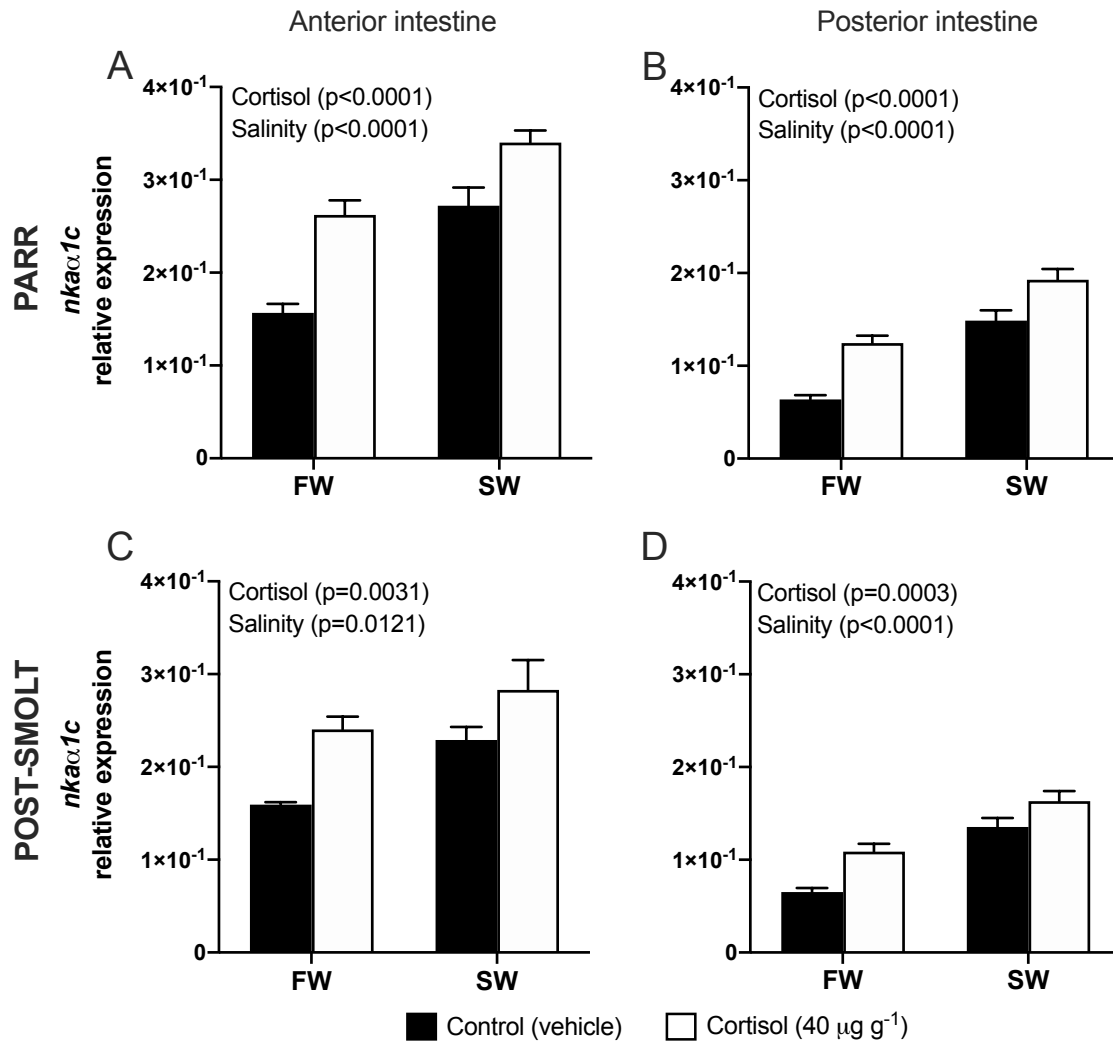


Figure 4.5. Relative expression of *nkaα1c* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=7-12$ for parr; $n=6-9$ for post-smolt). In the analysis, cortisol dose and salinity are the two considered factors (two-way ANOVA). Statistical significance accepted at $p<0.05$. Only significant effects are reported.

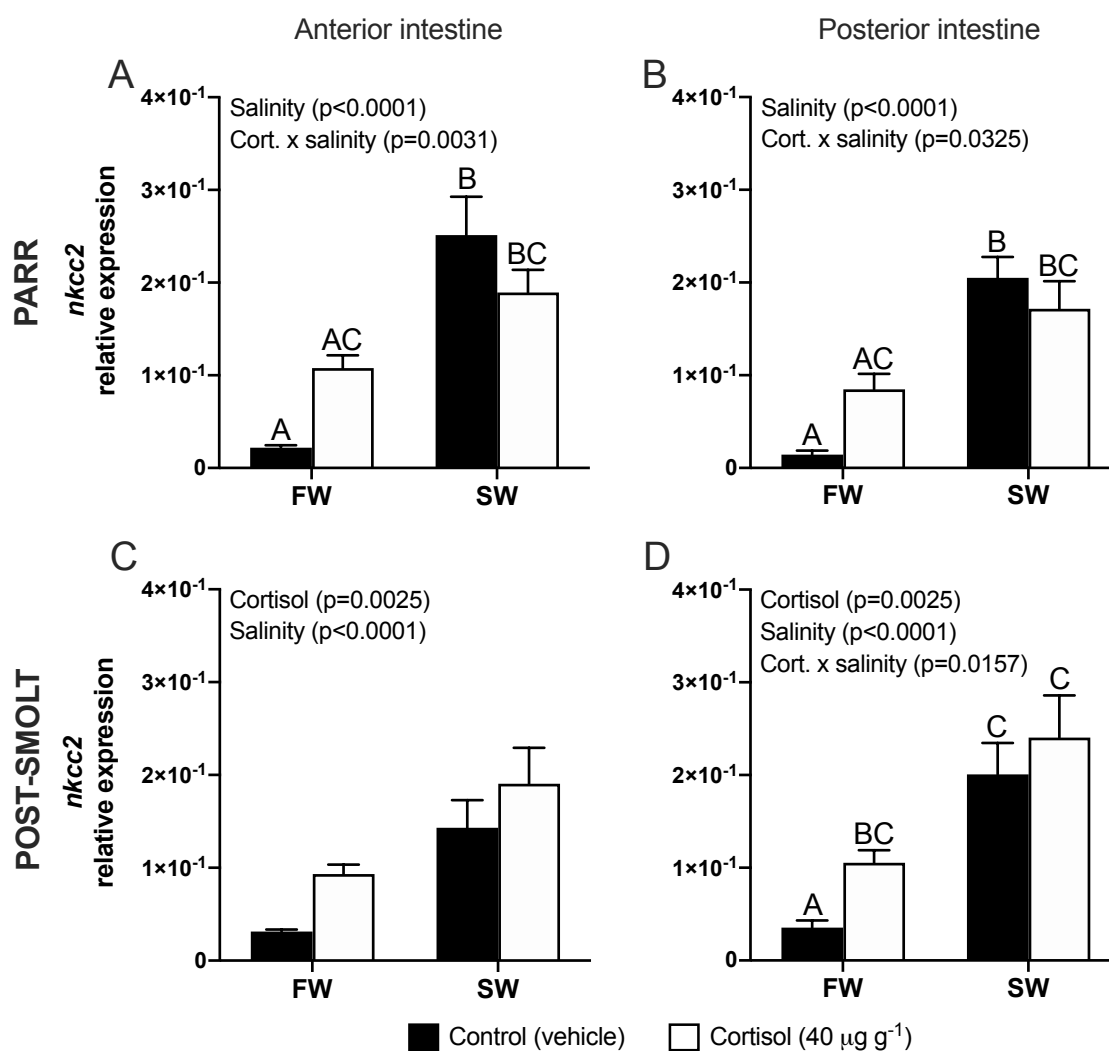


Figure 4.6. Relative expression of *nkcc2* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=7-12$ for parr; $n=7-9$ for post-smolt). When a significant interaction between cortisol dose and salinity was observed with two-way ANOVA, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

In parr, *slc26a3* mRNA levels were significantly affected by cortisol treatment and salinity in the anterior intestine, with an increase in mRNA levels observed in the SW cortisol group (Fig. 4.7A). In the posterior intestine, only salinity affected *slc26a3* mRNA abundance, with lower levels in SW for both control and cortisol groups (Fig. 4.7B). In post-smolts, salinity affected *slc26a3* mRNA levels in the anterior intestine, with higher levels in the SW groups (Fig. 4.7C). In the posterior intestine cortisol treatment significantly affected *slc26a3* mRNA levels causing a reduction of *slc26a3* abundance in FW and SW (Fig. 4.7D).

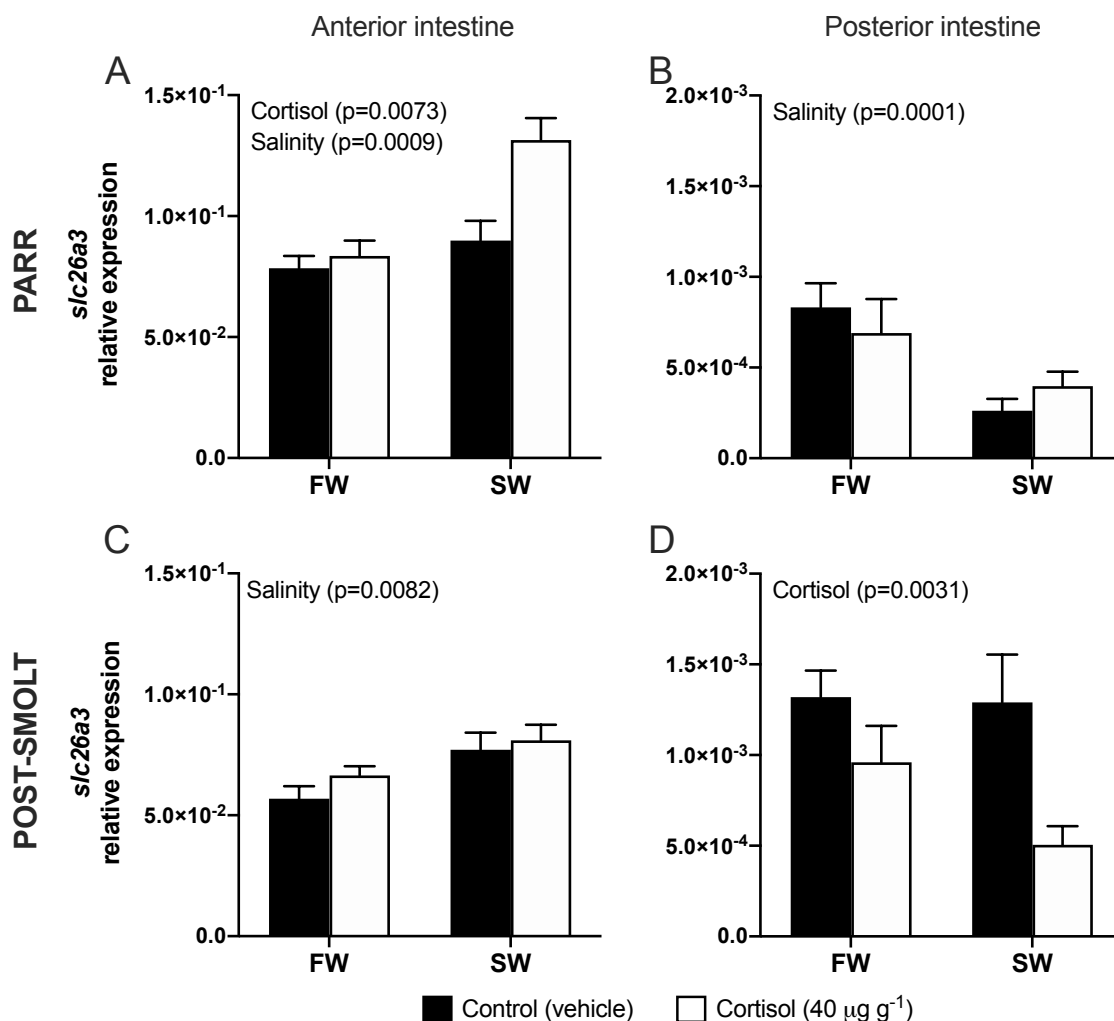


Figure 4.7. Relative expression of *slc26a3* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=7-12$ for parr; $n=8-9$ for post-smolt). In the analysis, cortisol dose and salinity are the two considered factors (two-way ANOVA). Statistical significance accepted at $p<0.05$. Only significant effects are reported.

Cortisol treatment considerably increased *slc26a6* mRNA levels, up to 8-fold, in the anterior intestine of FW parr (Fig. 4.8A, B). The transfer to SW caused a reduction of *slc26a6* mRNA levels in control and cortisol groups for both anterior and posterior intestine (Fig. 4.8A, B). In the posterior intestine, a significant interaction between cortisol treatment and salinity was also observed with lower levels due to SW exposure and higher levels due to cortisol. (Fig. 4.8B). In post-smolts, *slc26a6* mRNA levels in the anterior and posterior intestines were higher in the cortisol groups in both FW and SW (Fig. 4.8C, D). In the anterior intestine, cortisol-treated fish showed mRNA levels 35-fold and 16-fold higher than control groups in FW and SW respectively (Fig. 4.8C). The posterior intestine was also affected by salinity, with lower *slc26a6* mRNA levels observed in the SW groups (Fig. 4.8D).

In the anterior intestine of parr, both SW and cortisol increased *nbc1* mRNA levels (Fig. 4.9A). Higher *nbc1* levels in the posterior intestine were observed in SW groups (Fig. 4.9B). In post-smolts, the anterior intestine had higher *nbc1* mRNA levels in the cortisol-treated groups (Fig. 4.9C).

Both control and cortisol groups had significantly higher *atp6v1a* mRNA levels in SW than FW (Fig. 4.10A). *atp6v1a* mRNA levels in the posterior intestine of parr were significantly increased by cortisol treatment. Also, an interaction between cortisol treatment and salinity was observed, with *atp6v1a* mRNA levels significantly higher in the SW cortisol group than in the SW control (Fig. 4.10B). In post-smolts, *atp6v1a* mRNA levels were significantly affected by salinity, and interaction between cortisol treatment and salinity was observed (Fig. 4.10C); both control and cortisol groups were higher in SW than in FW (Fig. 4.10C). In the posterior intestine of post-smolts, *atp6v1a* mRNA levels were significantly elevated by cortisol treatment (Fig. 4.10D).

gn mRNA levels were significantly affected by cortisol treatment and salinity in the anterior and posterior intestines of parr. Also, interaction between cortisol and salinity was observed (Fig. 4.11A, B). Cortisol upregulated *gn* mRNA levels in FW fish, up to 3-fold in the posterior intestine. The transfer to SW caused a reduction of *gn* mRNA levels in the cortisol group, particularly in the posterior intestine (Fig. 4.11B). In post-smolts, *gn* mRNA levels in the anterior intestine were not affected by salinity or cortisol (Fig. 4.11C). However, the posterior intestine was affected by cortisol treatment and salinity and also showed significant interaction of the two factors. Cortisol treatment significantly increased *gn* mRNA levels in FW fish, and the transfer to SW caused a reduction of *gn* mRNA levels in the cortisol-treated group (Fig. 4.11D).

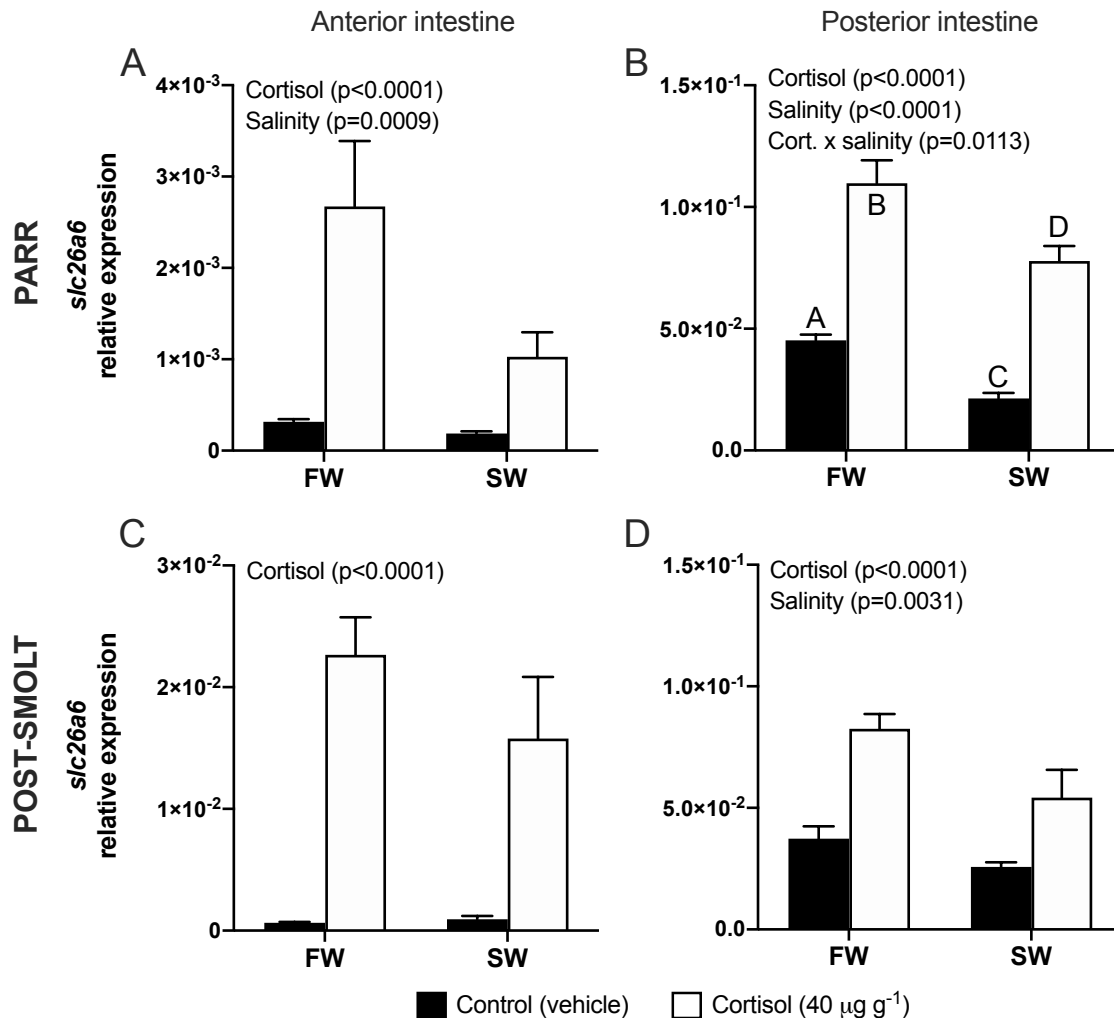


Figure 4.8. Relative expression of *slc26a6* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=7-10$ for parr; $n=8-9$ for post-smolt). When a significant interaction between cortisol dose and salinity was observed with two-way ANOVA, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

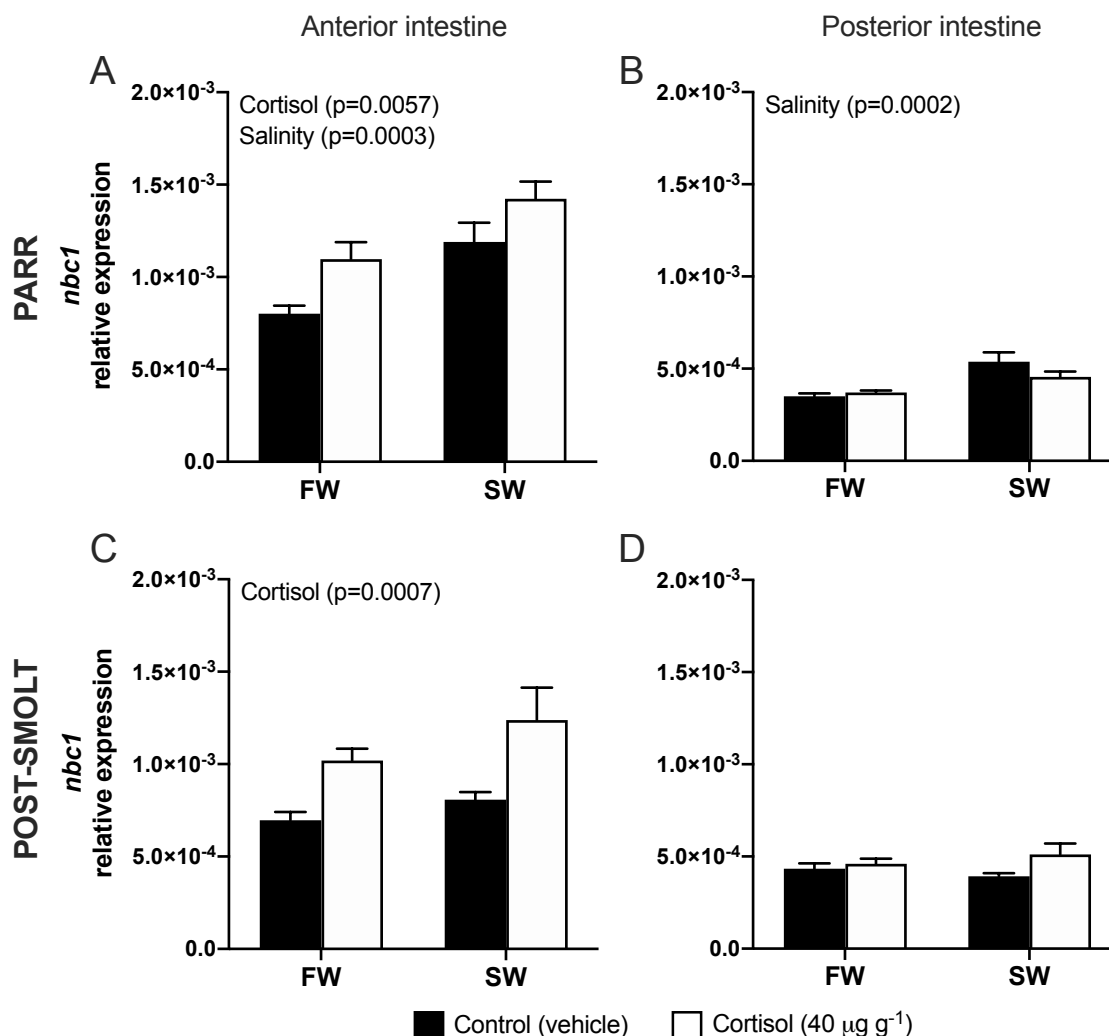


Figure 4.9. Relative expression of *nbc1* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=7-12$ for parr; $n=7-9$ for post-smolt). In the analysis, cortisol dose and salinity are the two considered factors (two-way ANOVA). Statistical significance accepted at $p<0.05$. Only significant effects are reported.

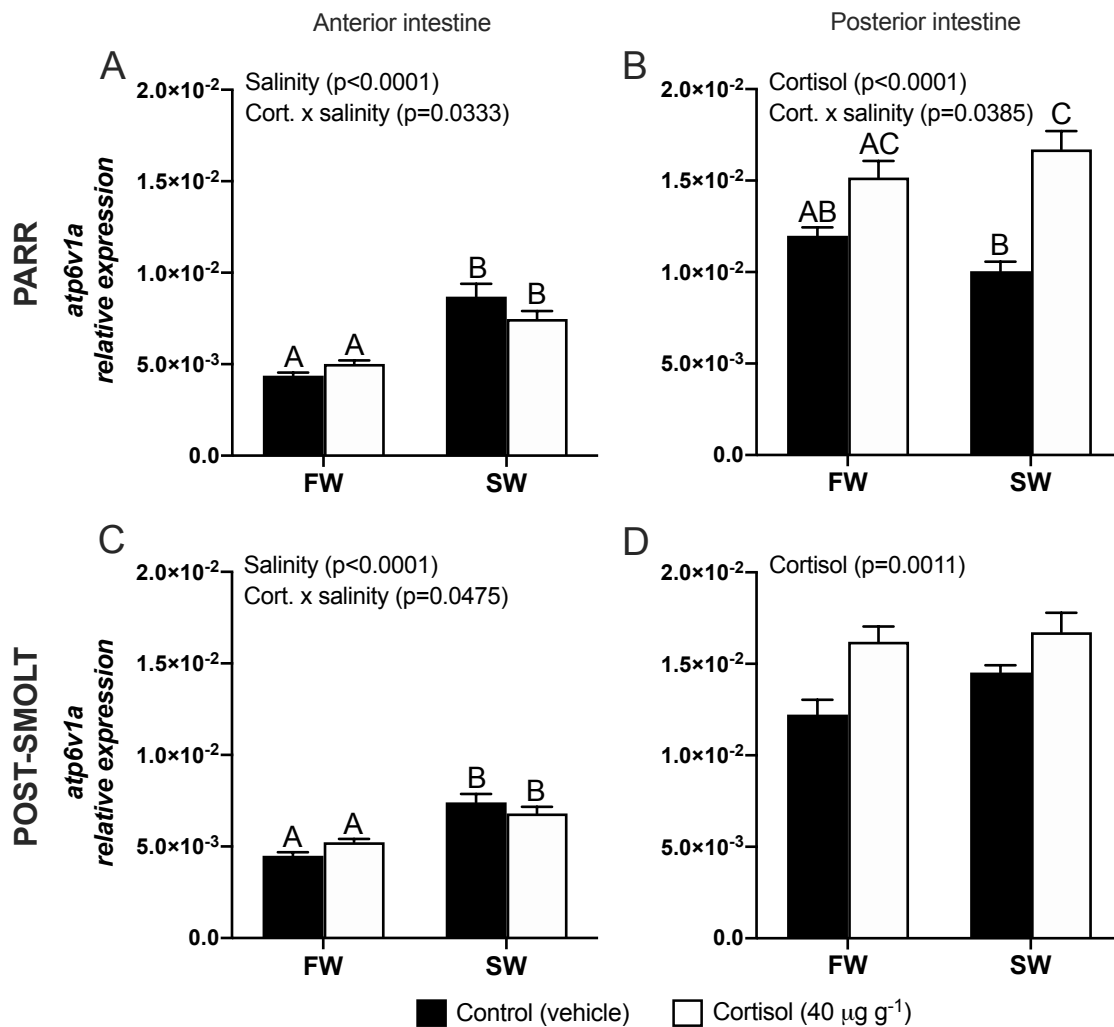


Figure 4.10. Relative expression of *atp6v1a* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=7-12$ for parr; $n=7-9$ for post-smolt). When a significant interaction between cortisol dose and salinity was observed with two-way ANOVA, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

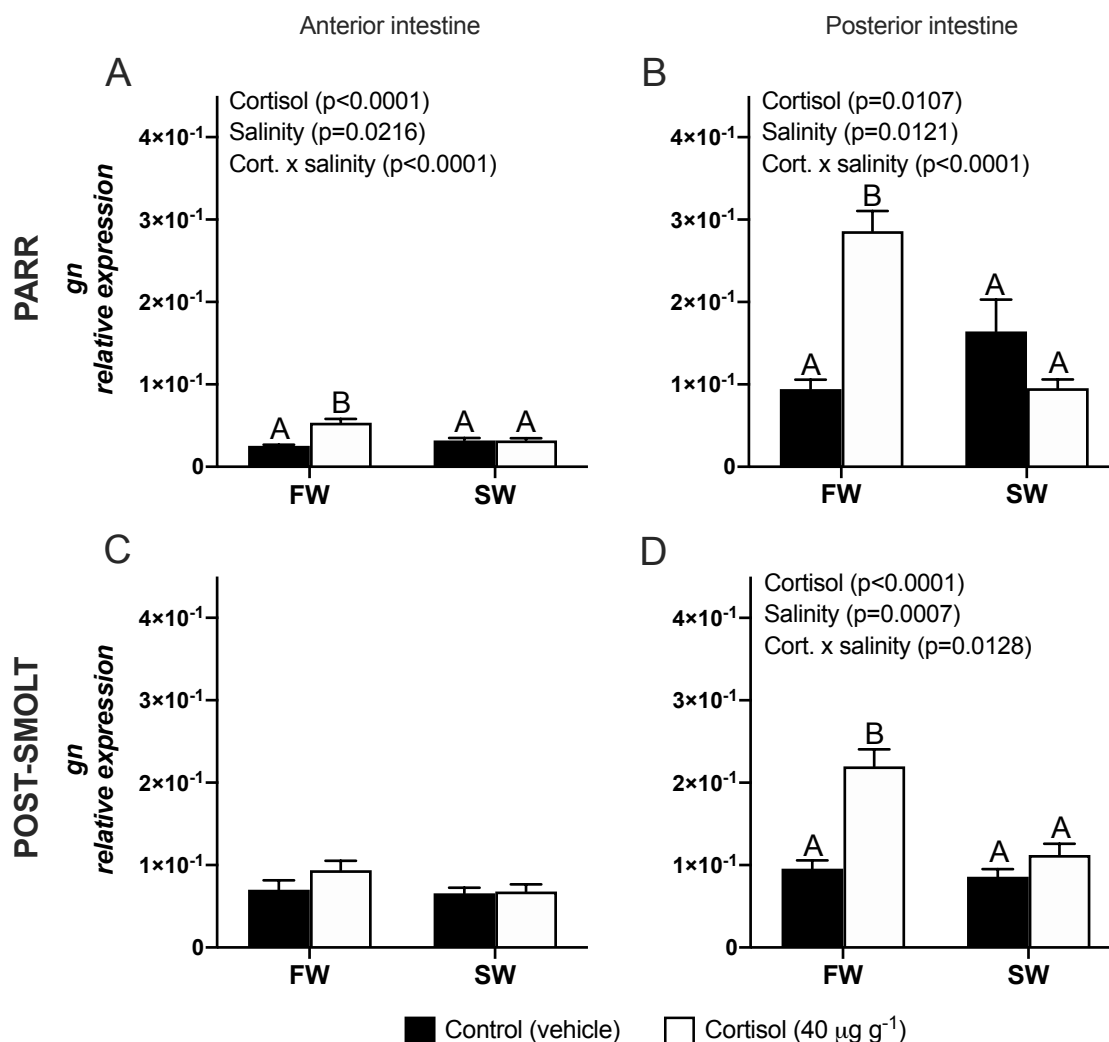


Figure 4.11. Relative expression of *gn* in the anterior and posterior intestines of parr (A, B) and post-smolt (C, D) Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*. Each bar represents the mean $\pm$ SEM ($n=6-11$ for parr; $n=8-9$ for post-smolt). When a significant interaction between cortisol dose and salinity was observed with two-way ANOVA, Bonferroni *post-hoc* test was carried out, and significant differences among experimental groups were indicated with different uppercase letters. Statistical significance accepted at $p < 0.05$. Only significant effects are reported.

In the posterior intestine of parr, and in both anterior and posterior intestines of post-smolts, *gr1* mRNA levels were higher in cortisol-treated fish in both FW and SW (Table 4.5). In parr, *gr2* mRNA levels in the posterior intestine were higher in the cortisol groups and lower after transfer to SW (Table 4.5). In post-smolts, *gr2* mRNA levels in both anterior and posterior intestines were lower in SW groups (Table 4.5). In parr, higher *mr* mRNA levels were observed in cortisol-treated fish, while lower levels were detected in SW groups (Table 4.5). In post-smolts, lower *mr* levels were observed after SW transfer in the anterior intestine (Table 4.5).

The regulation dynamics of the genes investigated in this study are summarized in a heatmap in Fig. 4.12.

Table 4.5. Relative expression of *gr1*, *gr2*, and *mr* in the anterior and posterior intestine of parr and post-smolt Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h. Data were normalized by the geometric mean between *18S* and *arpc1b*, and reported as mean $\pm$ SEM (n=7-12 for parr; n=7-9 for post-smolt). In the analysis, cortisol dose (F) and salinity (S) are the two considered factors (two-way ANOVA); no significant interaction between the two factors (F $\times$ S) was observed. Statistical significance accepted at $p < 0.05$. The abbreviation “ns” indicates not significant statistical differences.

				<i>gr1</i>	<i>gr2</i>	<i>Mr</i>
PARR	Anterior intestine	FW	Control	$3.30 \cdot 10^{-3} \pm 1.59 \cdot 10^{-4}$	$9.86 \cdot 10^{-4} \pm 3.20 \cdot 10^{-5}$	$5.85 \cdot 10^{-3} \pm 4.09 \cdot 10^{-4}$
			Cortisol	$3.24 \cdot 10^{-3} \pm 1.53 \cdot 10^{-4}$	$9.99 \cdot 10^{-4} \pm 4.58 \cdot 10^{-5}$	$5.84 \cdot 10^{-3} \pm 4.51 \cdot 10^{-4}$
		SW	Control	$3.35 \cdot 10^{-3} \pm 2.34 \cdot 10^{-4}$	$1.02 \cdot 10^{-3} \pm 5.07 \cdot 10^{-5}$	$4.95 \cdot 10^{-3} \pm 5.87 \cdot 10^{-4}$
			Cortisol	$3.88 \cdot 10^{-3} \pm 1.89 \cdot 10^{-4}$	$1.03 \cdot 10^{-3} \pm 4.76 \cdot 10^{-5}$	$5.22 \cdot 10^{-3} \pm 4.58 \cdot 10^{-4}$
					F (ns); S (ns); F $\times$ S (ns)	F (ns); S (ns); F $\times$ S (ns)
	Posterior intestine	FW	Control	$2.86 \cdot 10^{-3} \pm 1.17 \cdot 10^{-4}$	$8.26 \cdot 10^{-4} \pm 2.97 \cdot 10^{-5}$	$3.36 \cdot 10^{-3} \pm 1.50 \cdot 10^{-4}$
			Cortisol	$3.55 \cdot 10^{-3} \pm 1.38 \cdot 10^{-4}$	$9.80 \cdot 10^{-4} \pm 4.78 \cdot 10^{-5}$	$3.98 \cdot 10^{-3} \pm 2.15 \cdot 10^{-4}$
		SW	Control	$2.40 \cdot 10^{-3} \pm 1.84 \cdot 10^{-4}$	$7.62 \cdot 10^{-4} \pm 4.18 \cdot 10^{-5}$	$2.63 \cdot 10^{-3} \pm 3.25 \cdot 10^{-4}$
			Cortisol	$3.61 \cdot 10^{-3} \pm 2.12 \cdot 10^{-4}$	$8.66 \cdot 10^{-4} \pm 2.49 \cdot 10^{-5}$	$3.32 \cdot 10^{-3} \pm 1.36 \cdot 10^{-4}$
					F ($p < 0.0001$); S (ns); F $\times$ S (ns)	F ($p = 0.0008$); S ($p = 0.0161$); F $\times$ S (ns)
POST-SMOLT	Anterior intestine	FW	Control	$3.59 \cdot 10^{-3} \pm 1.92 \cdot 10^{-4}$	$1.03 \cdot 10^{-3} \pm 5.08 \cdot 10^{-5}$	$4.91 \cdot 10^{-3} \pm 4.85 \cdot 10^{-4}$
			Cortisol	$4.08 \cdot 10^{-3} \pm 2.37 \cdot 10^{-4}$	$1.16 \cdot 10^{-3} \pm 8.40 \cdot 10^{-5}$	$6.24 \cdot 10^{-3} \pm 5.85 \cdot 10^{-4}$
		SW	Control	$3.38 \cdot 10^{-3} \pm 2.04 \cdot 10^{-4}$	$8.86 \cdot 10^{-4} \pm 2.74 \cdot 10^{-5}$	$4.50 \cdot 10^{-3} \pm 4.95 \cdot 10^{-4}$
			Cortisol	$3.95 \cdot 10^{-3} \pm 2.67 \cdot 10^{-4}$	$1.01 \cdot 10^{-3} \pm 8.90 \cdot 10^{-5}$	$3.98 \cdot 10^{-3} \pm 3.04 \cdot 10^{-4}$
					F ($p = 0.0291$); S (ns); F $\times$ S (ns)	F (ns); S ($p = 0.0425$); F $\times$ S (ns)
	Posterior intestine	FW	Control	$3.17 \cdot 10^{-3} \pm 1.67 \cdot 10^{-4}$	$8.50 \cdot 10^{-4} \pm 3.76 \cdot 10^{-5}$	$3.21 \cdot 10^{-3} \pm 2.48 \cdot 10^{-4}$
			Cortisol	$3.70 \cdot 10^{-3} \pm 1.72 \cdot 10^{-4}$	$9.33 \cdot 10^{-4} \pm 1.72 \cdot 10^{-5}$	$3.95 \cdot 10^{-3} \pm 2.39 \cdot 10^{-4}$
		SW	Control	$2.94 \cdot 10^{-3} \pm 2.05 \cdot 10^{-4}$	$7.50 \cdot 10^{-4} \pm 4.09 \cdot 10^{-5}$	$2.94 \cdot 10^{-3} \pm 2.02 \cdot 10^{-4}$
			Cortisol	$3.75 \cdot 10^{-3} \pm 2.72 \cdot 10^{-4}$	$8.30 \cdot 10^{-4} \pm 6.25 \cdot 10^{-5}$	$3.19 \cdot 10^{-3} \pm 3.09 \cdot 10^{-4}$
					F ($p = 0.0036$); S (ns); F $\times$ S (ns)	F (ns); S (ns); F $\times$ S (ns)

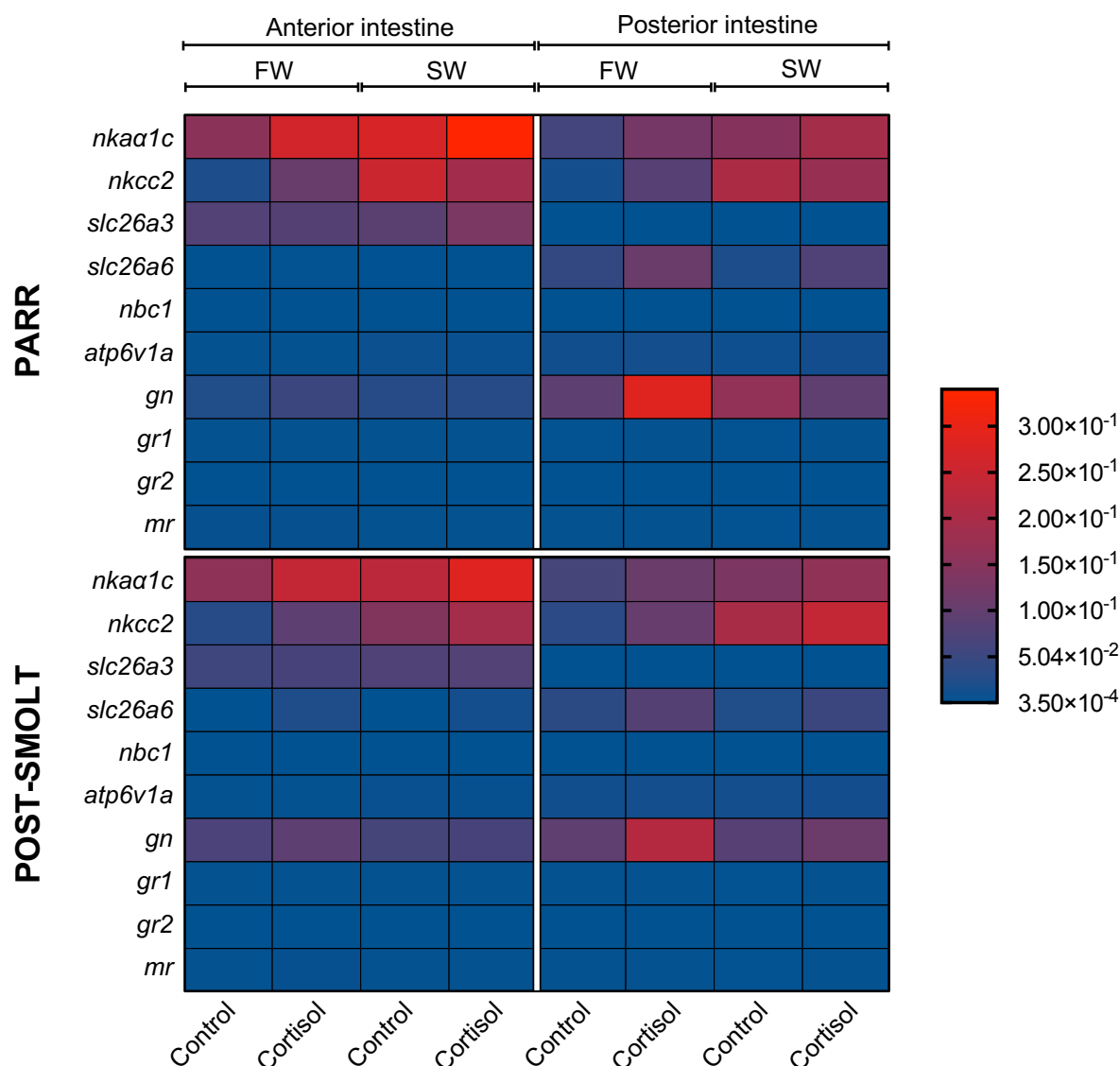


Figure 4.12. Heatmap summarizing the relative mRNA levels of the genes investigated in the anterior and posterior intestines of parr and post-smolt Atlantic salmon (*Salmo salar*). Fish were intraperitoneally implanted with cortisol at 0 (control) or 40 (cortisol) $\mu\text{g g}^{-1}$ body weight. After 12 d post-injection, fish were exposed to a FW-FW or FW-SW transfer (25 ppt for parr and 30 ppt for post-smolt) and sampled after 48 h.

4.4. DISCUSSION

In this study we investigated the role of cortisol on the development of hypo-osmoregulatory capacity in underyearling parr and post-smolts of Atlantic salmon. The focus of the study was directed toward the intestinal response and particularly the pathways involved in intestinal HCO_3^- secretion and carbonate precipitation. The results provide evidence that exogenous cortisol significantly enhances the hypo-osmoregulatory capacity in juveniles and positively affects the hypo-osmoregulatory

function of the intestine, including the ion transporters involved in the intestinal HCO_3^- secretion cascade.

In Atlantic salmon, the development of the hypo-osmoregulatory capacity required for ocean entry occurs during smoltification in FW. There are preparatory changes in the osmoregulatory organs that are regulated by endocrine factors, with cortisol playing a central role (Bisbal and Specker, 1991; McCormick *et al.*, 2019, 2000; Sundell *et al.*, 2003). In this study, cortisol implants effectively elevated circulating cortisol levels within the physiological range (McCormick *et al.*, 2000; Nilsen *et al.*, 2008) in both parr and post-smolts (Fig. 4.1). Cortisol enhanced SW performance as previously observed in Atlantic salmon and other species (Chang *et al.*, 2023; Mancera *et al.*, 2002; McCormick *et al.*, 2008; Pelis and McCormick, 2001). The beneficial action of cortisol was particularly evident in the underyearling parr, for whom exposure to 25 ppt SW represented a significant challenge that caused elevated plasma osmolality and chloride and mortality in the control group. The osmotic stress induced by the hyper-osmotic environment was considerably attenuated by prior cortisol treatment of parr, as evidenced by the absence of mortality during the 48h in SW and by the reduced levels of plasma osmolality and chloride concentrations, resembling those found in FW fish (Table 4.4). The improvement in the hypo-osmoregulatory capacity following cortisol treatment was likely causally related to the increased activity of the NKA observed in the gill and anterior intestine of parr and post-smolts, both in FW and SW (Fig. 4.4), in line with previous studies which demonstrated the direct action of cortisol on NKA (Takei and McCormick, 2013).

In SW, teleosts display elevated drinking rates as a strategy to counteract dehydration (Fuentes and Eddy, 1997; Perrott *et al.*, 1992; Tipsmark *et al.*, 2020). The ingested SW then undergoes processing along the GIT to reduce the osmotic pressure driving water absorption across the intestine (Marshall and Grosell, 2006). The intestinal fluid chemistry is a reflection of the absorptive and secretory mechanisms at work in the intestine of SW-acclimated teleosts (Grosell, 2014), and its analysis can provide valuable insights into the performance of the fish in SW (Gaetano *et al.*, 2023). In parr, the osmolality of the intestinal fluid was significantly lower in fish treated with cortisol (Table 4.2), highlighting the enhanced intestinal function mediated by cortisol. We also found that the intestinal fluid volume was lower in cortisol-treated parr in SW. These results are consistent with previous studies that have shown an enhanced

capacity for ion and fluid absorption of the intestine following cortisol treatment (Cornell *et al.*, 1994; Veillette *et al.*, 1995).

To our knowledge ours is the first study to examine the effect of cortisol on electrophysiological characteristics of the intestine of Atlantic salmon. SW exposure resulted in more negative TEP in post-smolts (Fig. 4.3A), which is consistent with greater ion uptake levels that are necessary for net fluid transport. Cortisol treatment resulted in less negative TEP values in FW but had no effect in SW (Fig. 4.3A). We also measured transepithelial resistance (R_t), and found no differences due to salinity, but that cortisol decreased resistance in FW (Fig. 4.3B). These results of R_t are similar to those found in rainbow trout by Sundell and Sundh, (2012) and indicate an important interaction between salinity and the impact of cortisol in the remodeling of the paracellular pathway.

In the intestine, the preparatory changes taking place during smoltification include increased fluid uptake rates (J_v) in the posterior intestine (Veillette *et al.*, 1993), and changes in the expression and localization of NKA (Breves *et al.*, 2022; Sundh *et al.*, 2014). In the intestine, J_v is sustained by the activity of the NKA, and both are stimulated by exogenous cortisol (Collie and Bern, 1982; Veillette *et al.*, 1993). In the intestine of Atlantic salmon, $Nka\alpha 1c$ has been proposed as the major functional NKA isoform (Sundh *et al.*, 2014; Tipsmark *et al.*, 2010), which transcriptional abundance increases in FW smolts and following SW transfer (Tipsmark *et al.*, 2010; Chapter 3 - Gaetano *et al.* in preparation), as also observed in this study. In a previous study in Atlantic salmon increased *nkaa1c* levels following cortisol treatment were observed only in the posterior region of the intestine (Tipsmark *et al.*, 2010). Here, we show that, in parr and post-smolts, cortisol regulates *nkaa1c* abundance also in the anterior intestine already in FW, providing evidence that cortisol drives the pre-adaptive changes involving the NKA in the intestine already at the transcriptional levels. Fluid absorption across the intestinal epithelium of teleosts is coupled with the absorption of NaCl which is largely driven by the apical NKCC2. Pre-adaptation during smoltification also include changes in expression and localization of this cotransporter (Breves *et al.*, 2022; Duarte *et al.*, 2023; Sundh *et al.*, 2014). In this study, we observed a salinity-dependence increase in *nkcc2* mRNA abundance in both intestinal regions (Fig. 4.6) as previously observed in Atlantic salmon and other teleosts (Sundh *et al.*, 2014; Watanabe *et al.*, 2011; Chapter 3 - Gaetano *et al.* in preparation), and we

demonstrate that cortisol stimulates the transcripts levels of this key cotransporter, particularly in FW.

A mechanism unique to SW-adapted teleosts is the precipitation of divalent cations as Ca(Mg)CO_3 as part of the intestinal HCO_3^- secretion cascade. The subsequent reduction in ion levels helps reduce total osmolality thereby facilitating water uptake by the intestine. The electrogenic anion exchangers Slc26a3 ($\text{nCl}^-/\text{HCO}_3^-$) and Slc26a6 ($\text{Cl}^-/\text{nHCO}_3^-$) (Mount and Romero, 2004) have been proposed as responsible for the high HCO_3^- secretion observed in the intestine of SW teleosts (Grosell *et al.*, 2001; Guffey *et al.*, 2011; Ruiz-Jarabo *et al.*, 2017b). The transcriptional levels of both *slc26a3* and *slc26a6* showed salinity dependence in the intestine of sea bream (Gregório *et al.*, 2013), red drum (Esbaugh and Cutler, 2016), and tilapia (Ruiz-Jarabo *et al.*, 2017b) in SW, with region-specific expression patterns (Alves *et al.*, 2019; Gregório *et al.*, 2013; Ruiz-Jarabo *et al.*, 2017b). In salmonids, limited information is available regarding the expression of Slc26a3 and Slc26a6 . In rainbow trout *slc26a6* expression did not show salinity dependence in the intestine (Boyle *et al.*, 2015). Here, both *slc26a3* and *slc26a6* showed a region-specific expression pattern in the intestine (Fig. 4.7 and 4.8) as previously observed in smolts (Chapter 3 - Gaetano *et al.* in preparation). Notably, cortisol implants increased *slc26a6* transcription by 35-fold in the anterior intestine of FW post-smolts.

The contribution of transepithelial transport of HCO_3^- and endogenous CO_2 hydration in providing substrate for apical secretion appear to vary among species (Grosell *et al.*, 2009b, 2005; Grosell and Genz, 2006) and between intestinal regions (Alves *et al.*, 2019). Transepithelial HCO_3^- transport relies on the basolateral electrogenic $\text{Na}^+/\text{HCO}_3^-$ cotransporter (NBC1) driven by the favorable Na^+ gradient, whose expression increases in the proximal regions of the intestine after exposure to higher salinity (Gregório *et al.*, 2013; Kurita *et al.*, 2008; Taylor *et al.*, 2010). In agreement with previous results in Atlantic salmon (Breves *et al.*, 2022), transcript levels of *nbc1* showed salinity-dependence in both intestinal regions of parr with a greater response in the anterior intestine in this study (Fig. 4.9). As in other species, the anterior intestine of parr and post-smolts had higher expression levels of *nbc1* that could be related to the higher secretory capacity attributed to the anterior intestine of teleosts, or alternatively could be due to a different contribution of HCO_3^- source (transcellular or endogenous) in the two intestinal regions. Furthermore, our data

demonstrate that exogenous cortisol has transcriptional control of *nbc1* in the anterior intestine of Atlantic salmon.

Within the intestinal epithelium, most metabolic CO_2 is converted to HCO_3^- , serving as an endogenous source of HCO_3^- for apical anion exchanges (Grosell, 2011). The hydration reaction of CO_2 also produces H^+ , which must be removed from the enterocyte to avoid acidification and allow further HCO_3^- production. A pathway for H^+ exit from the enterocytes is the vacuolar H^+ -ATPase (VHA), localized by immunoreactivity in the apical and basolateral side of the intestinal epithelium of several teleosts (Esbaugh and Cutler, 2016; Grosell *et al.*, 2007; Guffey *et al.*, 2011). The apical VHA holds significant functional importance in regulating HCO_3^- secretion and in reducing the osmotic pressure of the intestinal fluid, particularly in the distal regions of the intestine. The H^+ secreted by the VHA titrates part of the luminal HCO_3^- , producing H_2O and osmotically inert CO_2 that can re-enter the cell for hydration. HCO_3^- titration favors continued HCO_3^- secretion and Cl^- absorption via AEs, thus promoting further water absorption. In this study, we investigated the transcript levels of the VHA (*atp6v1a*) (Fig. 4.10). In the anterior intestine, *atp6v1a* is salinity-dependent, and increased levels were observed in the fish after 48h in SW as previously reported in the intestine of rainbow trout (Grosell *et al.*, 2007), indicating a relevant role of VHA in the osmoregulatory mechanisms in juveniles of Atlantic salmon. In line with this and other studies (Alves *et al.*, 2019; Gregório *et al.*, 2013; Ruiz-Jarabo *et al.*, 2017a), higher *atp6v1a* mRNA levels were observed in the posterior intestine of parr and post-smolts. Transcript levels of *atp6v1a* in the posterior intestine were upregulated by cortisol treatment. However, no salinity effect was observed, possibly linked to the involvement of VHA in the acid-base balance in FW animals, as suggested for tilapia (Seale *et al.*, 2014) and red drum (Esbaugh and Cutler, 2016).

In SW teleosts, the intestine provides fluid absorption to maintain whole-body water balance, but a secretory pathway also exists (Marshall *et al.*, 2002; Ruhr *et al.*, 2014). Studies in Japanese eel and Gulf toadfish demonstrated that the stimulation of the secretory capacity of the intestine is mediated by the guanylin peptides system and highlighted the importance of this system during SW acclimation (Ruhr *et al.*, 2014; Yuge *et al.*, 2006, 2003). Although the mechanisms of regulation and the physiological significance of the guanylin peptides still require investigation, a proposed role for the stimulation of the fluid secretion in the more distal segments of

the intestine is to facilitate the release of solids from the intestinal lumen, including the carbonate precipitates produced along the intestine in SW (Ando and Takei, 2015; Ruhr *et al.*, 2014). Considering the different levels of carbonate precipitates collected in the intestines of control and cortisol-treated fish (Fig. 4.2), we investigated the transcript levels of guanylin (*gn*) in Atlantic salmon. Of the three guanylin peptides identified in teleosts (guanylin, uroguanylin, and renoguanylin), guanylin (GN) is the intestine-specific peptide (Yuge *et al.*, 2003). It is produced by the goblet cells and is secreted in the intestinal lumen where it activates the apical guanylyl cyclase-C (GC-C) receptors (Yuge *et al.*, 2006, 2003). Using *in vivo*-like conditions in an Ussing chamber, mucosal application of Gn stimulated fluid secretion in the posterior intestine of Gulf toadfish and in the middle and posterior intestine of eels, where it also stimulated HCO_3^- secretion (Ando and Takei, 2015; Ruhr *et al.*, 2014; Takei *et al.*, 2019). We found abundant mRNA levels of *gn* in both intestinal regions of parr and post-smolts (Fig. 4.11), but these were not upregulated in SW animals as was observed in the Japanese eel (Yuge *et al.*, 2003). However, we did observe a 3-fold increase in *gn* transcription in the posterior intestine of cortisol-treated FW salmon, which to our knowledge is the first reported interaction of these two endocrine systems.

In fish, the genomic signaling action of cortisol is mediated by the activation of the glucocorticoid (GR) and mineralocorticoid (MR) receptors. Two functional Gr are present in teleosts, Gr1 and Gr2 (Bury *et al.*, 2003; Kim *et al.*, 2011), whereas a single Mr has been identified (Colombe *et al.*, 2000; Gilmour, 2005; Stolte *et al.*, 2008), each displaying distinct sensitivity to cortisol (Prunet *et al.*, 2006). The mRNA levels of *gr1*, *gr2* and *mr* observed in this study indicate a different regulation of these receptors in the intestine of Atlantic salmon in response to exogenous cortisol and SW exposure (Table 4.5). Previous studies in the gill of Atlantic salmon observed elevated levels of *gr* during smoltification, indicating an increased need for cortisol signalling during smoltification (Kiilerich *et al.*, 2007b). Our observation of upregulation of *gr1* in fish treated with cortisol (Table 4.5) indicates that this is also the case for the intestine. Increased *gr* mRNA levels were also found in the intestine of Mozambique tilapia (Takahashi *et al.*, 2006). In the present study, the response of *gr2* and *mr* was region and life-stage dependent, with both receptors exhibiting reduced mRNA levels after 48 h in SW. Given the hypo-osmoregulatory role of cortisol and the findings across different species in both intestine and gill (Chang *et al.*, 2023; Kiilerich *et al.*, 2011a;

Takahashi *et al.*, 2016; Tomy *et al.*, 2009), an increase in mRNA levels would be more expected than a reduction. However, some studies reported no difference in *gr* and *mr* mRNA levels (Kiilerich *et al.*, 2011a; McCormick *et al.*, 2008). Additionally, in the gill of Atlantic salmon, the response of *gr* and *mr* to SW exposure was found to be dependent on the stage of smoltification (Kiilerich *et al.*, 2007b). Our results, together with these previous observations, suggest that the regulation of cortisol receptors is species specific, and in the intestine of Atlantic salmon depends on life-stage and intestinal region.

In both parr and post-smolts, the concentration of the investigated ions (Cl^- , Ca^{2+} , Mg^{2+} , and HCO_3^-) was unchanged in the intestinal fluid (Table 4.4), but noteworthy, there was a reduction in the volume of intestinal fluid and in the quantity of carbonate precipitates collected in fish treated with cortisol (Fig. 4.2). It is somewhat paradoxical that cortisol clearly increased the underlying molecular mechanisms for intestine alkalization but that cortisol resulted in lower levels of precipitates in the intestine, more so if one considers that exogenous cortisol promotes drinking upon transfer to SW in Atlantic salmon (Fuentes *et al.*, 1996). However, it should be noted that we are simply measuring what is present in the intestine at a given time, and not the overall production. In other words, the intestine could be producing and excreting precipitates at a higher rate in response to cortisol. The greater excretion could be tied to elevated intestinal guanylin following cortisol treatment (Fig. 4.11) or elevated cortisol following SW exposure. A more integrative method of measuring precipitate production will be necessary to determine the true impact of cortisol on their production and elimination. It is also possible that the impact of cortisol to reduce intestinal monovalent ion levels may reduce the need for production of precipitates.

In this study, we show that even outside the smolt-window, juveniles of Atlantic salmon present some degree of hypo-osmoregulatory capacity when exposed to a hyper-osmotic environment. We also provide additional evidence for the relevance of cortisol in the development of hypo-osmoregulatory capacity in Atlantic salmon. While it is known that the intestine is a target of cortisol, our findings demonstrate that the mechanisms underlying the processing of the ingested SW in Atlantic salmon are positively regulated by cortisol. We also demonstrate that the molecular pathways associated with the HCO_3^- secretion cascade, previously characterized in other teleosts, are present in Atlantic salmon and that they are under the regulatory influence

of cortisol. Consistent with prior observations in Atlantic salmon, anterior and posterior intestines possess complementary set of osmoregulatory mechanisms, and the stimulatory action that cortisol mediates over the two regions reflects this complementarity. Overall, we conclude that cortisol plays a pivotal role in modulating intestinal function, enhancing the molecular pathways linked with the hypo-osmoregulatory mechanism in anterior and posterior intestine. Additionally, cortisol may regulate the production or excretion, or both, of the carbonate precipitates in the intestine of Atlantic salmon.

4.5. References

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CHAPTER

5

General discussion

5.1. General discussion

Teleosts are surrounded by a medium with ionic and osmotic characteristics different from those of their internal body fluids. Consequently, osmoregulatory mechanisms have evolved to ensure body homeostasis (Marshall and Grosell, 2006). Extensive work on gill has been carried out due to their relevance in osmoregulation (Evans *et al.*, 2005). Nevertheless, the GIT also plays a pivotal role. Marine teleosts indeed possess the extraordinary ability to get hydrated by drinking SW, owing to the capacity of the GIT to process the ingested SW and absorb water in the intestine (Grosell, 2014). The research project of this thesis investigated the development of the hypo-osmoregulatory capacity required to process the ingested SW in the intestine of juvenile Atlantic salmon following artificial stimulation of smoltification and salinity tolerance. In addition, the endocrine regulation of the intestinal function mediated by cortisol was investigated under natural-on-growing conditions.

The anadromous Atlantic salmon was chosen as subject of study because, being a migratory species, it represents an excellent model for investigating the development of the intestinal hypo-osmoregulatory mechanisms required in SW. Additionally, this species holds considerable commercial value and is subjected to intense aquaculture (FAO, 2022). A deeper understanding of its physiology could contribute to a further production optimization and to more sustainable aquaculture practices.

In several teleost species, including Atlantic salmon, morpho-functional specialization of the different segments of the intestine is present (Alves *et al.*, 2019; Gregório *et al.*, 2013; Grosell *et al.*, 2007; Ruhr *et al.*, 2014; Ruiz-Jarabo *et al.*, 2017; Seale *et al.*, 2014). Taking this into account, the focus was directed towards two distinct regions of the intestine, located after the point of insertion of the last pyloric caeca and identified in this thesis as anterior intestine and posterior intestine, to characterize their relative contribution to the hypo-osmoregulatory mechanisms in Atlantic salmon.

In the aquaculture, manipulation of the environmental factors and functional diets are used to stimulate smoltification, enhance SW performance, and improve overall fish welfare (Clarke *et al.*, 1996; Dawood *et al.*, 2018; Duncan and Bromage, 1998; Striberny *et al.*, 2021). Therefore, in **Chapter 2**, it was investigated how the stimulation of smoltification and salinity tolerance by mean of different photoperiods

(square-wave photoperiod, SP-LL, or continuous light, LL-LL), in combination or not with a transition diet enriched with salt mix and tryptophan modulate the intestinal function in Atlantic salmon. Also, the possibility of size-related response was investigated. In salmonids, hypo-osmoregulatory capacity is positively correlated with body size, and larger fish of a population show better salinity tolerance (McCormick and Saunders, 1987). Therefore, larger smolts are produced to transfer to sea-cage fish that are more robust and have higher salinity tolerance. In light of this, two different parr sizes at the beginning of the experiment were used, small (~40 g) and large (~130 g).

All treatments in **Chapter 2** successfully acclimated to SW. Still, the analysis of the intestinal fluid showed trends of osmolality and ion concentrations that suggest the possibility of adopting different osmoregulatory strategies depending on the group considered. Despite the ability of all groups to adapt in SW, the results indicated a better capacity to process the ingested SW for the small fish treated with a combination of short photoperiod and dietary treatment in FW. The chemistry of the intestinal fluid reflects the capacity of the fish to process the ingested SW (Grosell, 2014); therefore, its analysis may represent a valuable index to evaluate the intestinal hypo-osmoregulatory capacity in Atlantic salmon and other teleost species.

The Ussing chamber is an advantageous system to *in vitro* study the transepithelial transport of ions, nutrients and drugs across isolated tissues and cultured epithelial cells by mean of electrophysiological parameters such as TEP, I_{sc} and R_t (Clarke, 2009; Ussing and Zerahn, 1951; Vidyasagar and MacGregor, 2016). In **Chapter 2**, I_{sc} and R_t were used to functionally characterize the anterior and posterior intestines of Atlantic salmon. The basal I_{sc} , which represents the sum of the active ion transport across the epithelium, showed only marginal modulation in response to photoperiod. Instead, the R_t , which provides indication about tissue selectivity, viability, and integrity, showed significant modulation, especially in response to the short photoperiod. An overall reduction in R_t was observed, suggesting a preparatory reorganization of the tight junctions induced by the short-day, which potentially would favor water movement through the paracellular pathway.

When considering discrete regions, the pharmacological experiments in the Ussing chamber demonstrated the functional specialization existing between the anterior and posterior intestines of Atlantic salmon. Inhibition of the apical NKCC2 and NCC with bumetanide and HCTZ respectively caused a higher inhibition of the basal

I_{sc} in the anterior intestine, suggesting the presence of more functional transporters in this region than in the posterior intestine. Also, the stimulation of CFTR using bilateral FK+IBMX to stimulate endogenous cAMP differently affected the two regions. Moreover, the selectivity of anterior and posterior intestines appeared to be differently influenced by the environmental salinity. Specifically, the posterior intestine showed a more pronounced reduction in R_t after seven days in SW compared to the anterior intestine. This difference may reflect a greater need of a more permeable epithelium in this region of the intestine to enhance absorptive processes in SW also via the paracellular route.

The two size classes of fish used in the experiment adapted well to SW and responded similarly to the treatments used when smolt development and SW performance were analyzed (Striberny *et al.*, 2021). The results obtained in **Chapter 2** support the development of the intestinal hypo-osmoregulatory mechanisms required for SW in both fish sizes. However, the intestinal function in small fish appeared more responsive to the experimental factors investigated. Also, although small and large fish were not directly compared, the results suggest physiological differences between the intestines of the two sizes regardless of treatment, particularly in the functional characterization in the Ussing chamber. The epithelium of small fish seems to have more active transport, possibly due to a lower and less favorable surface/volume ratio. Higher absolute values of I_{sc} were measured in small fish, for both anterior and posterior intestines. Also, the response to the pharmacological treatments and the modulation of R_t by the light and diet suggest that the mechanisms acting in the two sizes of fish are potentially different.

Overall, the results obtained in **Chapter 2** revealed that the intestinal function of Atlantic salmon during the smoltification is modulated by photoperiod, diet, and fish size, which affect the development of the hypo-osmoregulatory machinery required for SW acclimation. Furthermore, the results highlighted the positive effect of short-photoperiod and transition diet on the intestinal hypo-osmoregulatory function and the functional difference existing between the anterior and posterior intestines of Atlantic salmon smolts.

In **Chapter 3**, anterior and posterior intestines were studied at the molecular level to characterize the mechanism underlying the intestinal function, focusing on pathways putatively involved in the osmoregulatory mechanisms. Following the functional results obtained in **Chapter 2** that showed higher responsiveness of small

fish toward photoperiod and diet, in **Chapter 3**, the molecular characterization was carried out only in small fish.

In **Chapter 3**, we demonstrated that the molecular pathways investigated, known to be involved in the intestinal hypo-osmoregulatory mechanisms in SW teleosts, are upregulated by exposure to the short photoperiod (SP-LL), particularly in the anterior intestine. The mRNA levels of three pivotal pathways of the hypo-osmoregulatory machinery were particularly upregulated by the short photoperiod in the anterior intestine of FW smolts: 1) the $\alpha 1c$ isoform of the NKA, which provide the electrochemical gradient required for ion movement; 2) the *nkcc2*, the major pathway for the absorption of ions in the intestine; 3) the *slc26a6*, one of the apical $\text{Cl}^-/\text{HCO}_3^-$ exchangers involved in Cl^- absorption and HCO_3^- secretion and consequently carbonate precipitation. These results indicate that exposure to the short photoperiod enhances the genetic response that facilitates the development of the molecular machinery underlying the intestinal function, better preparing the fish for the new hyper-osmotic environment. The diet enriched with the salt mix and tryptophan positively affected the mechanisms investigated, particularly in the fish treated with the short photoperiod, where the effect was enhanced.

The genetic response of the posterior intestine seemed lower to the treatments used, and a clear regionalization emerged. Confirming the functional data obtained in **Chapter 2**, anterior and posterior intestines showed a different transcriptional profile, suggesting a complementary role in handling the ingested SW.

From the genes investigated in **Chapter 3**, *nkcc2* and *slc26a6* emerged as potential molecular markers in the anterior intestine of FW smolts to evaluate the development of hypo-osmoregulatory capacity during smoltification.

Smoltification is an endocrine-regulated event, and cortisol is an important hormone in developing hypo-osmoregulatory mechanisms (McCormick, 2013). The intestine of Atlantic salmon is a target of cortisol (Cornell *et al.*, 1994; Fuentes *et al.*, 1996; Veillette *et al.*, 1995); however, little is known about the regulatory action mediated by cortisol in the transcellular mechanisms that mediate ion absorption in the intestine, particularly for the pathways involved in the HCO_3^- secretion and carbonate precipitation. In light of this and the molecular results obtained for AEs in **Chapter 3**, in **Chapter 4**, the action of cortisol on intestinal physiology was investigated in two life stages of Atlantic salmon outside the smolt-window,

underyearling parr and post-smolts, with particular focus on the HCO_3^- secretion cascade pathway.

The results demonstrate that even outside the smolt-window, Atlantic salmon juveniles present some degree of salinity tolerance, and that cortisol improves it, as shown by increased NKA activity in both the gill and anterior intestine and the overall better SW performance of fish treated with cortisol. At the transcriptional level, the intestinal mechanisms investigated were positively regulated by cortisol already in FW, suggesting that the peak of cortisol observed during smoltification (McCormick, 2013) not only stimulates the molecular mechanisms in the gill (Kiilerich *et al.*, 2007), but also induces the preparatory changes taking place in the intestine, with upregulation of the *nkaa1c* and *nkcc2*, and part of the pathways involved in the HCO_3^- secretion, specifically the mRNA levels of *slc26a6*, *nbc1*, *atp6v1a*.

As previously discussed, the chemistry of the intestinal fluid is a reflection of the mechanisms at work in the intestine of SW-acclimated teleosts (Grosell, 2014), and its analysis can provide valuable insights about the performance of the fish in SW. In **Chapter 4**, the transcriptional upregulation of the pathways investigated opposed to an unexpected similarity in ion composition between the intestinal fluid of control and cortisol-treated fish, and to a reduction of intestinal fluid volume and carbonate precipitates collected in the intestine of cortisol-treated fish. The results obtained in **Chapter 4** clearly indicate a cortisol-induced enhancement of the molecular pathways underlying hypo-osmoregulatory mechanisms in the intestine of Atlantic salmon. However, also suggest a potential role of cortisol in the production and/or excretion of carbonate precipitates, possibly linked to a higher speed or efficiency in the process of the ingested SW.

As observed for smolts (**Chapters 2 and 3** of this thesis), in parr and post-smolts, different transcriptional profiles exist in the anterior and posterior intestines, which are also differently regulated by cortisol (**Chapters 4**), providing evidence that further corroborate the regionalization of the hypo-osmoregulatory mechanisms in the intestine of Atlantic salmon.

As previously observed in other SW-acclimated teleosts (Ando *et al.*, 2014; Marshall *et al.*, 2002; Ruhr *et al.*, 2014), the results obtained in this thesis suggest the presence of a secretory pathway also in the intestine of Atlantic salmon, which could be relevant for recycling Cl^- and to avoid obstruction during the passage of solids in

the intestine. From the pharmacological experiments with FK and IBMX in **Chapter 2**, the possibility of a secretory pathway involving apical CFTR in the intestine of Atlantic salmon could be speculated. Also, the transcriptional results obtained in **Chapter 3** for *nkcc1*, similarly regulated to *cftr1*, and the positive pan-NKCC immunodetection observed in the basolateral side of the anterior and posterior intestines of Atlantic salmon (Duarte *et al.*, 2023) suggest the presence of a secretory pathway in the intestine of Atlantic salmon. Moreover, **Chapter 4** provided further evidence for the existence of this secretory pathway, supported by the high mRNA levels of guanylin (*gn*) in both FW and SW fish. A novel result is also the interaction of this endocrine factor with cortisol, particularly in the posterior intestine of FW fish, which would certainly deserve further investigation.

Overall, the results obtained in this thesis demonstrate that the intestine is an important target of the preparatory changes taking place during smoltification and that at the functional, biochemical, and transcriptional levels, the intestine of Atlantic salmon is modulated by external stimuli such as light, salinity, and diet, as well as endocrine factors like cortisol.

5.2. References

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CHAPTER

6

Conclusions

6.1. Conclusions

The development of this project allowed to increase the current knowledge about the changes that take place in the intestine of Atlantic salmon during smoltification and SW acclimation. As well as improving scientific knowledge about intestinal physiology, the results obtained have the potential to contribute to the development of sustainable aquaculture.

The main conclusions of the work are the following:

1. Regardless of photoperiodic history, dietary treatment, and fish size, Atlantic salmon smolts can develop hypo-osmoregulatory competence required for SW acclimation, demonstrating the high flexibility and adaptability of this species towards stimuli.
2. Exposure to a short-day period is the most promising strategy to stimulate smoltification. The light signal enhances the molecular pathways involved in intestinal hypo-osmoregulatory mechanisms already in FW and, at the functional level, allows better processing of the ingested SW after transfer into the sea.
3. Dietary stimulation with a salt mix and the amino acid tryptophan is not able alone to stimulate smoltification and the suite of intestinal responses needed for optimal SW performance. Still, it synergizes with the short photoperiod improving its action.
4. Cortisol enhances salinity tolerance in out-season juveniles and positively modulates the osmoregulatory function of the intestine, including the pathways involved in the intestinal HCO_3^- secretion cascade.
5. The intestine of Atlantic salmon presents a region-specific expression and regulation of the genes involved in hypo-osmoregulatory mechanisms, resulting in a complementary role of the anterior and posterior intestine.
6. To evaluate hypo-osmoregulatory capacity during smoltification, the transcriptional levels of the apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, *nkcc2*, and of the apical $\text{Cl}^-/\text{HCO}_3^-$ exchanger, *slc26a6*, in the anterior intestine are potential molecular markers in FW fish. Also, upon transfer to SW, the intestinal fluid composition may represent an advantageous index to evaluate SW performances in smolts of Atlantic salmon.

CHAPTER

7

Conclusiones

7.1. Conclusiones

El desarrollo de esta Tesis Doctoral ha permitido aumentar el conocimiento actual sobre los cambios que tienen lugar en el intestino del salmón del Atlántico durante el proceso de esmoltificación y la aclimatación al agua de mar. Los resultados obtenidos, además de mejorar el conocimiento científico sobre la fisiología intestinal del salmón del Atlántico, presentan el potencial de contribuir al desarrollo de una acuicultura más sostenible.

Las principales conclusiones de la presente Tesis Doctoral son las siguientes:

1. Los ejemplares smolts de salmón del Atlántico, independientemente de la historia fotoperiódica, el tratamiento dietético y el tamaño, pueden desarrollar la capacidad hipo-osmorreguladora necesaria para la aclimatación al agua de mar, demostrando la alta flexibilidad y adaptabilidad de esta especie hacia los estímulos.
2. La exposición a un fotoperiodo de días cortos es la estrategia más prometedora para estimular el proceso de esmoltificación. La señal luminosa potencia las vías moleculares involucradas en los mecanismos hipo-osmorreguladores intestinales en los ejemplares mantenidos en agua dulce y, a nivel funcional, permite un mejor procesamiento del agua de mar ingerida tras la transferencia al mar.
3. El tratamiento dietético con una mezcla de sal y el aminoácido triptófano no es capaz, por sí solo, de estimular el proceso de esmoltificación y la serie de respuestas intestinales necesarias para una óptima aclimatación a agua de mar. Sin embargo, dicho tratamiento potencia estos efectos cuando se combina con el fotoperiodo corto.
4. El cortisol mejora la tolerancia a la salinidad en juveniles fuera de temporada de esmoltificación y estimula positivamente la función osmorreguladora del intestino, incluyendo las vías involucradas en la cascada de secreción intestinal de HCO_3^- .
5. El intestino del salmón del Atlántico presenta una expresión y regulación específicas de la región de los genes involucrados en los mecanismos hipo-

osmorreguladores dependiente de la región. Esto origina un papel complementario del intestino anterior y posterior a nivel osmorregulador.

6. Los niveles de transcripción del cotransportador apical $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ (*nkcc2*), y del intercambiador apical $\text{Cl}^-/\text{HCO}_3^-$ (*slc26a6*) en el intestino anterior son posibles marcadores moleculares para evaluar la capacidad hiposmorreguladora durante la esmoltificación en ejemplares aclimatados a agua dulce. Además, la composición del fluido intestinal puede representar, tras la transferencia a agua de mar, un parámetro adecuado para evaluar el rendimiento en agua de mar en los ejemplares smolts del salmón del Atlántico.

APPENDIX

Supplementary Table 1. Ranking of candidate reference genes in the anterior intestine and posterior intestine of Atlantic salmon (*Salmo salar*) obtained with NormFinder analysis. Lower stability values indicate higher expression stability.

ANTERIOR INTESTINE			POSTERIOR INTESTINE		ANTERIOR & POSTERIOR INTESTINE	
Ranking	Gene	Stability value	Gene	Stability value	Gene	Stability value
1	<i>actb</i>	0.104	<i>ubap2</i>	0.310	<i>arpc1b</i>	0.260
2	<i>arpc1b</i>	0.125	<i>ef1a</i>	0.338	<i>ubap2</i>	0.270
3	<i>18S</i>	0.152	<i>arpc1b</i>	0.366	<i>ef1a</i>	0.299
4	<i>ef1a</i>	0.160	<i>b2m</i>	0.423	<i>actb</i>	0.365
5	<i>ubap2</i>	0.185	<i>actb</i>	0.439	<i>b2m</i>	0.394
6	<i>b2m</i>	0.186	<i>18S</i>	0.473	<i>18S</i>	0.403
Best combination	<i>18S-actb</i>	0.100	<i>ef1a-arpc1b</i>	0.194	<i>18S-actb</i>	0.182

LIST OF CONTRIBUTIONS

PUBLICATIONS

Gaetano, P.*, Duarte, V.*, Striberny, A., Hazlerigg, D., Jørgensen, E.H., Campinho, M.A., Fuentes, J. (2023). Photoperiod and dietary treatment in freshwater modulate the short-term intestinal response to seawater in Atlantic salmon (*Salmo salar*). Aquaculture. <https://doi.org/10.1016/j.aquaculture.2023.739316>

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ORAL COMMUNICATION

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POSTER

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