Development of Na^+, K^+ ATPase enzyme activity and expression patterns of sulfate transporters in gills, intestine and kidney during smoltification and SW acclimation in Atlantic salmon (*Salmo salar* L.)

For the Fulfilment of the Master Degree in Aquaculture Biology

By

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Abstract

Parr-smolt transformation (smoltification) is an important developmental stage that entails physiological changes preparing the smolt for a marine life. Development of seawater tolerance (functionally linked to changes in the gills, intestine and kidney) is arguably one of the most important changes associated with smolt quality. Producing high quality smolts is a critical factor for successful salmon farming.

Surprisingly, few studies have addressed concurrent changes in osmoregulatory functions of gills, intestine and kidney in smolts. Juvenile Atlantic salmon subjected to a 6 week short day photoperiod (12 hours light:12 hours dark) followed by a 7 week long day (24 hours light) showed a classic smoltification related decrease in condition factor 260 day degrees (d.d) after continuous light was turned on, while smolt index increased steadily throughout smoltification. Gill Nka enzyme activity increased significantly at 260 d.d, reaching peak levels at 450 d.d, while intestinal and kidney enzyme activity increased at 350 d.d, remaining high at 450 d.d. This indicates that both intestine and kidney require longer time to acquire full SW capacity under intensive out of season smoltification protocols. Nka activity in gills and intestine remained high after SW transfer, contrasting a transient decrease in kidney Nka activity. This probably reflects an extra initial acute need for short time reduction of water loss via the kidney after abrupt SW exposure.

The use of increased salinities in land-based recirculation facilities has been associated with unfortunate episodes of acute mortalities, probably due to accumulation of hydrogen sulphide (H_2S). Mitigating actions, such as removing sulfate from the water, may also compromise osmoregulatory functions. Searches in the salmon genome and phylogenetic analysis revealed annotated and non-annotated sequences of solute carrier family 13 (Slc13) and 26 (Slc26), including Slc13a1 (intestine), Slc26a6a (gills, intestine, kidney), Slc26a6b (intestine, kidney), Slc26a6c (kidney) and Slc26a1 (intestine, kidney). An additional repertoire of Slc26a6a (Slc26a6a1 and Slc26a6a2) and Slc26a1 (Slc26a1a and Slc26a1b) paralogues appears to be present after the salmonid-specific fourth vertebrate whole-genome duplication. The preparatory increase in kidney specific *slc26a6a1* and *slc26a1a* mRNA levels in addition to the gill specific decrease of *slc26a6a2* mRNA levels during smoltification and SW transfer suggests an important role of these sulfate transporters in the regulatory shift from absorption to secretion moving from FW to SW. The mRNA abundance of *slc26a6b*, *slc26a6c* and *slc26a1b* remained stable, with no significant differences over time and between parr and smolts.

The demonstration of different Nka patterns in gills, intestine and kidney warrants more comprehensive investigations during smolt development, including all three organs. This is substantiated by the discovery of potential SO_4^{2-} transporters in all three organs. More research is needed in the kidney considering the predominant role it has in handling divalent ions in SW.

Content

Glossary (alphabetic order)

Anguilla Japonica – Japanese eel

Esox lucius – Northern pike

Hemopoietic tissue – Blood producing part of the kidney

Nephrocalsinosis – calcium precipitation in the kidney

Oncorhynchus mykiss – Rainbow trout

Takifugu Obsurus – Obscure pufferfish

Podocytes – Modified epithelial cells (glomerulus)

Renal physiology – physiology of the kidney at the level of nephrons

Salmo salar – Atlantic salmon

Smoltification - parr-smolt transformation

Biological abbreviations list

AA – Afferent arteriole (blood vessel)

ADP – Adenosine diphosphate

AM – Assay mixture

AQP – Aquaporins

ATP - Adenosine Trisphosphate

BLAST - Basic Local Alignment Search Tool

cAMP – cyclic adenosine monophosphate

CFTR - apical cystic fibrosis transmembrane conductance regulator

ClC-K – Chloride channel kidney specific

CT – Collecting tubule (nephron)

CA – Carbonic anhydrase

DS – Distal segment (nephron)

d.d – Day degrees

EA – Efferent arteriole (blood vessel)

FW – Freshwater

FXYD – FXYD domain-containing transport regulator

GFR – Glomerulus filtration rate

G – Glomerulus

HSS – Haemorrhagic smolt syndrome

ILAB – Industrilabratoriet i Bergen

LDH - Lactic Dehydrogenase

mOsm/L – milliosmole per liter

NADH - β-Nicotinamide Adenine Dinuclotide

NCC – Na^+/Cl^- cotransporter

NKA – Na^+, K^+ -ATPase

NKAa1 – NKA α-1 isoform

NKCC1 – Na^+, K^+, $2CL^-$ cotransporter 1

NKCC2 - Na^+, K^+, $2CL^-$ cotransporter 2

NS – Neck segment (nephron)

PEP - phosphenylpyruvate

PS – Proximal segment (nephron)

PK - Pyravate Kinase

qPCR - Quantitative Polymerase Chain Reaction

RAS – Recirculating aquaculture systems

RBC – Red blood cells

RIN – RNA integrity number

SW – Seawater

SLC26A6 – Solute carrier family 26 member 6

SLC26A1 – Solute carrier family 26 member 1

SLC13A1 – Solute carrier family 13 member 1

Ss4R – Salmonid specific fourth vertebrate whole genome duplication

UFR – Urine filtration rate

Note on nomenclature

Gene/protein fish - gene: *slc26a6* (italicized, small letters), protein/enzyme: Slc26a6 *(*first letter cap*).*

General nomenclature - reference to the transporter or function in general, not referring to either the gene (RNA, DNA, mRNA) or protein/enzyme (levels, location) specifically, are written in capital letters (E.g. Na^+, K^+ $2Cl^-$cotransporter 1, NKCC1).

References

Literature having more than tree authors are written *et al.,* in the text and in reference list.

Introduction

1.1 Natural and out of season smolt development

Atlantic salmon (*Salmo salar*) displays an anadromous lifecycle, with juveniles migrating out to sea where they grow before returning to their natal river and reproduce. One critical developmental stage in their lifecycle is the parr-smolt transformation (smoltification), which entails morphological, behavioural, and physiological changes that prepare the smolt to enter the marine environment (Hoar, 1988). This developmental process is largely stimulated by environmental cues such as photoperiod, temperature, salinity, turbidity and flow rate (Hoar, 1988). During smoltification, salmon gradually lose their parr marks, become silvery, slimmer and begin downstream migratory and schooling behaviour (Björnsson, Stefansson and McCormick, 2011). Preparatory osmoregulatory changes in the gills, kidney and intestine are vital for salmon acclimation to seawater (SW) (Nilsen *et al.*, 2007; 2008; Tipsmark *et al.*, 2010, 2011; Kato *et al.*, 2011; McCormick, Farrell and Brauner, 2013; Sundh *et al.*, 2014). Developing a working knowledge of the osmoregulatory capabilities of the fish during smolt development and acclimation to SW has been essential for successful production of Atlantic salmon in Norway.

Norwegian salmon farming has been subject to an unprecedented growth over the last 45 years. Norway is the leading producer of Atlantic salmon worldwide, with an annual production of approximately 1.1 million tons in 2019, resulting in an export value of approximately 72.5 billion Norwegian kroner (Norwegian Seafood Council, 2019 (seafood.no)). A key component of this success has been the increased understanding and knowledge of the Atlantic salmon smoltification process and improved definition of the mechanisms (Björnsson and Bradley, 2007; Björnsson, Stefansson and McCormick, 2011). One critical success factor is the implementation of light regimes (photoperiod), enabling cultivators to control smolt development and produce “out of season” smolts. This is achieved by altering the daylength, with 6 weeks of shorter days (winter signal, 12h Light:12h Dark) followed by 4-6 weeks of longer days (summer signal, 24hLight) (Stefansson *et al.*, 1991; Porter *et al.*, 2001; Ebbesson *et al.*, 2008). However, the industry is rapidly intensifying smolt protocols, producing larger smolt directly compromising the physiological capabilities of the fish. Thus, the need for redirecting focus on the osmoregulatory capacity and integrative role of gills, intestine and kidney has become increasingly important.

1.2 The osmoregulatory role of gills, intestine and kidney

Osmoregulation is essential for proper cell function in all organisms, independent of terrestrial or aquatic environments. Fish live in an aquatic environment that is either hypo-osmotic or hyper-osmotic and are thus more vulnerable to changes in body fluids compared to terrestrial animals (Takei et al., 2014). Teleost fish regulate salt and water balance through the cooperative efforts of the gills, kidney and intestine in order to maintain a plasma osmolality range of 300 – 325 mOsm/kg (Evans, Piermarini and Choe, 2005; Marshall and Grosell, 2006). To achieve this, they adopt different strategies to maintain osmotic and ionic homeostasis in freshwater (FW) (< 0.5 ppt (3-5 mOsm/kg)) and seawater (SW) (>30 ppt (roughly 1000 mOsm/kg)) environments.

1.2.1 Hyper-osmoregulation (FW)

In FW teleosts, ion retention and water excretion are vital to maintain homeostasis (Evans, Piermarini and Choe, 2005). Most fish in FW have a small positive plasma membrane electrical potential in the gill that allows anion (Cl^-) uptake. Further, since the concentration gradient is so large, both Na^+ and Cl^- ions are actively transported across the gill epithelium (Potts, 1984; Evans, Piermarini and Potts, 1999). Specialized ionocytes in the gill epithelium enable uptake of NaCl via the apical V-type H^+ -ATPase that is linked to an apical Na^+ channel, apical Cl^-/HCO_3^- exchanger and a basolateral Na^+, K^+ -ATPase (NKA) transporter (Marshall, 2002). In the intestine, absorption occurs across enterocytes via apical Na^+, K^+, $2Cl^-$ cotransporter 2 (NKCC2), Na^+/Cl^- cotransporter (NCC) and basolateral NKA transporter, creating the electrochemical gradient necessary for Na^+ and Cl^- transport in enterocytes (Colin *et al.*, 1985; Sundell and Sundh, 2012; Sundh *et al.*, 2014). In the kidney, large volumes of dilute urine are excreted, and only minor ion levels are found in the urine of FW fish. Typically, this includes 5-20 mM of NaCl and other ions being at most a few mM, and often in the sub millimolar range (Hickman and Trump, 1969). However, a portion of the Na^+ and Cl^- are reabsorbed, facilitated by Nka enzyme activity and NKCC transporters (Nishimura, Imai and Ogawa, 1983; Dantzler, 2003; Braun and Dantzler, 2011). Very little is known about the handling of divalent ions in FW.

1.2.2 Hypo-osmoregulation (SW)

Seawater contains a high abundance of Mg^{2+}, SO_4^{2-}, Ca^{2+}, K^+ ,Na^+ and Cl^- (Edwards and Marshall, 2012), which causes teleost fish to osmotically lose water from the environment, primarily across the gills (Evans et al 2005). To compensate they need to drink seawater, thus loading the blood with NaCl that is actively secreted across gills and skin epithelia (Takei *et al.*, 2014). In SW, the electrical potential of the plasma membrane in the gills is large enough to facilitate cation secretion and active transcellular transport of Cl^-, by the combined action of the basolateral NKA transporter and Na^+, K^+, $2Cl^-$ cotransporter 1 (NKCC1), apical cystic fibrosis transmembrane conductance regulator (CFTR)

anion channel and paracellular transport of Na^+ (Marshall, 2002; Marshall and Grosell, 2006; Hwang, Lee and Lin, 2011). In the intestine, water transport has been tightly linked to the active absorption of Na^+ and Cl^- (Hickman, 1968a), powered by the basolateral NKA transporter while the cotransport system NKCC2 facilitates absorption of Cl^- and K^+ (Grosell, 2010; Sundh *et al.*, 2014). The NKA and NKCC2 transporter is highly active in the intestine of euryhaline fishes following SW transfer. The importance of this is illustrated by the upregulation of messenger ribonucleic acid (mRNA) expression of *nka* and *nkcc2* in the intestine (Colin *et al.*, 1985; Cutler *et al.*, 2000; Esbaugh and Cutler, 2016). In addition, Mg^{2+}, SO_4^{2-} and Ca^{2+} are dominant in intestinal fluid and it has been proposed that the intestine has a key role dealing with divalent ions (Pelis and Renfro, 2003; Kodzhahinchev, Biancolin and Bucking, 2018). In the kidney, minute volumes of concentrated urine are produced in order to conserve water (Hickman, 1968b). The main electrolytes secreted in the kidney are Mg^{2+} and SO_4^{2-}, abundantly found in the urine, but electrochemical gradients for K^+ and Na^+ allow Cl^- entry across the basolateral membrane (Cliff and Beyenbach, 1992; Katoh *et al.*, 2008). As in the gills and intestine, the NKA transporter in the kidney is crucial, being linked to numerous transport pathways (Beyenbach, 2004). Although the gills removes most of the excess Na^+ and Cl^- the kidney contributes around 5 % of monovalent ion removal (Teranishi and Kaneko, 2010). However, the kidney is imperative for removing excess divalent ions (Mg^{2+}, SO_4^{2-}, Ca^{2+}) in SW environments (Flik *et al.*, 1996; Chandra, Morrison and Beyenbach, 1997; Renfro, 1999; Beyenbach, 2004).

1.2.3 Euryhalinity

There is no doubt that osmoregulation in both FW and SW are essential for regulation and require all three osmoregulatory organs to readily maintain both osmotic and ionic levels (figure 1A and 1B). However, osmoregulation is energy demanding and transitions between different salinities require substantial remodelling of transport pathways in gills, intestine and kidney. Therefore, given the variation in salinity throughout the lifecycle of an Atlantic salmon, the osmoregulatory pathways in all three organs need extra plasticity (Stefansson *et al.*, 2008; McCormick, Farrell and Brauner, 2013; Kültz, 2015). Notably, adequate changes in the three osmoregulatory organs are pivotal for the performance and survival of Atlantic salmon transitioning to SW. Research of osmoregulatory changes occurring during smoltification has predominantly targeted the gills (Björnsson and Bradley, 2007), particularly ionocyte development and key osmoregulatory enzymes and ion transporters such as the Nka enzyme, NKA α-1 subunit isoforms, CFTR I and NKCC1 transporters (Tipsmark *et al.*, 2002; Nilsen *et al.*, 2007; Hiroi and McCormick, 2012). As such, several of these are now used as a key indicator for smolt development. Conversely, the osmoregulatory role of the intestine and kidney during smoltification have received less attention. Nevertheless, several studies have addressed intestinal changes during smoltification revealing important findings of several transport pathways

(Veillette, Sundell and Specker, 1995; Sundell *et al.*, 2003; Sundell and Sundh, 2012; Sundh *et al.*, 2014; Takei *et al.*, 2014). However, the majority of studies on the kidney focus primarily on stenohaline fish, especially in SW, and with few addressing salmonids (Renfro and Pritchard, 1983; Beyenbach, Petzel and Cliff, 1986; Beyenbach, 1995, 2004; Renfro, 1999; Pelis *et al.*, 2003; Marshall and Grosell, 2006). Therefore, there is a noticeable requirement to elucidate transport mechanisms in the kidney, increasing the understanding of the complete osmoregulatory machinery in fish.

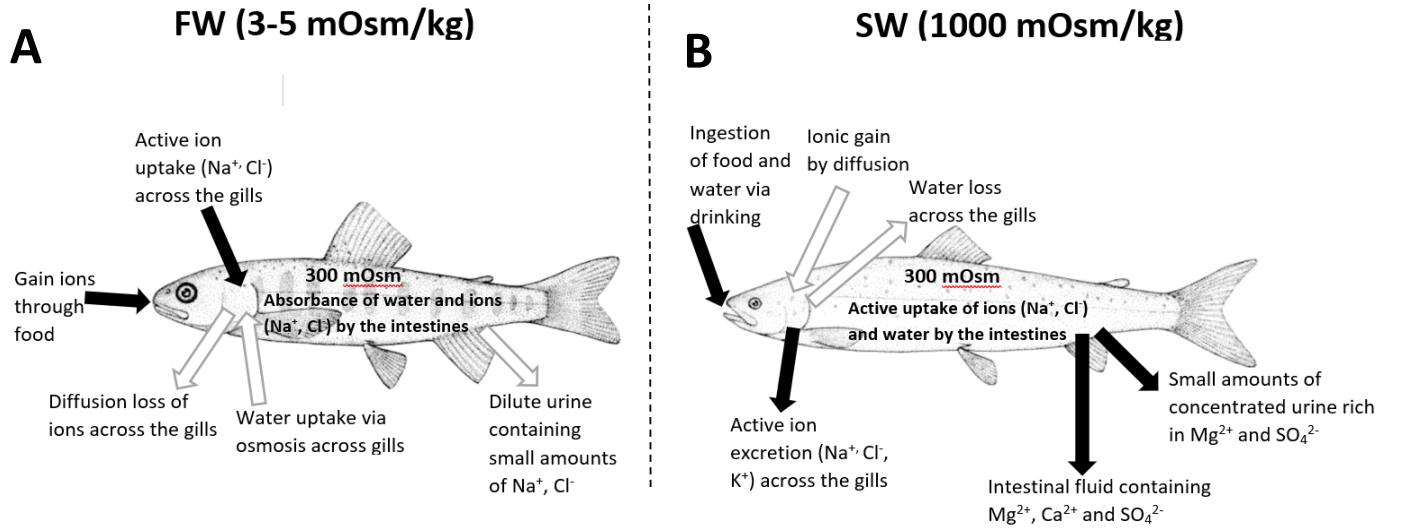

Figure 1: Overview of water and salt regulation in all three osmoregulatory organs for salmonids in FW (A) and SW (B). ***A:*** *In FW, fish are subject to osmotic gain from the water and diffusional loss of ions across gills and other permeable surfaces. The water gain is countered by the kidney producing large volumes of dilute urine containing small amounts of NaCl, while loss of ions is regained through ion uptake in the gills and intestinal tract (Na^+, Cl^-).* ***B:*** *In SW, fish are subject to osmotic loss of water and diffusional gain of ions across gills and other permeable surfaces. Osmotic loss of water is countered by drinking SW followed by passive or active uptake of water and ions through the intestinal tract (Na+, Cl-). The excess ions such as Na+ and Cl- in the extracellular fluids are transported by the blood to ionocytes in the gills and skin where they are secreted/excreted. Excess divalent ions are secreted/excreted through small volumes of concentrated urine in the kidney, especially rich in SO42- and Mg2+. Additionally, intestinal fluid contains Mg2+, Ca2+ and SO42-. Modified after Marshall and Grosell (2006) and Edwards and Marshall (2012); pictures: retrieved from public domain.*

1.3 Anatomical characteristics and transport pathways in the kidney

1.3.1 Anatomical characteristics of the kidney

In their seminal review, Hickman and Trump (1969) provided a detailed overview of the evolution and anatomy of the teleost kidney. Based on both microscopic observation and studies on isolated tubules, the nephron of euryhaline fishes is divided into sections of different lengths; glomerulus (excluding aglomular fish), proximal segments I and II (marine teleosts have a third proximal segment), intermediate segment (only present in freshwater teleosts), the distal segment (sometimes missing in marine teleosts), the collecting tubule and the collecting duct (Hickman and Trump, 1969). The anatomical and regulatory properties of these segments may differ slightly depending on the

salinity to which the fish is acclimated, or if the animal is transitioning between salinities. In general, euryhaline species, such as the Atlantic salmon, display very similar anatomical attributes largely analogous of those in stenohaline species (Braun and Dantzler, 2011; Dantzler, 2016). In salmonids, glomeruli and tubular segments are tangled and in close connection with hemopoietic tissue, which is especially true in the anterior part, while urine producing nephrons are substantially more numerous towards the posterior part of the kidney (Anderson and Loewen, 1975; Resende *et al.*, 2010). In the glomerulus, a network of glomerular capillaries filter plasma through small pores called podocytes consisting of visceral and parietal layers and into the Bowman`s capsule (Brown, Taylor and Gray, 1983; Resende *et al.*, 2010) (figure 2). Thereafter, the primary urine passes the short neck segment and is sequentially modified by reabsorption and secretion processes as it passes through the proximal segment, distal segment and collecting tubule before it enters the collecting ducts (Hickman and Trump, 1969) (figure 3). The absorption or secretion of ions in FW and SW environments are accomplished by many proposed transport pathways in the proximal, distal and collecting tubule in the kidney.

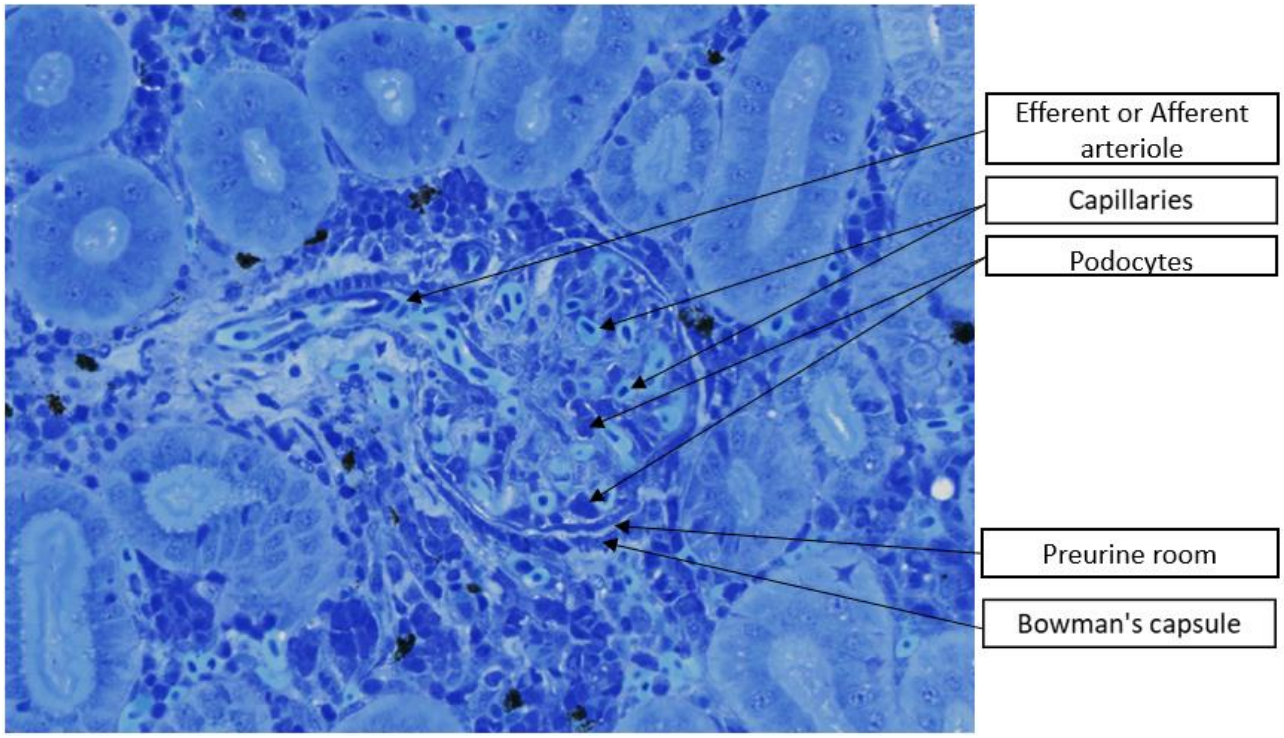

Figure 2: ***Anatomical structures of the glomerulus in the kidney of Atlantic salmon (Salmo salar).*** *Kidney tissue sections stained with toluidine blue (Appendix 3) allow visualisation of efferent and afferent arterioles, capillaries, Bowman's capsule, podocytes and pre-urine room.*

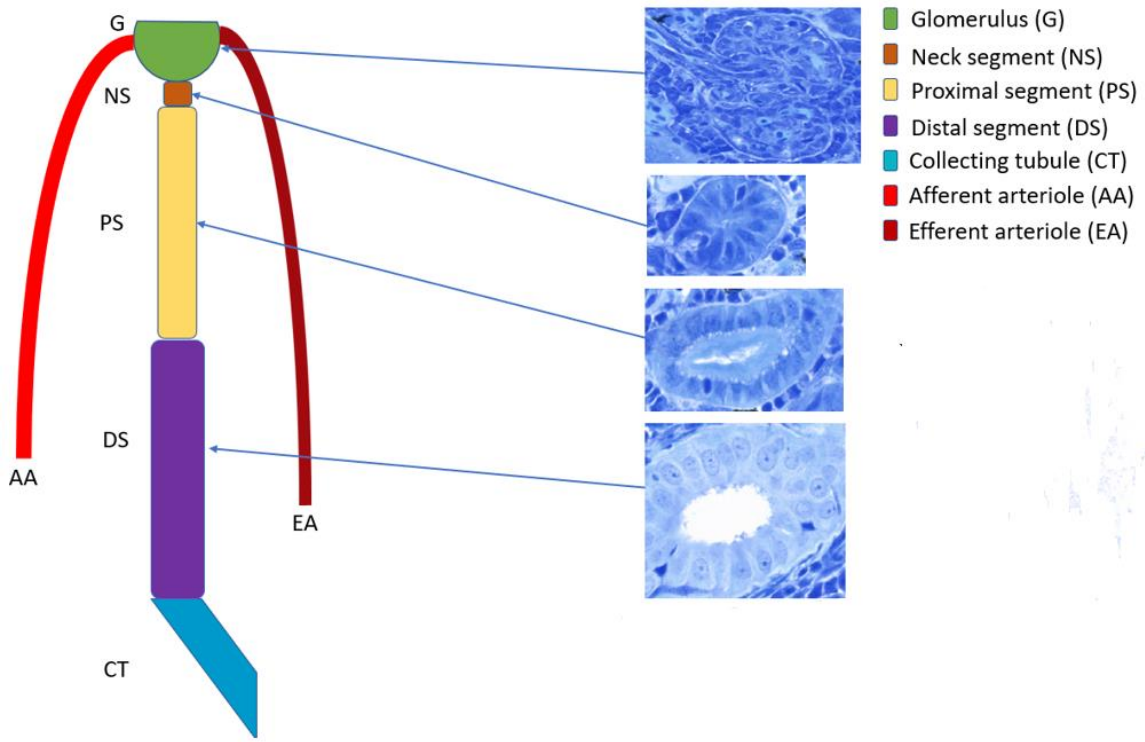

Figure 3: Anatomical overview of the nephron in Atlantic salmon. *Kidney tissue from Atlantic salmon was cut and stained with toluidine blue before examination (Appendix 3). The figure represents the general anatomy of the nephron with corresponding histological cuts of the following segments: Glomerulus (G), Neck segment (NS), Proximal segment (PS), Distal segment (DS), Collecting tubule (CT) (Appendix 3). Hence, no histological overview is given for Collecting tubule (CD), Afferent (AA) and Efferent arterioles (EA).*

1.3.2 Transport pathways in the kidney of FW acclimated teleosts

In FW, ions are scarce and osmolarity is very low. Therefore, fish experience a continuous osmotic influx of water and diffusive loss of major ions through the gills/skin. The kidney counters this by filtering large volumes of blood in the glomeruli, thereby maintaining high Glomerular Filtration Rates (GFR) of 4-16 milliliter/kilogram/hour (ml//kg/h) and elevated Urine Flow Rates (UFR) of approximately 1-6 ml/kg/h, ensuring excretion of large volumes of dilute urine (20-50 mOsm/L) (Hickman and Trump, 1969; Beyenbach, 1995). Production of the dilute urine is possible due to the impermeable features of the distal tubule and downstream regions, including the bladder, enabling the reabsorption of precious salts (mainly Na^+ and Cl^-) while limiting the accompanying osmotic reabsorption of water. Moreover, in the proximal tubule I, glucose, macromolecules and Na^+ and Cl^- are reabsorbed as water follows (Hickman and Trump, 1969; Marshall and Grosell, 2006). Although minor secretion of Mg^{2+} and SO_4^{2-} occurs (Nishimura, Imai and Ogawa, 1983), most divalent ions such as Mg^{2+}, SO_4^{2-} and Ca^{2+} are reabsorbed in the proximal tubule II, the longest portion (~50%) of the nephron. Other important ions, such as Na^+, Cl^-, K^+ and HCO_3^-, are also reabsorbed in the proximal tubule II, together with water (Cliff and Beyenbach, 1992; Dantzler, 2003). Even though ions generally are reabsorbed in the kidney on an overall basis in most reports on fasted FW acclimated

fish, there is evidence that dietary ion intake can cause shifts from reabsorption to secretion of both monovalent and divalent ions (Oikari and Rankin, 1985; Curtis James and Wood, 1991; Cliff and Beyenbach, 1992; Wood and Shuttleworth, 1995; Bucking, Landman and Wood, 2010). Nevertheless, the primary function of the kidney in FW is to reabsorb most of the filtered solutes while excreting excess water.

1.3.3 Transport pathways in the kidney of SW acclimated teleosts

In SW, ions are abundant, some at toxic levels (e.g. Mg^{2+}, Ca^{2+}, SO_4^{2-}), and water is lost by osmosis through the gills/skin. The fish replace the water loss by drinking, despite incurring additional Na^+ and Cl^- loading which accompanies this enteric water absorption. In general, both urine volume and composition are very different in SW and FW species. As a result, the kidney typically exhibits low GFR (0.5-2.0 ml/h/kg) in SW fish, reflecting greatly reduced numbers of functioning glomeruli (Schmidt-Nielsen and Renfro, 1975; Brown et al., 1978), and lower UFR (0.2-0.3 ml/h/kg) than FW fish, producing a urine which is isotonic (300-410 mOsm/L), to the blood plasma in which the main electrolytes excreted are Mg^{2+}, SO_4^{-2} and Ca^{2+} (Hickman, 1968b; Hickman and Trump, 1969; Renfro, 1999; Beyenbach, 2004). Additionally, the distal segment appears to be reduced (Hickman and Trump, 1969). Most secretion of Na^+ and Cl^- primarily occurs in the early proximal segment (proximal segment I) suggesting facilitation by the NKA transporter, NKCC cotransport isoforms and apical Cl^- channel (Beyenbach, 1986). Hence, the urine produced is made isosmotic largely due to reabsorption of NaCl in the distal segment (Dantzler, 2003). Further reabsorption of Na^+ and Cl^- appears to occur in the urinary bladder, leaving high concentrations of divalent ions in the urine (Beyenbach and Kirschner, 1975). Secretion of divalent ions (Mg^{2+}, Ca^{2+} and SO_4^{-2}) are primarily believed to occur in the late proximal segment (proximal segment II) of SW teleosts (Beyenbach, 1995).

The preceding overview summarizes our general understanding of how the kidney functions at a macro level in FW and SW (figure 4A and 4B). For additional details, the reader is referred to Hickman and Trump (1969), Curtis and Wood (1991), Wood and Patrick (1994), Nishimura and Fan (2003), Dantzler (2003), Beyenbach (2004), Marshall and Grosell (2006) and Dantzler (2016). While the current research provides a valuable insight into renal functions it does not elucidate how renal function is altered during smoltification or following SW transfer in salmonids. Furthermore, very few studies have verified this using emerging molecular methods, and it is surprising that so few studies have addressed this in salmonids.

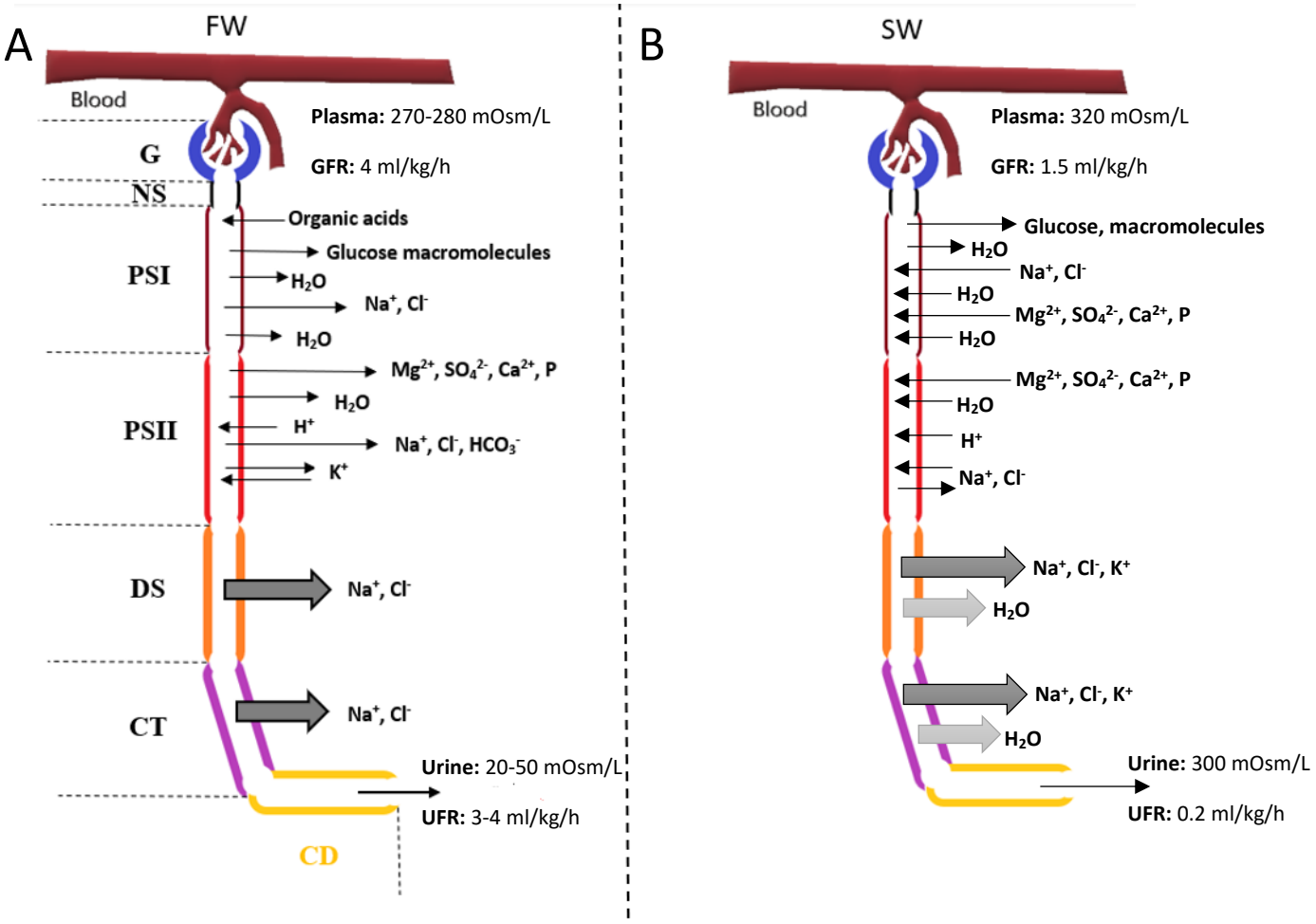

Figure 4: Overview of water and ion movement in FW teleost nephron (A) and SW teleost nephron (B). A: *In a FW fish kidney, most ions are reabsorbed, and water follows by diffusion. Therefore, impermeable features of the distal and collecting duct can reabsorb Na^+ and Cl^- without the diffusion of water. The glomerulus filtrates (GFR) roughly 4-16 milliliter/kilogram/hours (ml/kg/h) of plasma (270-280 mOsm/L) while producing (UFR) roughly 1-6 ml/kg/h of dilute urine (20-50 mOsm/L).* ***B:*** *In a SW fish kidney most ions are secreted and water follows by diffusion. To countenance water loss and dehydration in SW fish distal and collecting duct must be more permeable enabling effective Na^+ and Cl^- while water can follow by diffusion. The glomerulus filtrates (GFR) roughly 0.2-1.5 ml/kg/h of plasma (320 mOsm/L) while producing (UFR) roughly 0.2-0.3 ml/kg/h of concentrated urine (300 mOsm/L) rich in SO_4^{2-} and Mg^{2+}. Different sections of the nephron are displayed as follows: Glomerulus (G), Neck segment (NS), Proximal segment I (PSI), Proximal segment II (PSII), Distal segment (DS), Colleting tubule (CT) and collecting duct (CD). The same differentiation of segments applies for model B. Keep in mind that this nephron model is largely based on euryhaline teleost in FW or SW environments that often have a distal segment commonly found in FW teleost and do not possess a third proximal segment commonly found in SW teleost. Model modified from the paper of Hickman and Trump (1969) and Marshall and Grosell (2006).*

1.4 Kidney function and genetic studies in salmonids

1.4.1 Renal function in salmonids

Our knowledge about renal physiology and transporter mechanisms stem largely from studies on either FW or SW acclimated fish. Despite one study reporting Nka activity in the kidney during smoltification (McCartney, 1976) and another from different salinities (McCormick, Moyes and Ballantyne, 1989), our knowledge about changes in renal physiology during salmonid smoltification is limited. In wild Atlantic salmon, a rapid increase in UFR, thus urine production, was reported during smoltification (Eddy and Talbot, 1985) and in adult FW acclimated Atlantic salmon (Talbot *et al.*, 1989). In contrast, a rapid decrease in UFR, as well as changes in plasma and urine concentrations of Na^+, Cl^- and Mg^{2+}, has been reported in FW Atlantic salmon smolts transferred to SW (Talbot, Stagg and Eddy, 1992). Despite new evidence about the role and function of Aquaporins (AQP), NKCC and SLC26A1 transporters in the salmonid kidney (Katoh *et al.*, 2006, 2008; Engelund and Madsen, 2011, 2015), virtually nothing has been reported about transporters regulating divalent ions (Mg^{2+}, SO_4^{2-} and Ca^{2+}) in the region. While understanding such transport pathways, in particular the Atlantic salmon, it is important to consider the salmonid specific genome duplication event since it may have major implications in molecular studies.

1.4.2 Salmonid specific fourth vertebrate whole genome duplication (Ss4R)

Atlantic salmon have gone through a salmonid specific fourth vertebrate whole genome duplication (Ss4R), resulting in a large genomic reorganization, highlighting the species as a particularly interesting study in an evolutionary perspective (Lien *et al.*, 2016). Genome duplication events can generate new genetic material for mutation, drift and selection to act upon, promoting phenotypic diversity (Kondrashov *et al.*, 2002; Kellogg, 2003). Studies of Atlantic salmon are especially appealing since they often have paralog genes that adopt a similar or new function in relation to the ancestral gene (Houston and Macqueen, 2019). As such, there are three main theories concerning the outcome of such paralogous genes; 1) the level of genetic product is maintained by both paralogs having similar or same function (dosage balance model), 2) the role of the ancestral gene is subdivided between duplicates and both sequences and expression patterns are altered (subfunctionalization), and 3) one or both of the duplicates gain functions not present in the ancestral gene (neofunctionalization) (Warren *et al.*, 2014). In salmonids, paralog retention rate can range 25-75 % (Bailey, Poulter and Stockwell, 1978). This might underline an important role for paralogous genes (duplicates) in explaining the remarkable plasticity of salmon adapting to different environments.

Ultimately, the limited knowledge on divalent transport in the kidney and the remarkable plasticity of Atlantic salmon acclimatizing to both FW and SW highlights the importance of more research on the osmoregulatory functions in the kidney. Emerging technology in Norwegian smolt facilities currently attempts to remove sulfate ions from the water to reduce hydrogen sulfide related mortalities (Fiskehelserapporten, 2019). Therefore, this indicates a requirement for understanding sulfate (SO_4^{-2}) transport pathways in salmonids, especially Atlantic salmon considering the importance of this species in Norwegian aquaculture production.

1.5 Sulfate (SO_4^{2-}) transporters in the kidney

In FW environments SO_4^{-2} concentrations are low (e.g. ~ 0.3 mM) while in SW environments SO_4^{-2} is the second most abundant anion (approx. 30 mM), often 30-50-fold and higher than what is found in plasma (Edwards and Marshall, 2012). Most teleost fish maintain plasma SO_4^{-2} levels close to 1mM, regardless of SO_4^{-2} levels in the external environment, so a regulatory shift from renal reabsorption to secretion is needed in migratory species moving from FW to SW (Watanabe and Takei, 2011a). The kidney is considered the main place of SO_4^{-2} excretion, primarily by involving several transport pathways to secrete SO_4^{-2} via urine (Beyenbach, 1995; Renfro *et al.*, 1999; Watanabe and Takei, 2012). Fish do this by concentrating and excreting roughly 97 % of SO_4^{2-} in the urine (Watanabe and Takei, 2012). Work on isolated proximal tubules suggests that SO_4^{2-} is secreted into the lumen of nephrons against its electrochemical potential (Beyenbach, 2004). Moreover, basolateral transportation of SO_4^{2-} is accomplished via exchange transport utilizing intracellular OH^-, subsequently transported apical from the cell into the tubular lumen in exchange for HCO_3^- (Renfro, 1999). SO_4^{2-} transport is largely dependent on pH across both basolateral and apical surfaces and apparently SO_4^{2-} transport can be directed via a Cl^- gradient in fish kidney's (Renfro and Pritchard, 1983; Renfro *et al.*, 1999), which does not occur in the mammalian kidney (Burckhardt and Burckhardt, 2003).

Recently, sulfate transporters from the solute carrier family 26 (SLC26) and family 13 (SLC13) involved in the absorption and secretion of SO_4^{-2} have been categorized and localized in the kidney of two euryhaline species eel and pufferfish (Nakada *et al.*, 2005; Kato *et al.*, 2009; Watanabe and Takei, 2011b). Especially interesting are the solute carrier family 26 member 1 (SLC26A1), 6 (SLC26A6A, B and C) and family 13 member 1 (SLC13A1). While the SLC26A6A have been suggested to be important in SW and the SLC13A1 is important in FW, the SLC26A1, SLC26A6B and SLC26A6C appear to be active both in FW and SW (Kato *et al.*, 2009; Kato and Watanabe, 2016). The Slc26a6a is the most likely candidate for the major apical SO_4^{-2} transporter in proximal tubule I, which participate in SO_4^{-2} secretion in fish kidney (Kato et al., 2009; Watanabe and Takei, 2011)

(figure 5B). Reabsorption of SO_4^{-2} in FW adapted eels is controlled by the combined effort of the apical Slc13a1 and the basolateral Slc26a1 isoforms in the proximal tubules (Nakada et al., 2005) (figure 5A). In addition, the Slc26a6b (proximal II) and Slc26a6c (proximal I) has been proposed as apical transporters that are active in both FW and SW environments (Kato *et al.*, 2009; Watanabe and Takei, 2011b) (figure 5C), while basolateral the Slc26a1 in proximal tubule II has been proposed to be active in both FW and SW (Nakada *et al.*, 2005; Watanabe and Takei, 2011b) (figure 5). Currently nothing is known about this transport in Atlantic salmon.

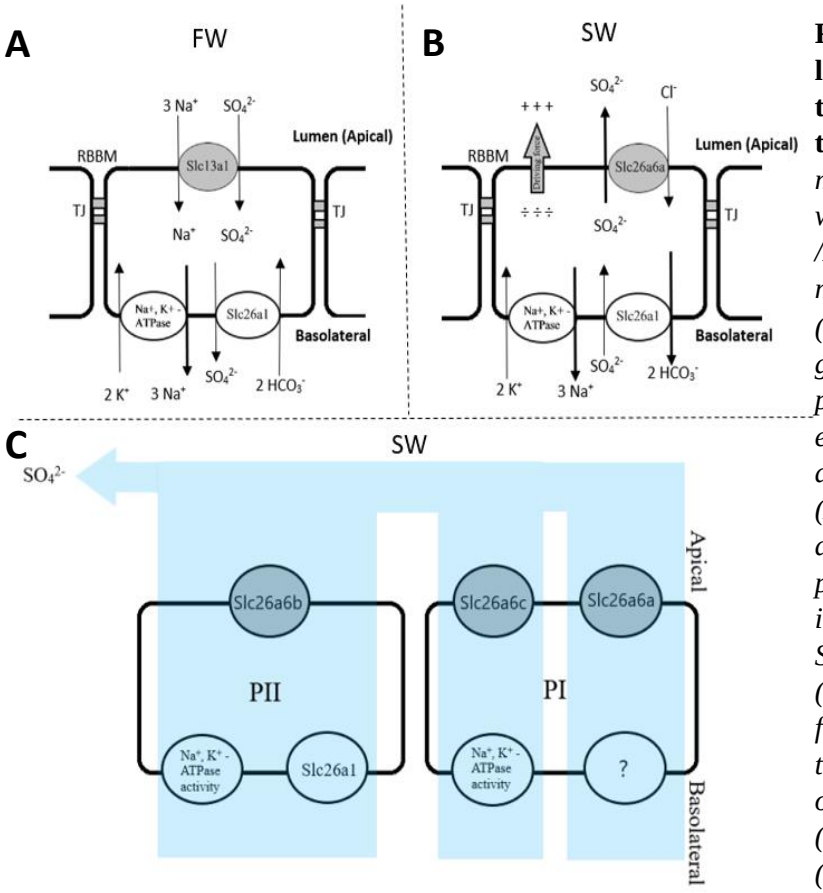

Figure 5: Schematic overview showing localization and mechanism of SO_4^{2-} transport in FW (A) and SW (B and C) teleosts. ***A:*** *Slc13a1 are located on apical membranes, co-transporting $3Na^+/SO_4^{2-}$, while Slc26a1 are an electroneutral SO_4^{2-}/HCO_3^- exchanger located on basolateral membranes, driven by Na^+, K^+-ATPase (NKA) creating a transmembrane Na^+ gradient. This transport is located on proximal tubule cells.* ***B:*** *Slc26a6a is an electrogenic SO_4^{2-}/Cl^- exchanger located apically, while the Slc26a1 similar to FW (A) exchanges SO_4^{2-}/HCO_3^- but in opposite direction. NKA creates an electronegative potential in the cell, favoring the transport in SW.* ***C:*** *The apical Slc26a6c and Slc26a6a are located in proximal tubule I (PI), while Slc26a6b and Slc26a1 are found apical and basolateral in proximal tubule II (PII), respectively. Models based on Nakada, 2005 (FW), Kato et al., 2009 (SW) and Watanabe and Takei, 2011b (SW).*

1.6 Current issues in smolt production

The use of Recirculation Aquaculture Systems (RAS) technology has transformed smolt production in recent years (Dalsgaard *et al.*, 2013). In RAS, most of the production water is reused after removing metabolic biproducts while alkalinity and oxygen are added to the system (Bergheim *et al.*, 2009). As more RAS facilities emerge and the industry intensifies their production protocols, growing ever larger smolts increases demand for more comprehensive knowledge on physiological processes during smolt production (Calabrese et al., 2017). The smoltification process culminates with a period where smolts fully prepare for the marine environment. The consequences of missing such a 'smolt window' leads to lower marine survival. Therefore, producing high quality smolt is critical for successful culture and thus frequent variation in smolt quality (stunted growth, high annual transfer

mortality (16%)) is of great concern (Fiskehelserapporten, 2019). Additionally, increased incidents of production disorders such as mineral precipitation (nephrocalsinoses) in the kidney (Fivelstad *et al.*, 2018), haemorrhagic smolt syndrome (HSS) (Byrne *et al.*, 1998) and other issues related to growth, health and general performance (Skov, 2019) have raised concerns surrounding potential adverse effects on smolt physiology. Therefore, it is problematic that so little effort is put into assessing the transport capacity of all three osmoregulatory organs during and after smoltification considering these production disorders. Thus, one may argue that a more holistic approach is required for the industry`s assessment of smolt quality where gills, intestine and kidney all should be included.

1.7 Objective

The current study aims at increasing our understanding and knowledge of smolt development and SW tolerance in Atlantic salmon in the hopes of improving smolt quality and seawater survival. First, I will address changes in Nka enzyme activity in all three osmoregulatory organs (gills, intestine and kidney) during smoltification (**Objective 1**) and after abrupt SW transfer (**Objective 2**). Then key SO_4^{2-} transporters will be identified and characterized in these organs (**Objective 3**). Secondly, mRNA abundance of candidate SO_4^{2-} genes in these organs will be measured during smoltification and after SW transfer (**Objective 4**). Although the main focus will be on the kidney, as knowledge is more limited, the study aspires to also address changes in gills and intestine in an attempt to reflect both the individual and collective efforts of these organs to the overall osmoregulatory capacity during smoltification and after SW transfer. These objectives aim to answer the following predictions:

Objective 1. Determine the Nka enzyme activity in the three osmoregulatory organs during smolt development

Increased Nka enzyme activity is commonly found in the gills of Atlantic salmon during smoltification and is used as the main indicator for smolt development (Mccormick *et al.*, 1998; Nilsen, Ebbesson and Stefansson, 2003; Björnsson and Bradley, 2007; Stefansson *et al.*, 2008). Intestinal Nka activity has been shown to increase during smoltification (Sundell *et al.*, 2003), while in the kidney one study has demonstrated an increase during peak smoltification (McCartney, 1976). Based on these notions, I predict that Atlantic salmon will increase the overall Nka enzyme activity in all osmoregulatory organs during smolt development

Objective 2. Determine the Nka enzyme activity in the three osmoregulatory organs after SW transfer

In SW a large body of evidence shows an increase to or at least maintenance of high Nka enzyme activity after SW transfer in both gills (McCormick, Moyes and Ballantyne, 1989; Nilsen *et al.*, 2007; McCormick *et al.*, 2013) and intestine (Sundell *et al.*, 2003; Sundell and Sundh, 2012; Sundh *et al.*, 2014). In contrast very few studies have addressed the kidney in Atlantic salmon. A gradual increase in salinity showed no apparent difference in Nka enzyme activity in Atlantic salmon kidney (McCormick, Moyes and Ballantyne, 1989), and it has been suggested that kidney Nka activity will decrease after abrupt SW transfer (McCartney, 1976). This may reflect the requirement to reduce GFR and UFR in SW (Hickman and Trump, 1969; Talbot *et al.*, 1989) and thus a reduction in overall tubular activity. I predict that both gills and intestine will increase or maintain high Nka enzyme activity levels after abrupt SW transfer. Further, I also predict that a potential increase in kidney Nka activity during smoltification is followed by a reduction of Nka enzyme activity after abrupt SW transfer.

Objective 3. Identification and characterization of key SO_4^{2-} transporters in Atlantic salmon

The studies addressing sulfate regulation have predominantly found key transporters in the kidney (Katoh *et al.*, 2006; Kato *et al.*, 2009; Watanabe and Takei, 2011b) and a few in the intestine (Watanabe and Takei, 2011b). Additionally, studies demonstrate sulfate influx across the gills (Watanabe and Takei, 2012), yet no transporter candidates have been suggested. Given the Ss4R event in salmonid species I predict that several paralog genes may be present in Atlantic salmon. I also predict that sulfate transporters are present in the gills, intestine and kidney but are more predominantly found in the kidney of Atlantic salmon.

Objective 4. Gene expression patterns of SO_4^{2-} transporters during smoltification and after SW transfer

Given that the kidney is the predominant organ secreting and removing excess SO_4^{2-} in SW environments (Kato *et al.*, 2009; Watanabe and Takei, 2011a) I predict that SO_4^{2-} transporters involved in tubular secretion in the kidney will be upregulated after SW transfer. The preparatory changes for a marine life during smoltification suggest that also transporters involved in secretion of SO_4^{2-} in SW may be upregulated before SW transfer.

2. Material and methods

2.1 Fish stock, experimental design and sampling protocol

2.1.1 Fish stock

Juvenile Atlantic salmons (*Salmo salar* L.) of AquaGen stock were obtained from the Aquatic Laboratory of Bergen. All fish were delivered as eyed egg to Industrilabratoriet i Bergen (ILAB) from for further development. The survivors hatched at egg yolk stage and were grown out first as fry, then the fish were grown to parr using commercial fish feed. These stages followed standard and commercial production protocol until the start of the experiment. At the start of the experiment Atlantic salmon parr were approximately 30 grams and were continually supervised and fed commercial fish feed in both tanks from September 4th 2019, supervised by ILAB.

2.1.2 Experimental design

FW phase

Experimentally, the study relied on applying a classic square wave photoperiodic induction of smoltification (Stefansson *et al.*, 1991). The control tank (parr) was kept at a 12 hour darkness and 12 hour light (12D:12L; winter signal) photoperiod regime during the whole experimental period while the other experimental tank (smolt) was changed to a 24L hour light regime initiating the smoltification development. Both tanks (1m^3, 400 L rearing volume) where kept in freshwater (*Salinity;* 1-2 ‰, *Temp;* 10±0.23 °C, *oxygen outlet water;* >80 %, *Flow rate;* 0.6 L/kg/min) and the tanks were always covered with a lid. This ensured minimal disturbance in the tanks during the entire developmental period. After initiation of the experiment on September 4th, sampling (12 individuals per group) from both parr group and smolt group was conducted after 12 days (16th September, 120 d.d), 26 days (30th September, 260 d.d), 35 days (9th October, 350 d.d), and 45 days (18th October, 450 d.d) in FW (figure 6).

SW phase

Smolts in FW were transferred to SW in three separate tanks (1m^3 160 L rearing volume: *Salinity*; 32 ‰, *Temp*; 9.2±0.3 °C, *oxygen outlet water:* <80%, *Flow rate*; 0.6 L/kg/min) on the 20th of October and sampled after 1 day (21th October, 480 d.d), 2 days (22th October, 490 d.d), and 38 days after SW transfer (25th November, 830 d.d) (figure 6). Parr control fish in FW were only sampled after 83 days (830 d.d) on November 25th, (figure 6). The parr group was intentionally disturbed in the tank to ensure similar handling stress for both fish groups.

2.1.3 Sampling protocol

For each sampling, 12 juvenile salmons were collected from each group, first 12 parr and then 12 smolts (only smolts were sampled after 1 and 2 days in SW). All fish were quickly dip-netted out of the tanks and anesthetized using a lethal dose of tricaine methanesulphonate (100 mg l^{-1} MS222; Sigma, St Louis, MO, USA). Blood was collected from the caudal vein and stored on ice until centrifugation (4^0C, 3000xg, 5 min) then plasma aliquots were frozen. Thereafter, fork length and body weight were measured, before fish were placed on ice and gills, kidney and intestine were dissected out and preserved in different media depending on the later applications. At all representative timepoints (figure 6), the different preserving and storage methods were 1) for NKA activity measurement: SEI buffer, -80°C (gills/kidney) and intestinal SEI buffer, -80°C (gut), 2) for gene expression analysis: RNAlater, -80°C (gills/kidney) and fresh frozen, -80°C (gut), 3) for protein analysis: fresh frozen, -80°C for all tissues. In addition, gills/kidney/gut tissue was collected for immunohistochemistry, but only after 12 days (sampling 1), 45 days (sampling 4), 1 day in SW (sampling 6) and 38 days in SW (sampling 7). For that application, tissues were fixed in 4% paraformaldehyde at 4°C prior to embedding, cutting and staining. Water samples from both FW and SW tank were retrieved after the experiment was terminated, for ion composition analysis.

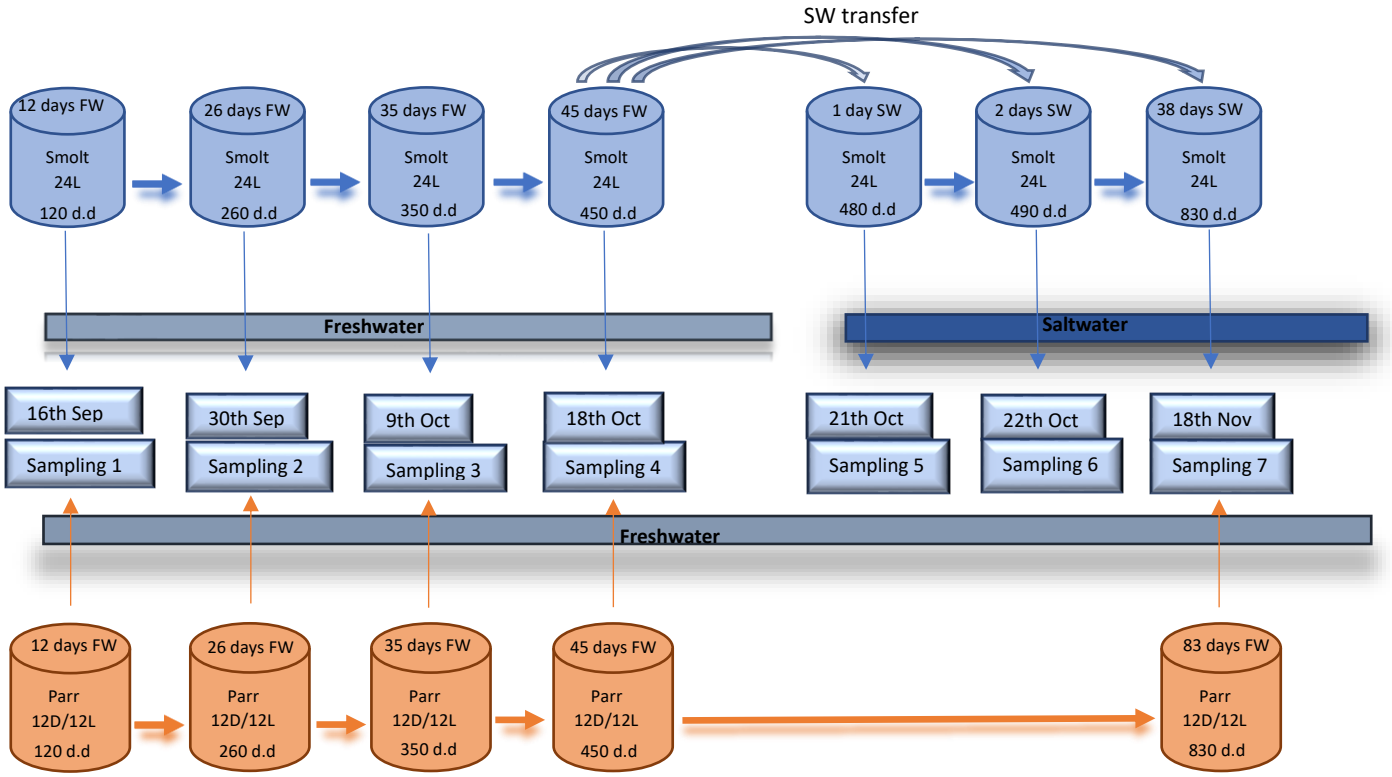

Figure 6: Experimental setup of the smolt development study. *Two different tanks, one control (parr group) and one experimental group (smolt group) were sampled at 7 representative timepoints: sampling 1 (120 d.d), sampling 2 (260 d.d), sampling 3 (350 d.d), sampling 4 (450 d.d) and sampling 7 (830 d.d) (n=12). Hence, no sampling was conducted for the parr group at 1 day and 2 days SW as a sampling in this group had been conducted only 3 days prior but was sampled again after 83 days in FW. Smolt group were transferred to SW in three separate tanks for sampling after 1 day SW, 2 days SW and 38 days in SW (n=12).*

2.2 Condition factor and morphology (smolt characteristics)

2.2.1 Condition factor

Fulton's condition factor was calculated according to the following formula:

$$CF = W/L^3$$

where CF is condition factor, W is wet weight (g) and L is fork length (cm)

2.2.2 Morphology (smolt characteristics)

Each fish was given a smolt score/index based on several criteria ranging from 1 to 5, based on the silvering characteristics, parr marks, body colour, tail colour and shape (table 1, illustrated in Appendix 1e).

Table 1. *Overview of the smolt score criteria*

CRITERIA	SCORE OF 1	SCORE OF 5
SILVERING CHARACTERISTICS	*No silvering*	*Complete silvering body*
PARR MARKS/RED DOTS	*Present*	*Not present*
BODY COLOUR	*Dark green*	*White ventrally and dark dorsally*
TAIL COLOUR AND SHAPE	*Light dark and round shape*	*Completely/almost dark and fork shape*

2.3 Analytical analysis

Tissue from the gills, intestine and kidney was used for several analytical methods; Nka activity (verification of smolt development) and gene expression (verification of mRNA abundance for the selected transporters). Samples were also preserved in conditions adequate for protein analysis and immunohistochemistry, for further analysis. In addition, blood plasma was used for quantification of key ions during smoltification and SW transfer. However, as a result of the Covid-19 situation, plasma, protein and immunohistochemistry analysis unfortunately had to be excluded from the master thesis.

2.3.1 Nka enzyme activity (gills, intestine, kidney)

The Nka activity in gills, intestine and kidney was measured in a Spark multimode microplate reader (Tecan, Mannedorf, Switzerland) under strict temperature control (25°C) according to the microassay method of McCormick, 1993.

An assay mixture (AM: 5.0 U/ml Pyravate Kinase (PK) and 4.0 U/ml Lactic Dehydrogenase (LDH), 2.8 mM phosphenylpyruvate (PEP), 0.22 mM β-Nicotinamide Adenine Dinuclotide (NADH), 0.7 mM Adenosine Trisphosphate (ATP) and 50 mM immidazole) was prepared, either without (AM) and with (AM-O) 0.5 mM Oubain (ATPase enzyme inhibitor). The assay medium was completed by adding salt solution to achieve final concentrations of 50 mM immidazole, 189 mM NaCl, 10.5 mM $MgCl_2$ and 42 mM KCl in both the AM and AM-O mixtures. Prior to analyzing samples, the AM solution was quality assessed by running a 10 µl triplicate standard curve from 0 to 20 nmol ADP per well using the slope of the endpoint. Standard curve in the kinetic reading should be within 17-19 mOD nmole ADP per well.

During sampling, the intestine was collected in a different buffer than the gills and kidney. Gill and kidney were preserved in SEI buffer (250 mM Sucrose, 10 mM Na_2EDTA, 50 mM Imidiazole) while the intestine samples were preserved in intestinal SEI buffer (200 mM glycine, 45 mM EDTA, 50 mM EGTA, 300 mM sucrose, 50 mM imidazole; for each 10 mL batch of intestinal SEI buffer 1 tablet of Complete™ protease inhibitor cocktail (04693124001 Roche)) was used to prevent degradation of proteins/enzymes during lysis and homogenization. Intestine were obtained by making two incisions, one posterior of the pyloric caeca and one anterior of the rectum to sample the entire length of the proximal and distal sections of the intestine. Then, the intestine was cut open longitudinally with the luminal side facing upwards. Next, the luminal side was gently rinsed with intestinal SEI buffer before the mucosa was gently scraped off with a glass microscope slide. The mucosal sample was then weighed (wet weight) and transferred to an Eppendorf tube already containing 100 µl of the intestinal

SEI buffer and immediately placed on dry ice. The mucosal sample was about 1/10 of the total buffer volume (roughly 10 mg). All sampling of the intestinal tissue was carried out on ice. The Nka activity assay was then completed using the same method used for the gills and kidney. All three tissues were performed in collaboration with another master student (Sigval Myren). Sigval and myself assayed the gill tissue in collaboration, while I carried out the analysis of the Nka enzyme activity in the kidney while Sigval performed, optimized and analyzed the intestinal Nka protocol.

Prior to analysis, gill filaments (n=4-6, McCormick, 1993), intestinal (10 mg, intestinal SEI buffer) and kidney tissue (5-8 mg, see Appendix 2a) were thawed on ice and homogenized (10 s using a motor pestle (VWR 431-0100) in 125 µl containing 80 % (v/v) SEI buffer and 20 % (v/v) SEID buffer (0.5 % (w/v) Na deoxycholate acid in SEI buffer). Cell debris were precipitated by centrifugation (Eppendorf 5424 R, Hamburg, Germany) for 1 min, at 5000 g and 4 °C. Then 10 µl quadruplicates of supernatant were loaded in a 96-well Nunc microplates (nunc plate #269620, 732-2746, VWR), while triplicates of supernatant were added in Costar plates (CLS9017, Sigma-Aldrich) to measure protein concentration.

Then 200 µl of AM or AM-O solution were added to each half of the replicates samples, then absorbance was measured at 340nm at 25°C with 10 s intervals over 60 cycles for a total of 10 min. The linear rate of NADH disappearance output of the machine was expressed as mOD/10µl/min (mOD = milli optical density unit) and calculated using the standard curve slope according to the formula:

$$\frac{Na^+/K^+\text{-}ATPase\ (mOD/10\mu l/min)}{ADP\ standard\ curve\ (mOD/nmole\ ADP)} = nmoles\ ADP/10\mu l/min$$

Protein concentration in the samples was determined using the Pierce BCA Protein Assay kit (Thermo fisher Scientific, Massachusetts, USA). Two hundred µl of reagents (50 parts A:1 part B) from the Pierce BCA Protein Assay kit (Thermo fisher Scientific, Massachusetts, USA) were added to each triplicate sample and covered with parafilm, shaken for 30 seconds and incubated 30 min at 37°C in a INCU-Line digital incubator (IKA). After cooling for 2-3 min, the absorbance was measured in the Spark multimode microplate reader at 562nm. The final enzyme activity was then calculated accordingly:

$$\frac{nmoles\ ADP/10\mu l/min}{\mu g/10\mu l\ protein} = \mu moles\ ADP/mg\ protein/min$$

$$(\mu moles\ ADP/mg/min)(60\ min) = \mu moles\ ADP/mg\ protein/hour$$

The Nka enzyme activity is reported as µmoles ADP per mg protein per hour.

2.3.2 Sulfate (SO_4^{2-}) transporters in the Atlantic salmon genome

2.3.2.1 Protein sequences and alignment

The Atlantic salmon (*Salmo salar*) homologues of Slc13a1, Slc26a1, and Slc26a6 were identified by running a BLAST search on the NCBI database using the protein sequences identified in Japanese Eel (*Anguilla japonica)* and Obscure Pufferfish (*Takifugu obscurus*) (details in Appendix 4). The selection of sequences chosen for further analyze was based on three criteria; 1) The *E value*, a number that describes how many times you would expect a match by chance in the database of that size (The lower the E value the more significant the match), 2) *Percent identity*, a number that describes how similar the query sequence is to the identified sequence (how many amino acid characters are identical); the higher the percent the more significant the match and 3) *Query cover*, is the percentage of the query sequence (target species) overlapping with the reference sequence (reference species). The cover is 100% if the query sequence covers the whole reference sequence (Query cover > 70% was considered high). For each of the three transporter families, Atlantic salmon sequences that had the lowest E-value, highest percent identity and the highest query cover were selected and aligned in Seaview (http://doua.prabi.fr/software/seaview) with the already annotated genes from teleost species representing the diversity of the group (including the Japanese eel and the obscure pufferfish), as well as other groups of vertebrates, in order to perform a phylogenetic analysis. This analysis shows how the newly sequenced group compares with the already annotated sequences from other species, and thus can be assigned an identity based on their homology. The alignment was performed with the help of the CLUSTALW algorithm in Seaview. Then, to select the most informative areas of this alignment for this kind of analysis (containing similar enough but not too identical amino acid stretches), the Gblocks tool (included in Seaview) was run of the alignment, using default parameters. The resulting new alignment, containing only the selected blocks, was then submitted to a Maximum Likelihood phylogenetic analysis (also in Seaview - check parameters). The generated tree was visualized and edited using the FigTree tool (https://www.softpedia.com/get/Science-CAD/FigTree-AR.shtml). Ultimately a phylogenetic interpretation of the transporters in an evolutionary context was constructed for all named transporters. To confirm the identity of the genes, especially in cases when salmon-specific duplications were suspected, another approach was used, a synteny analysis. The principle was to compare the chromosomal environment of each gene with the one the phylogenetic analysis concluded it corresponded to in the Norhern pike (*Esox lucius*), a closely related species to Atlantic salmon that did not undergo a fourth round of whole genome duplication. The gene environment for each transporter in the pike was visualized using Genomicus (https://www.genomicus.biologie.ens.fr/genomicus-99.01/cgi-bin/search.pl) and the genes around

the salmon transporters were manually analyzed on the genome browser of NCBI. The figure was then made using InkScape (Appendix 4).

2.3.2.2 Primer design, efficiency and tissue distribution

For each identified sulfate transporter, the corresponding genomic and mRNA sequences were retrieved (Table 2, appendix 4). The genomic and mRNA sequences were aligned using Seaview (http://doua.prabi.fr/software/seaview) to locate the position of introns and identify unique regions in each sequences that could serve as potential primers. The primer design for all transporters was then carried out in collaboration with the supervisors. To design the primers, the BioRad guide to design qPCR primers was used (https://www.bio-rad.com/en-no/applications-technologies/qpcr-assay-design-optimization) and the properties of the primers were calculated and checked using OligoCalc (http://biotools.nubic.northwestern.edu/OligoCalc.html). The primers were chosen according to match the following parameters: 1) for at least one in the pair: spanning exon-intron junctions to reduce the potential of amplification of genomic DNA instead of cDNA; 2) having a GC content of 50-60% and one or several Gs or Cs at the 3' of the primer for increased binding stability to the target; 3) having a melting temperature (Tm) around 60°C (the annealing temperature for the qPCR assay); 4) avoiding secondary structures, self-complementarity or complementarity between the two primers (avoid primer-dimers and effects that would lower the efficiency of the PCR). In addition, the distance between the primers was chosen to have an amplicon length between 75 and 200 bp, so it would be long enough to be able to be differentiated from primer-dimers yet not too long so the efficiency would be optimal. A complete overview for each primer set and their parameters can be found in Appendix 5a.

To test the specificity and efficiency of all primer sets, and provide a first indication of their relative tissue distribution, test qPCRs were performed on a pool of cDNA for five different tissues: gills, kidney, urine bladder, liver and intestine, both in FW and SW conditions. The specificity was confirmed by the presence of a single melting curve (single amplicon) and the most relevant tissues of interest for each gene (significant expression or first clues of high difference between FW and SW) were selected based on the Ct value (Ct value <30). The overview and results of the tissue distribution for all the different genes mentioned can be found in Appendix 5c.

To then calculate the PCR efficiency for each primer pair for each selected tissue (kidney, gills and intestine), and determine the optimal dilution for the cDNA to use for all the experimental samples, a two-fold dilution series (1:10, 1:20, 1:40, 1:80 and 1:160) of each cDNA pool was prepared in order to establish a dilution curve. The mean Ct value from each dilution was plotted against the logarithmical cDNA diluted concentrations and the primer efficiency (E) was determined based on

the slope of the regression line generated from this dilution curve. For a complete overview of the dilution curves, primer efficiency and melting curves, see appendix 5c.

To determine the optimal reference gene(s) for kidney and gills, three genes were tested on a range of samples covering the whole experiment : *gapdh*, *ef1a* and *b-actin*. Based on several tests incorporated in RefFinder (Xie *et al.*, 2012): BestKeeper (Pfaffl *et al.*, 2004), Normfinder (Andersen, Jensen and Falck Ørntoft, 2004), Genorm (Vandesompele *et al.*, 2002), the comparative delta-Ct method (Silver *et al.*, 2006) (Appendix 5d), *ef1a* was used for normalization of the expression for the genes of interest. For gills, the same tests were completed, and the same conclusion was made and *ef1a* was also selected as most stable reference gene here. Intestine had to be excluded for further qPCR analysis (insufficient RNA integrity) and only the tissue distribution based on Ct values was performed for this tissue.

2.3.3 RNA isolation, quantification and integrity

2.3.3.1 Total RNA isolation using TRI Reagent

Total RNA from intestine samples was isolated using the TRI Reagent method reported by Chomczynski and Sacchi (1987). Gut samples was fresh frozen at -80^{0}C and was directly put in 1 ml Tri Reagent (T9424, Sigma-Aldrich) and homogenized using ceramic spheres (Bertin Technologies BERT03961-1-103, distributed by VWR) and the Precellys 24 tissue homogenizer (Bertin Technologies) to disrupt cells and dissolving cell components. After 5 min at room temperature, 200 µl of chloroform (C2432, Sigma-Aldrich) was added and the samples vortexed and centrifuged for 15 min at 4°C, 21 130 x RCF (g) (Centrifuge 5424 R, Eppendorf). The aqueous phase (RNA) was transferred to separate tubes and 500 µl of isopropanol (I9516, Sigma-Aldrich) was added. Tubes were inverted 5 times and left at RT for 10 min followed by a 10 min centrifugation at 4°C, 21 130 x RCF (g). Supernatant was decanted and the pellet was washed with 500 µl 80 % EtOH, centrifugated for 5 min, 7600 x g, 4°C and supernatant was removed. Lastly, samples were centrifugated using flash spin to remove residual EtOH, air dried for 5-10 min and resuspended in 50 µl sterile molecular grade water (Thermo Fisher Scientific). All samples were precipitated using 1/10 volume of 3 M NaAc (pH 5,2, S7899 Sigma-Aldrich) and 2,5 vol of -20°C 100 EtOH and left at -80°C overnight. Samples were then centrifugated for 30 min, 21 130 x g, 4°C before the RNA pellet was washed and resuspended (only 20-50 µl) as described above.

2.3.3.2 Total RNA isolation with Qiasymphony Robot

Kidney and gill samples stored in RNAlater at -80°C were thawed on ice and approximately 20-25 mg of tissue was homogenized in 600 µl of RLT plus buffer and Reagent DX (Qiagen Qiasymphony mRNA extraction kit) using ceramic spheres and the Precellys 24 tissue homogenizer as described

above. Total RNA was extracted using the Qiasymphony Robot (Qiagen) in conjunction with the QIAsymphony RNA kit, following manufacturers protocol (Qiagen). Isolated total RNA was diluted in 100 µl (kidney) and 50 µl (gills) of ultra-pure water and stored at -80°C.

2.3.3.3 Quantification, purity and integrity of total RNA

Quantification and testing of RNA concentrations for kidney, gill and intestine tissue were accurately measured using the Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific) applying the Qubit™ RNA HS Assay Kit protocol (Invitrogen™, Thermo Fisher Scientific). The integrity of total RNA in samples was measured with an Agilent 2100 expert analyzer (Agilent technologies) using the Agilent RNA 6000 Nano kit (Agilent Technologies). Total RNA integrity was classified and assigned an RNA Integrity Number (RIN) ranging from 1 to 10, with 1 being the lowest integrity and 10 being the highest integrity (Schroeder *et al.*, 2006). All samples tested had a RIN value exceeding 8,6 and based on previous experience (Pers. comm. Dr. Tom Ole Nilsen) it was assumed to be representative for all the samples. As previously mentioned, intestine was excluded as the integrity number was inadequate.

2.3.3.4 Complementary DNA (cDNA) synthesis

A total RNA amount of 1500 ng (kidney) and 500 ng (gills) was diluted in a total volume of 11 µl ultra-pure water before Oligo(dT)$_{20}$ primer (2,5 µM) and dNTP mix (0,5 mM) were added, resulting in a total volume of 13 µl. After incubation at 65℃ for 5 min on the C1000 Touch Thermo Cycler (Bio-Rad Laboratories), samples were placed on ice for one min to reduce formation of secondary structures. A master mix consisting of First strand Buffer ((20% (v/v) + (Tris-HCL (250 mM at pH 8,3), KCL (375 mM), $MgCl_2$ (15 mM))), dithiothreitol (DTT; 5mM), RNaseOUT™ Recombinant RNase Inhibitor (40 U) and SuperScript™ III Reverse Transcriptase enzyme (RT; 200 U) were added to the samples, resulting in a final volume of 20 µl. The complementary DNA (cDNA) synthesis was then carried out according to the manufacturers (Invitrogen) thermocycler protocol: first step at 50 ℃ for 60 min, followed by an inactivation at 70 ℃ for 15 min and then down to 4 ℃ before storage at -20 ℃ until Quantitative Polymerase Chain Reaction (qPCR) analysis to determine mRNA abundance.

2.3.4 Real time quantitative Polymerase Chain Reaction (qPCR)

Real time quantitative PCR (qPCR) was carried out to measure mRNA abundance of s*lc26a1a* (kidney), s*lc26a1bX1* (kidney). s*lc26a1bX3* (kidney), *slc26a6a1* (kidney), *slc26a6a2* (gills), *slc26a6b* (kidney) *and slc26a6c* (kidney) in all kidney and gill samples (table 2). All pipetting was automated using the Microlab STARlet pipetting robot. The cDNA was diluted 1:20 (gills, 25 ng/µl) and 1:30 (kidney, 50 ng/µl) with ultra-pure water (intestine were excluded, inadequate RNA integrity). All

qPCR reactions were carried out in a 96-well plate (Bio-Rad Laboratories) using 6,25 µl iTaq™ Universal SYBR® Green Supermix (2x) (1x final concentration) (Bio-Rad Laboratories), 0,5 µl (5 µM) forward and reverse primer mix (final concentration 200 nM), cDNA 2,5 µl (gills; 2 ng/µl, kidney; 4 ng/µl) and nuclease free H_2O in a total volume of 12,5 µl. The reactions were run in a C1000 Touch™ Thermo cycler, CFX96™ Real-Time PCR detection System and CFX Manager software (software version 3.1) (Bio-Rad Laboratories) with the following thermal cycle program (figure 7). For functional properties and melt curves for all primers see Appendix 5a and 5b.

Table 2: Target and reference genes/primers for kidney and gills qPCR. *Overview of primer sequences used to measure mRNA abundance of slc26a1a, slc26a1bX1, slc26a1bX3, slc26a6a1, slc26a6a2, slc26a6b, slc26a6c and the reference genes ef1a in the kidney and gill samples.*

Gene	Primer sequence (5'-3')	mRNA reference
slc26a1a (kidney)	GTAGAGCGAGTTGGTTGTGAGG GCTGTGCTCCCACACTTCG	XM_014129156.1
slc26a1bX1 (kidney)	GTTGGCTGTAAGTGTGAGGGAC CCTCTGGAAGTGGTAGGCTG	XM_014138168.1
slc26a1bX3 (kidney)	GTGACACATGTTGGCTGAGCAC GCTTCGTCTTCAGGATGGCC	XM_014138170.1
slc26a6a1 (kidney)	CTCATCTCCTACTACGGCAACCTG CTGGGAGACTTCAGCCCTCTG	XM_014134693.1
slc26a6a2 (gills)	GACCTGAAATTGAACCAGACGGCC GTGTGTGTCGTTGACGGAGTTC	XM_014192131.1
slc26a6b (kidney)	ACAGAGAGGTGCTGGATGAGGG GGGGACAGAACACCTCACTGAC	XM_014135170.1
slc26a6c (kidney)	GTACTGGATGAGCAGAGACTGGAGG GCCTGGGTACAGTACATCTGAAGGACTC	XM_014132723.1
ef1a (kidney, gills)	CCCTGTGGAAGTGGCTGAAG CATCCAAGGGTCCGTATCTCTT	Olsvik et al. 2013

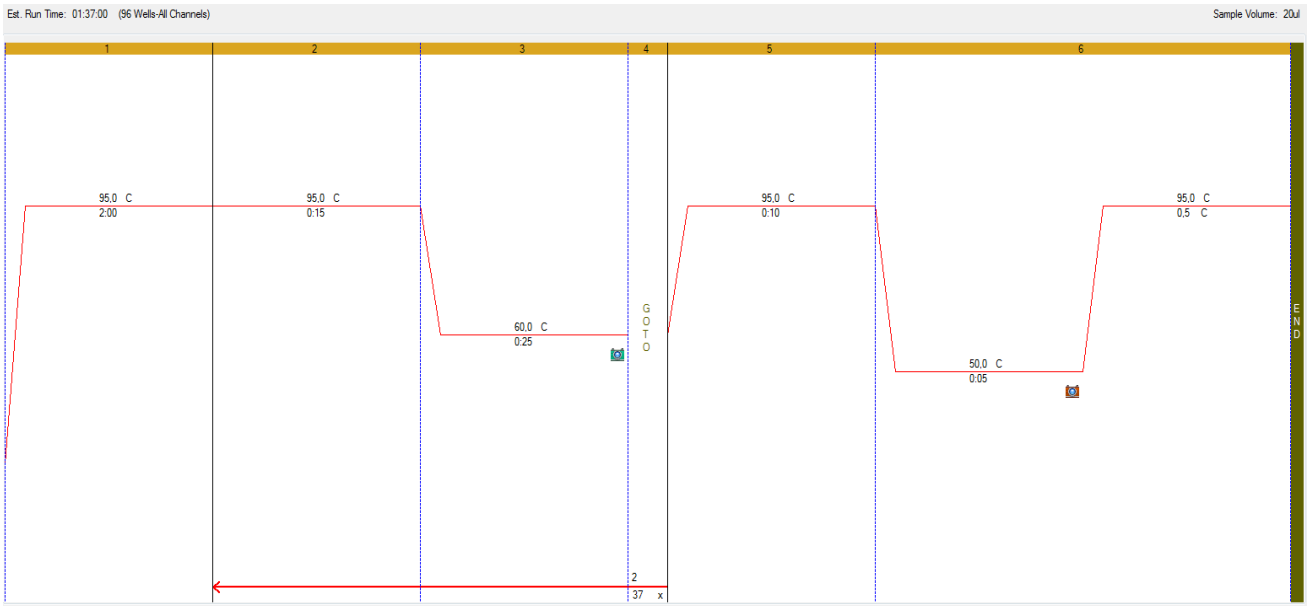

Figure 7: Thermal cycle program overview for qPCR. *Step 1: the temperature was set to 95℃ for 2 min for initial denaturation of the cDNA. Thereafter, the PCR cycle consisted of two steps: step 2, the temperature stayed at 95℃ for 15 sec (denaturation) before step 3, the temperature decreased to 60℃ for a 25 sec incubation (annealing and extension). The GOTO instruction (step 4) ensured the looping of the program 37 times, to result in 38 cycles. Each annealing/extension step was completed with a measurement step (green camera pictogram) for detection of fluorescent light from the SYBR green. After the 38 cycles, a program for generating a melting curve was initiated. First the temperature reached 95℃ for 10 sec (step 5, denaturation of all PCR products), decreased to 50℃ for a 5 sec incubation (re- hybridization) and gradually increased again to 95℃ through 0,5℃ increments every 0,5 sec (step 6), while at each increment the fluorescent signal was measured.*

For the entire experiment a total of 480 samples, covering kidney tissue (n=120), gill tissue (n=120 and gut tissue (n=240, excluded), were randomized and utilized for the measure of relative mRNA abundance of s*lc26a1a* (kidney), s*lc26a1bX1* (kidney), s*lc26a1bX3* (kidney), *slc26a6a1* (kidney), *slc26a6a2* (gills), *slc26a6b* (kidney) *and slc26a6c* (kidney). The complete primer list (including splice variants) can be found in Appendix 5a. All the samples where then analyzed in duplicates for relative mRNA abundance for all the target genes and selected reference genes including a control referred to as the non-template control (NTC) and a common sample that were used for intercalibration among plates comprised of a cDNA pool. The mean Ct value of the sample duplicates were used for the quantification of mRNA abundance, using the reference gene(s) value(s) for normalization. The following formulas were used for calculations (Pfaffl, 2001) :

I) Primer efficiency:

$$E = 10^{(-1/slope)}$$

II) Relative quantification of *slc13a1, slc26a1a, slc26a1bX1, slc26a1bX3, slc26a6a1, slc26a6a2, slc26a6b, slc26a6c:*

$$\frac{(E_{target})^{\Delta Ct_{mean\ target}}}{(E_{ref})^{\Delta Ct_{mean\ ref}}}$$

E_{target}…... The qPCR efficiency of target gene (*slc26a1a, slc26a1bX1, slc26a1bX3, slc26a6a1, slc26a6a2, slc26a6b and slc26a6c)*

E_{ref}…… The qPCR efficiency of the reference gene (*ef1a)*

$\Delta Ct_{mean\ target}$ …… The mean Ct value of the target gene in duplicates (*slc26a1a, slc26a1bX1, slc26a1bX3, slc26a6a1, slc26a6a2, slc26a6b and slc26a6c)*

$\Delta Ct_{mean\ ref}$ …… The mean Ct value of the reference gene (*ef1a)*

Standardizing the relative mRNA abundance……The final calculation of relative mRNA transcription was multiplied with a factor of 500 for all genes to give a more tangible number when interpreting the results.

2.4 Statistical analysis

All statistical analysis was performed using RStudio (Rstudio version 1.2) utilizing the following packages: Rtools, dplyr, ggplot2, car and emmeans. The distribution of all responses (length, weight, condition factor, smolt index, Nka activity, mRNA abundance) were initially checked with histograms. Linear models (Two-Way ANOVA) analysis were fitted between each response variable and the predictor variable group, day degrees or sampling and their interaction. For all response variables (see above) two different models were fitted to the data set. The first model used Group and Day degrees as the predictor variables while the second model used Group and Sampling as predictor variables. For each model different diagnostics were run to validate the models graphically to assess normality (q-q plots), influential outliers (Residuals vs Leverage with Cook`s distance) and non-linear patterns (Residuals vs fitted plots) or homogeneity of variance (scale-location plots). A Levine's test was used in case of any uncertainties from plots about the homogeneity of variance over time and among groups. In cases were the model assumptions were not met, the response variable was either log transformed, or square root transformed and tested again. If the model assumption was still not met, a generalized linear model (glm) for non-normal response variables was applied (family: Gamma and Gaussian). Once the best fitted model was selected, a significant model was applied using a Tukeys`s HSD post-hoc test to identify significant differences between groups. P-values lower than 0,05 ($p < 0,05$) was deemed a statistically significant datapoint and marked with asterisk (between groups) accordingly; $p < 0,05$ (*), $p < 0,01$ (**) and $p < 0,001$ (***). Non-identical letters where used

for significant difference between timepoints/samplings in each group. The data in all graphs represents the means of each group and ± of the standard error of means (SEM).

3. Results

3.1 Length, Weight, Condition factor and Smolt index

3.1.1 Fork length (cm) of juvenile Atlantic salmon during smoltification and SW phase

The average fork length in the smolt group displayed a gradual increase from 13,90±0,07 centimeters (cm) at 260 day degrees (d.d), reaching 15,68±0,16 cm after 35 days (350 d.d) ($p<0,001$) and 16,54±0,14 cm after 45 days (450 d.d) ($p<0,001$) during the FW phase (figure 8). Fork length was elevated after 38 days in SW (19,30± 0,18; $p<0,0001$). The average fork length in the parr group increased slightly, albeit significantly, from 14,42±0,13 cm to 15,18±0,18; $p<0,05$ between 35 days (350 d.d) and 45 days (450 d.d), reaching 15,18±0,18 cm ($p<0,05$) after 83 days (830 d.d) in FW ($p<0,001$) (figure 8).

Average fork length was significantly ($p <0,001$) higher in the smolt group compared with the parr group between 26 days (260 d.d) and after 38 days in SW (830 d.d) (figure 8).

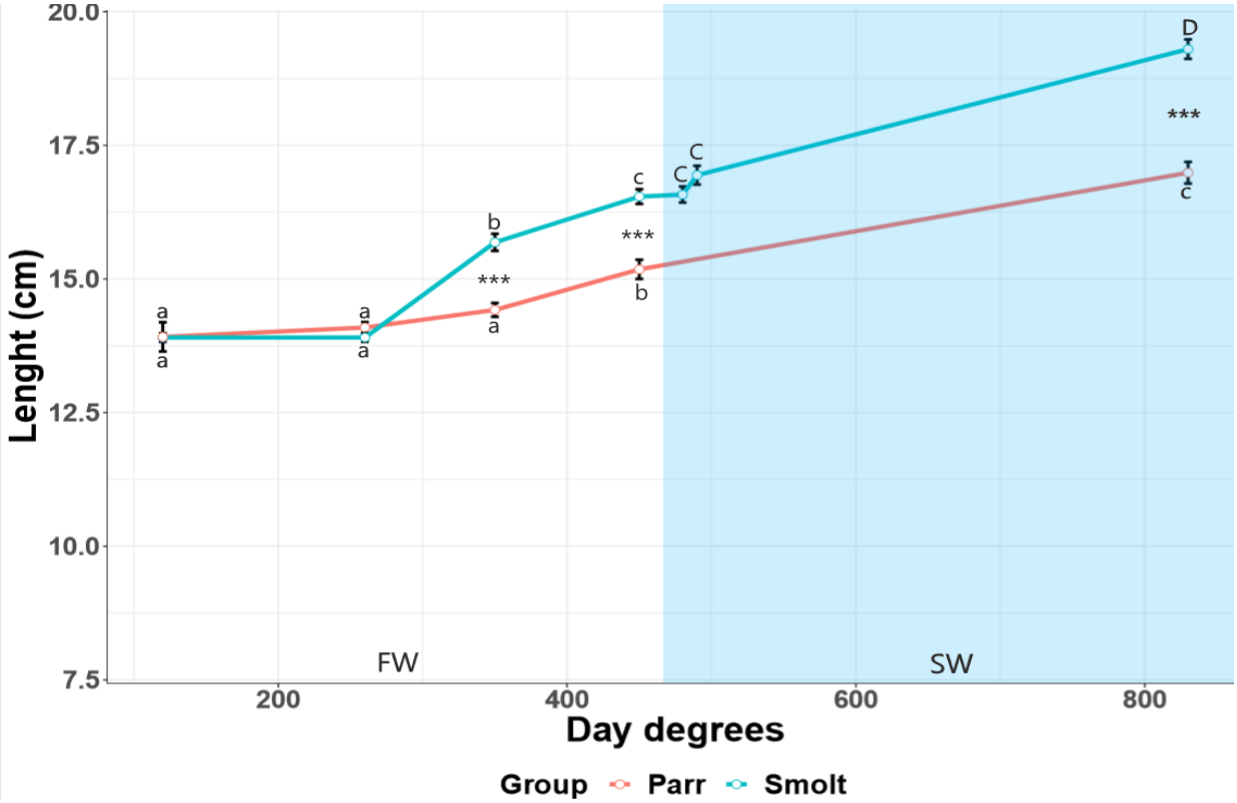

Figure 8: Fork length (centimeters, cm) of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * p<0,05; **p<0,01 and ***p<0,001 indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10-12.*

3.1.2 Weight (grams, g) of juvenile Atlantic salmon during smoltification and SW phase

The weights in the smolt group displayed a gradual increase from 38,6±1,11 grams (g) after 26 days (260 day degrees (d.d)) to 51,5±1,49 g after 45 days (450 d.d) ($p<0,001$) during the FW phase (figure 9). The weights further elevated after 38 days in SW (78,2± 3,38 g; $p<0,0001$). The weights in the parr group also increased, from 32,3±0,51 g to 42,8±1,33 g; $p<0,0003$ between 12 days (120 d.d) and 45 days (450 d.d), reaching 58,4±0,18 g ($p<0,0001$) after 83 days (830 d.d) in FW ($p<0,001$) (figure 9).

Weights were significantly higher in the smolt group compared with the parr group at all representative timepoints, except at 12 days (120 d.d) and 26 days (260 d.d) in FW (figure 9).

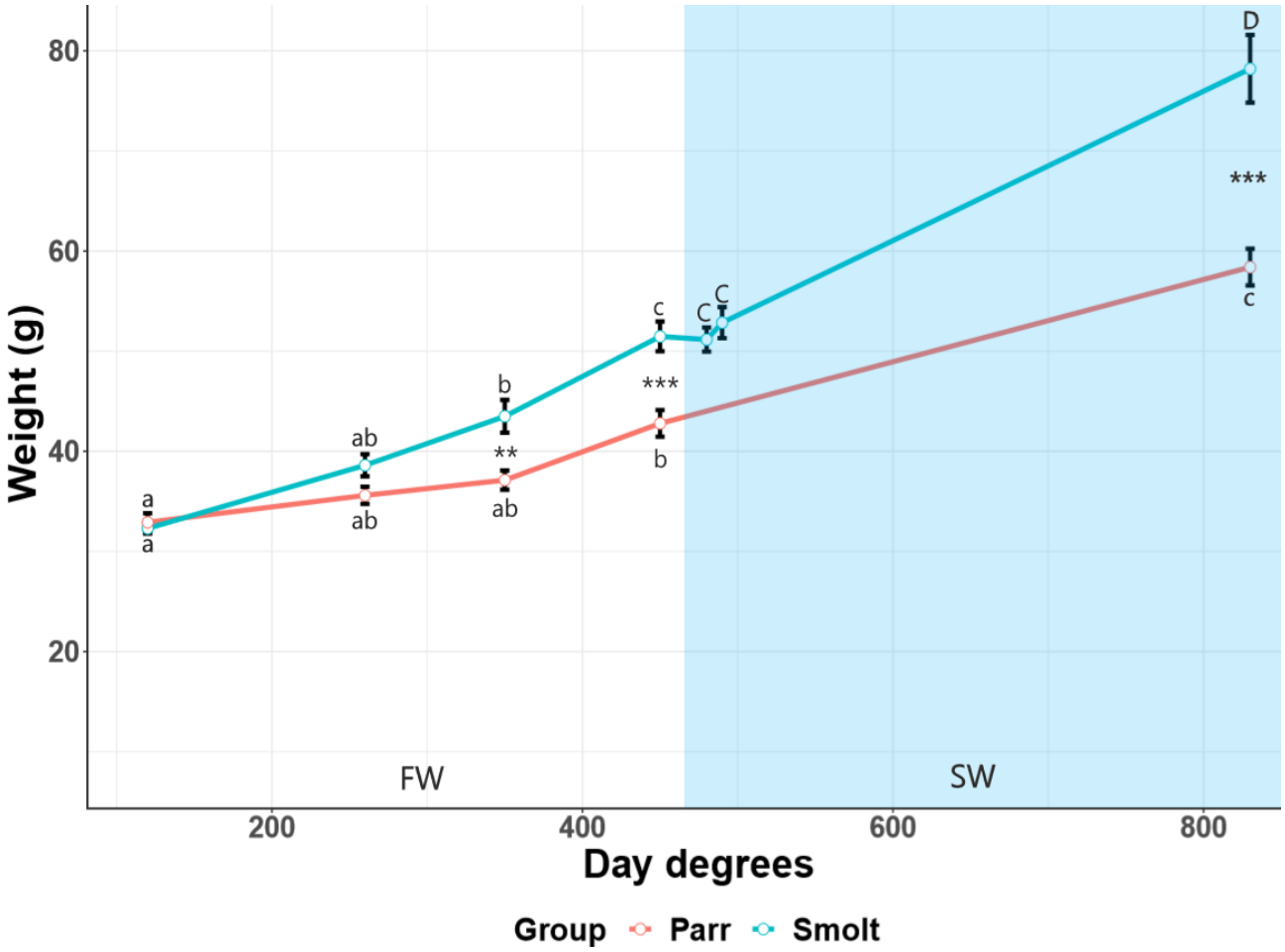

Figure 9: Weight (grams, g) of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * p<0,05; **p<0,01 and ***p<0,001 indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10-12.*

3.1.3 Condition factor calculations in juvenile Atlantic salmon during smoltification and SW phase

The condition factor in the smolt group displayed a gradual decrease from 1,20±0,011 after 26 days (260 day degrees (d.d)) to 1,15±0,015 after 45 days (450 d.d) ($p<0,0432$) during the FW phase (figure 10). Condition factor was reduced further after 38 days in SW (830 d.d) but not significantly from last timepoint in SW. The condition factor in the parr group increased slightly, albeit significantly, from 1,18±0,017 to 1,27±0,010; $p<0,0016$ between 12 days (120 d.d) and 26 days (260 d.d) in FW (figure 10).

Condition factor was significantly lower in the smolt group compared with the parr group at all representative timepoints, except at 12 days (120 d.d) in FW (figure 10).

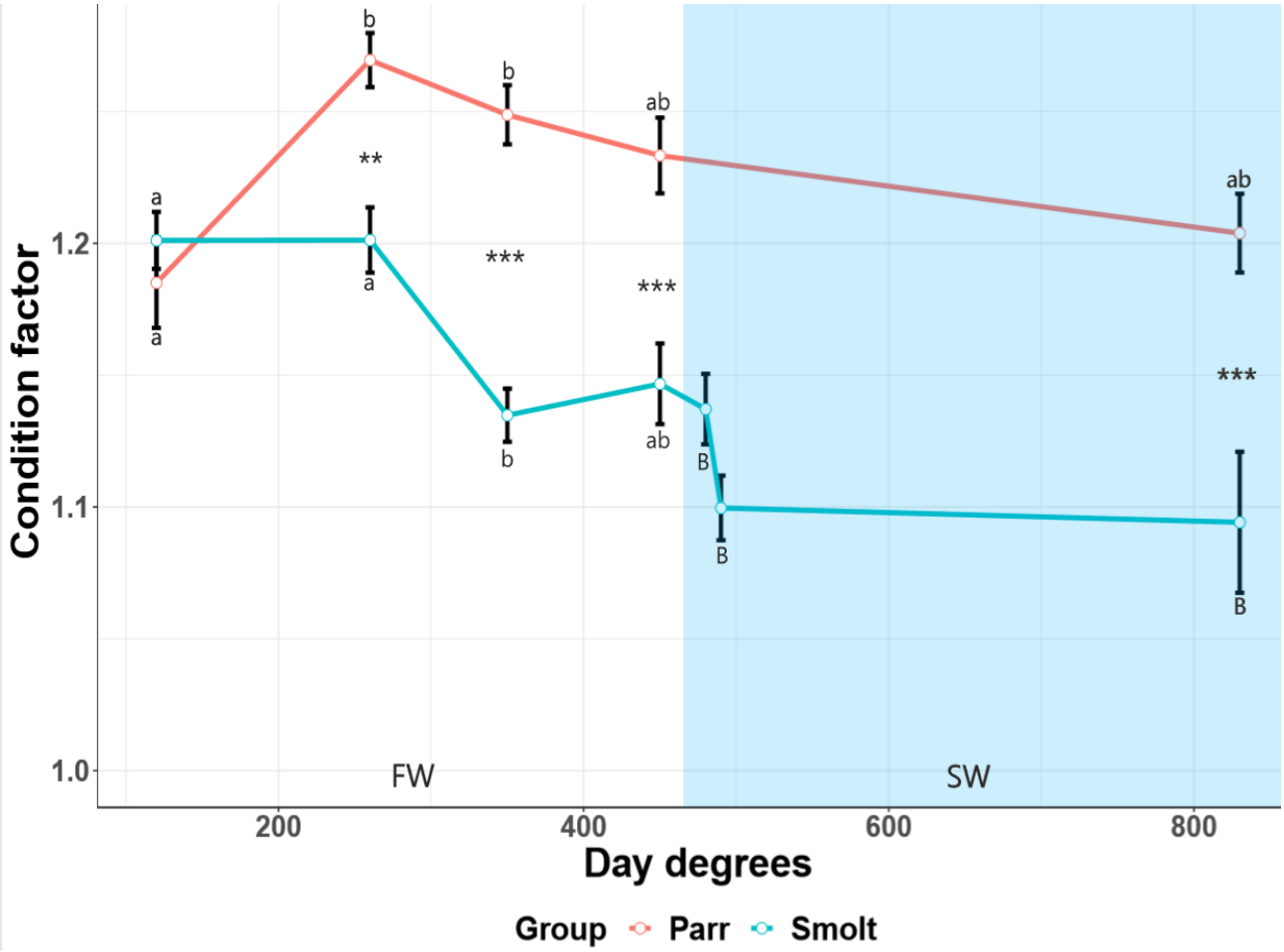

Figure 10: Condition factor of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; ** $p<0,01$ and *** $p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10-12.*

3.1.4 Smolt index in juvenile Atlantic salmon during smoltification

The smolt index in the smolt group increased from 1,49±0,057 after 12 days (120 day degrees (d.d)) to 3,11±0,12 ($p<0,0001$) after 26 days (260 d.d), displaying the highest smolt index 4,93±0,031 ($p<0,0001$) after 45 days (450 d.d) in FW (figure 11). In contrast, smolt index remained low in the parr group during the entire 45-day period in FW (figure 11).

The smolt group displayed consistently higher smolt index than those observed in the parr group (figure 11).

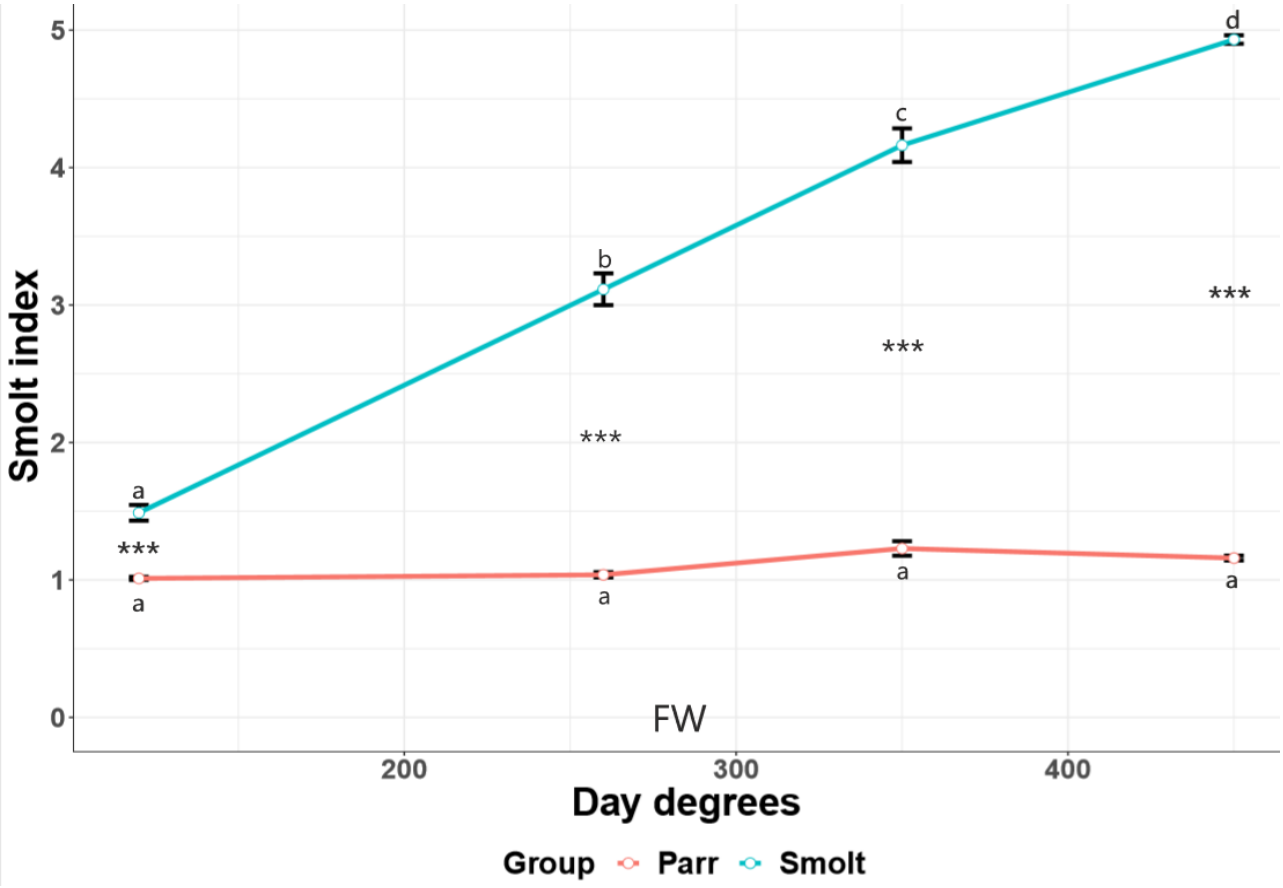

Figure 11: Smolt index of juvenile Atlantic salmon parr and smolts in freshwater (FW). *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW. Asterisk * p<0,05; **p<0,01 and ***p<0,001 indicate significant differences between groups at each timepoint. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10-12.*

3.2 Nka enzyme activity gills, intestine and kidney

3.2.1 Gill Nka enzyme activity levels in juvenile Atlantic salmon during smoltification and SW phase

The Nka enzyme activity levels in the smolt group increased from 5,93±0,68 µmoles µmoles ADP/mg protein/hour after 12 days (120 day degrees (d.d)) to 12,98±0,75 ($p<0,0001$) after 26 days (260 d.d), reaching peak activity levels of 17,19±0,76 µmoles ADP/mg protein/hour ($p<0,0001$) after 45 days (450 d.d) in FW phase (figure 12). Gill Nka activity was slightly elevated after 38 days in SW (20,17±0,73; $p<0,0274$). In contrast, gill Nka enzyme activity levels in the parr group after 12 days (120 d.d) remained low until a slight, albeit, significant increase from 4,08±0,33 to 7,99±1 µmoles ADP/mg protein/hour ($p<0,0006$) between 45 days (450 d.d) and 83 days (830 d.d) during the FW phase (figure 12).

The smolt group displayed consistently higher gill enzyme activity levels than those observed in the parr group (figure 12).

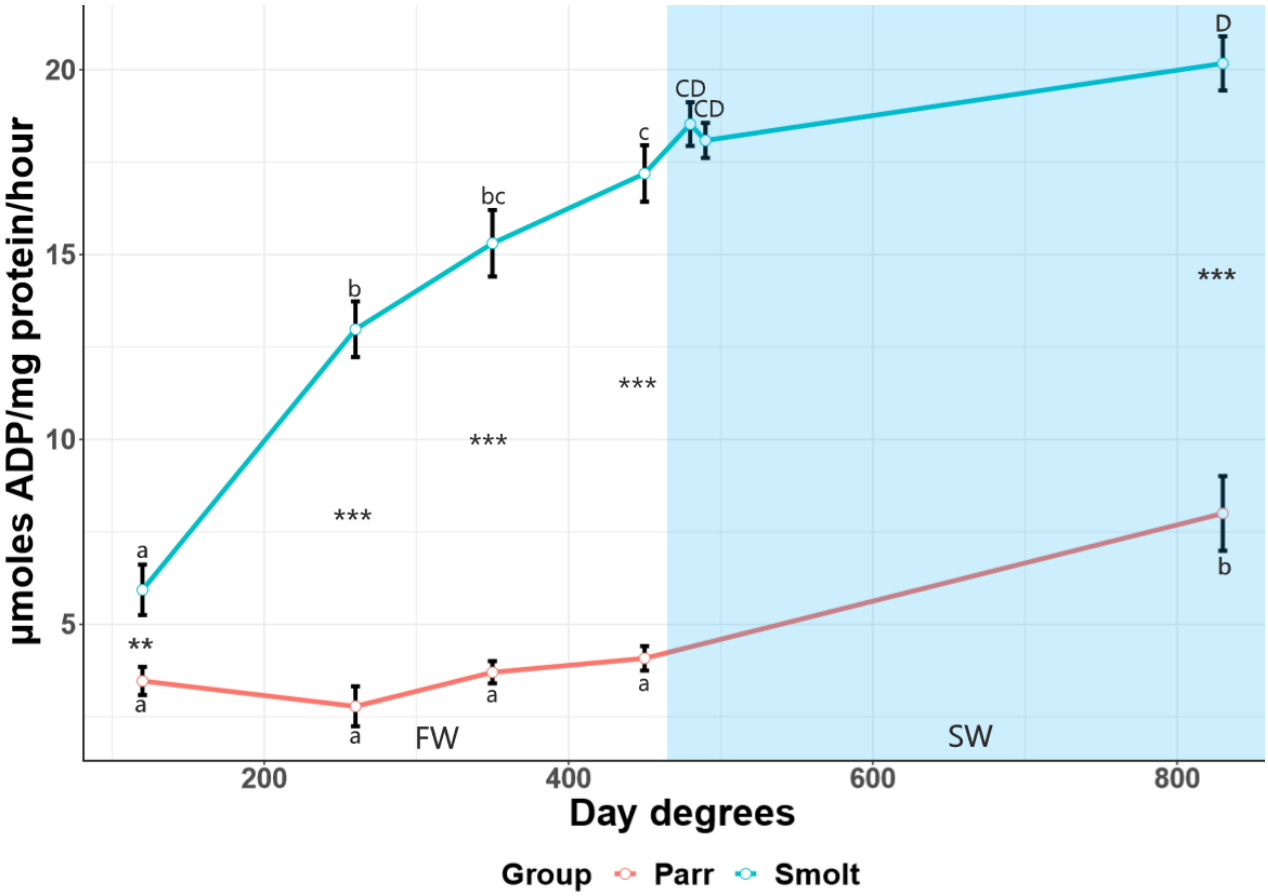

Figure 12: Gill Nka enzyme activity levels (µmoles ADP/mg protein/hour) of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * p<0,05; **p<0,01 and ***p<0,001 indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10-12.*

3.2.2 Proximal intestine Nka enzyme activity in juvenile Atlantic salmon during smoltification and SW phase

The proximal intestinal Nka enzyme activity levels in the smolt group increased from 6,48±0,78 µmoles ADP/mg protein/hour after 12 days (120 day degrees (d.d)) to 14,4±1,2 ($p<0,0357$) after 26 days (260 d.d) reaching peak activity 22,8±1,81 ($p<0,0001$) after 35 days (350 d.d) in FW phase (figure 13). An increase in Nka activity was observed from 14,5±2,16 µmoles ADP/mg protein/hour after 45 days in FW (450 d.d) to 23,1±3,1 after 1 day in SW (490 d.d), thus not significant before 38 days in SW (830 d.d) reaching 27,7±3,89 ($p<0,0070$). In contrast proximal intestine Nka activity in the parr group remained low until a significant increase was observed from 2,54±0,30 to 18,6±1,55 µmoles ADP/mg protein/hour ($p<0,0013$) between 45 days (450 d.d) and 83 days (830 d.d) in FW (figure 13).

The smolt group displayed consistently and significantly higher proximal intestinal Nka enzyme activity levels than those observed in the parr group (figure 13).

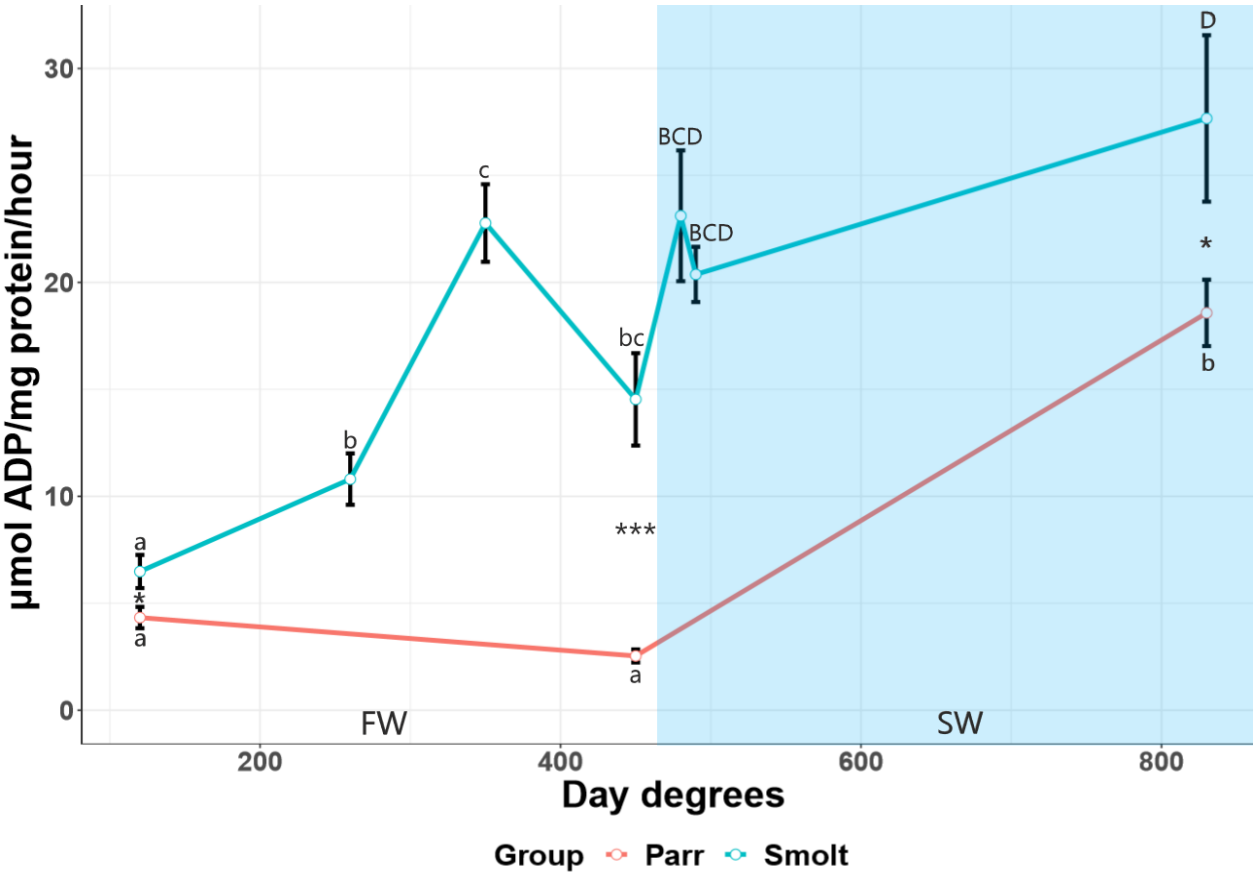

Figure 13: Proximal intestinal Nka enzyme activity levels (µmoles ADP/mg protein/hour) of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; ** $p<0,01$ and *** $p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=9-12.*

3.2.3 Distal intestine Nka enzyme activity in the of juvenile Atlantic salmon during smoltification and SW phase

The distal intestinal Nka enzyme activity levels in the smolt group increased from 2,15±0,32 µmoles ADP/mg protein/hour after 12 days (120 day degrees (d.d)) to 4,39±0,82 ($p<0,0357$) after 26 days (260 d.d) reaching peak activity 10,7±1,18 ($p<0,0001$) after 35 days (350 d.d) in FW phase (figure 14). The Nka activity remained high and was prolonged after SW transfer (figure 14). In contrast distal intestine Nka activity in the parr group remained low, until a slight, but significant increase was observed from 1,30±0,18 to 4,14±0,68 µmoles ADP/mg protein/hour ($p<0,0004$) between 45 days (450 d.d) and 83 days (830 d.d) in FW (figure 14).

The smolt group displayed consistently higher proximal intestinal Nka enzyme activity levels than those observed in the parr group, except at 12 days (120 d.d) (figure 14).

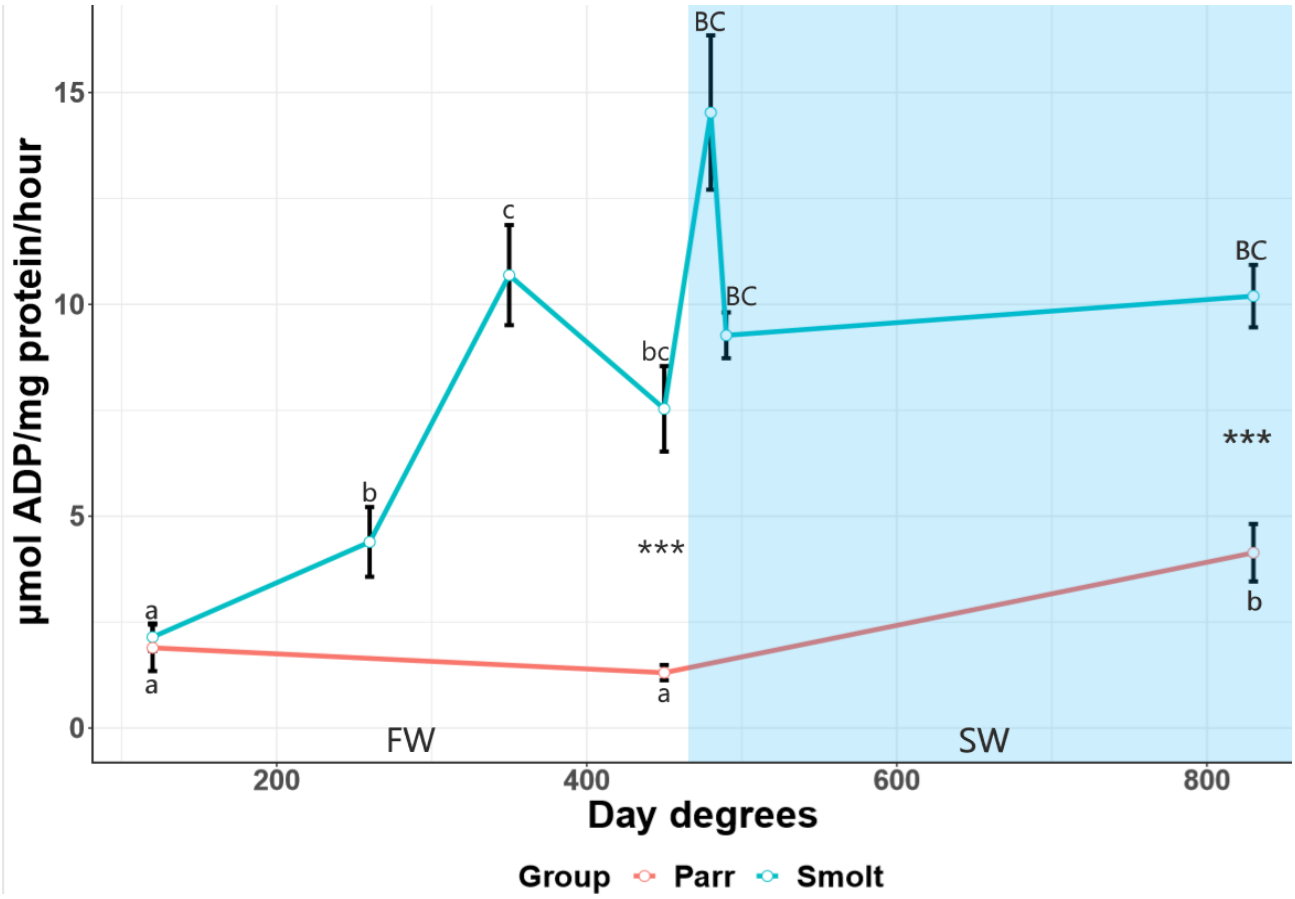

Figure 14: Distal intestinal Nka enzyme activity levels (µmoles ADP/mg protein/hour) of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; ** $p<0,01$ and *** $p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=9-12.*

3.2.4 Kidney Nka enzyme activity levels in juvenile Atlantic salmon

The kidney Nka enzyme activity levels in the smolt group increased from 14,47±0,70 µmoles ADP/mg protein/hour after 26 days (260 day degrees (d.d)) to 19,30±0,88 ($p<0,0002$) after 35 days (350 d.d) in FW phase (figure 15). Kidney Nka activity rapidly decreased from 20,03±0,66 µmoles ADP/mg protein/hour after 45 days (450 d.d) to 13,98±0,70 ($p<0,0001$) after 1 day in SW and remained low after 2 days in SW (figure 15). A significant increase was observed in the smolt group from 14,29±0,47 µmoles ADP/mg protein/hour to 19,78±0,83 ($p<0,0001$) between 2 days (480 d.d) in SW and 38 days (830 d.d) in SW (figure 15). In contrast, kidney Nka enzyme activity levels in the parr group remained low until a significant decrease was observed from 15,88±0,38 after 45 days (450 d.d) in FW to 11,17±1,04 µmoles ADP/mg protein/hour ($p<0,0002$) after 83 days (830 d.d) in FW (figure 15).

The smolt group displayed higher kidney Nka enzyme activity levels than those observed in the parr group after 35 days (350 d.d) FW, 45 days (450 d.d) FW and 38 days SW (830 d.d) (figure 15).

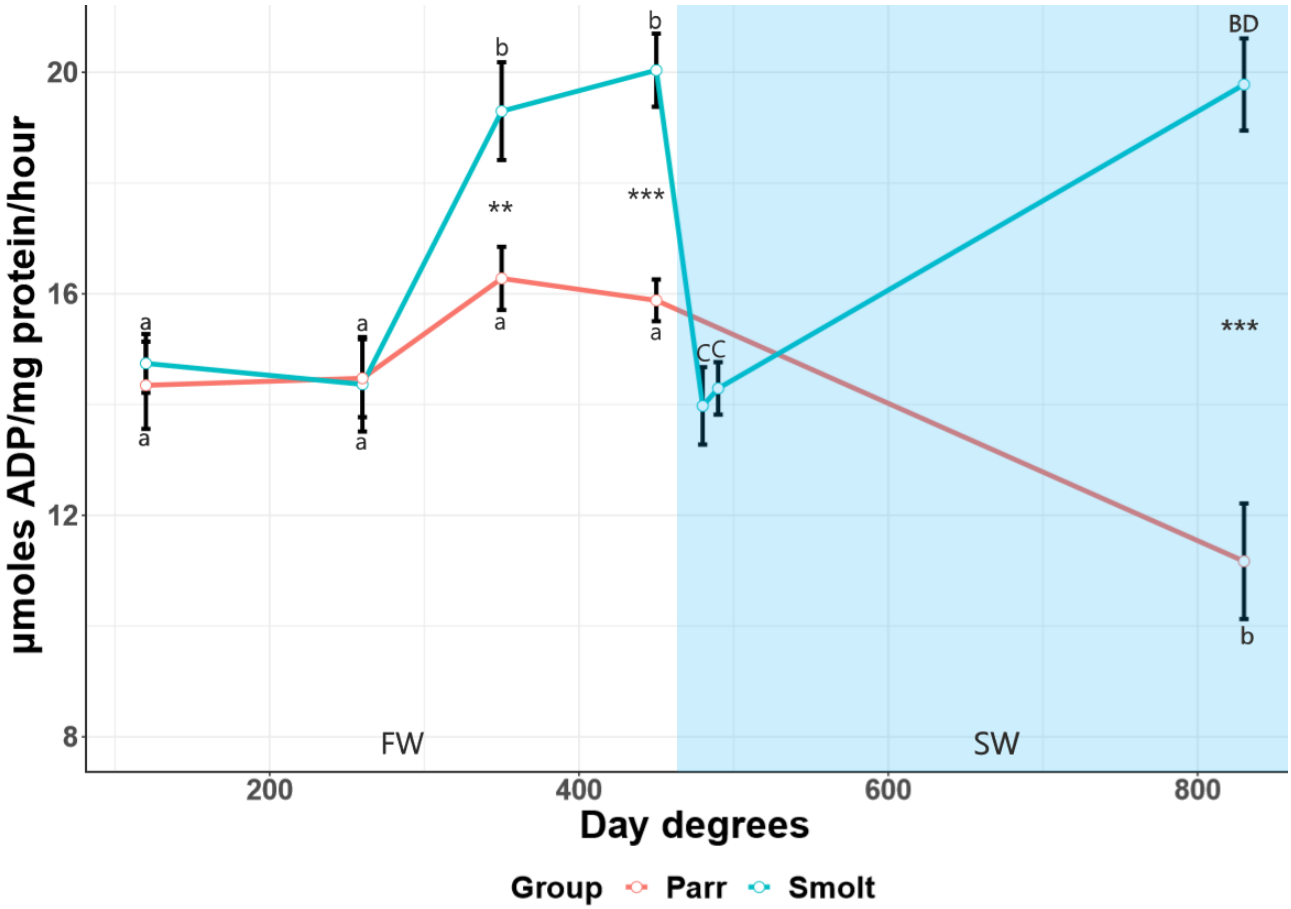

Figure 15: Kidney Nka enzyme activity levels (µmoles ADP/mg protein/hour) of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; ** $p<0,01$ and *** $p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10-12.*

3.3 Results phylogenetic tree

3.3.1 Phylogenetic tree for the Slc13a1 transporter

The candidates for salmon Solute carrier family 13 member 1 (Slc13a1) orthologues, identified by BLAST using the previously annotated Slc13a1 amino acid sequence in Japanese eel (*Anguilla japonica* - *Nakada et al., 2005*), were aligned to a set of representative teleosts and vertebrates and submitted to a phylogenetic analysis. The resulting phylogenetic tree is presented on figure 16 (the corresponding alignment is presented on appendix 4). A single salmon gene grouped within the vertebrate Slc13a1 group, with as closest relative the Northern pike (*Esox lucius*) Slc13a1 – a position consistent with the evolutionary relationship between these species.

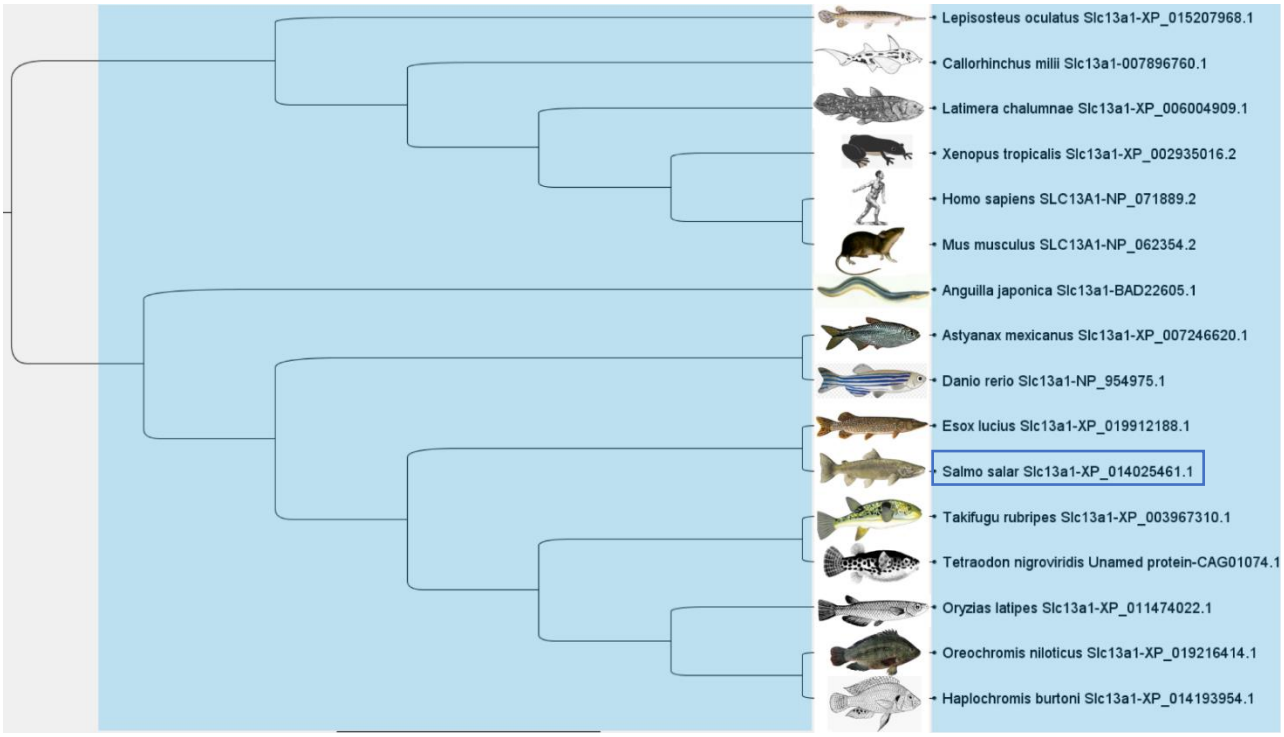

Figure 16: Phylogenetic analysis placing the Atlantic salmon candidate for a Slc13a1 homologue within the Slc13a1 family. *Phylogenetic tree presenting the phylogenetic relationship between the protein sequences of Slc13a1 of Atlantic salmon (Salmo salar) and a set of other vertebrate species, using Figtree as graphical viewer. Protein sequences from fish species are written with first letter upper-case (Slc13a1), whereas mammals are written with all letters in upper case (SLC13A1). Pictures of all species are retrieved from public domain or fish base (www.fishbase.org).*

3.3.2 Phylogenetic tree for the Slc26a1 transporter

The best candidates for salmon Solute carrier family 26 member 1 (Slc26a1) orthologues, identified by BLAST using the previously annotated Slc26a1 amino acid sequence in Japanese eel (*Anguilla japonica - Nakada et al., 2005*), were aligned to a set of representative teleosts and vertebrates and submitted to a phylogenetic analysis. The resulting phylogenetic tree is presented on figure 17 (the corresponding alignment is presented on appendix 4). Two salmon genes grouped within the vertebrate Slc26a1 group, each pairing with a Rainbow trout (*Oncorhynchus mykiss)* gene, and the group containing these four sequences had as closest relative the single Northern pike (*Esox lucius*) Slc26a1. This position was consistent with the evolutionary relationship between the Northern pike and indicated that the two salmon genes, thereafter, named Slc26a1a and Slc26a1b, are the result of a salmonid-specific duplication.

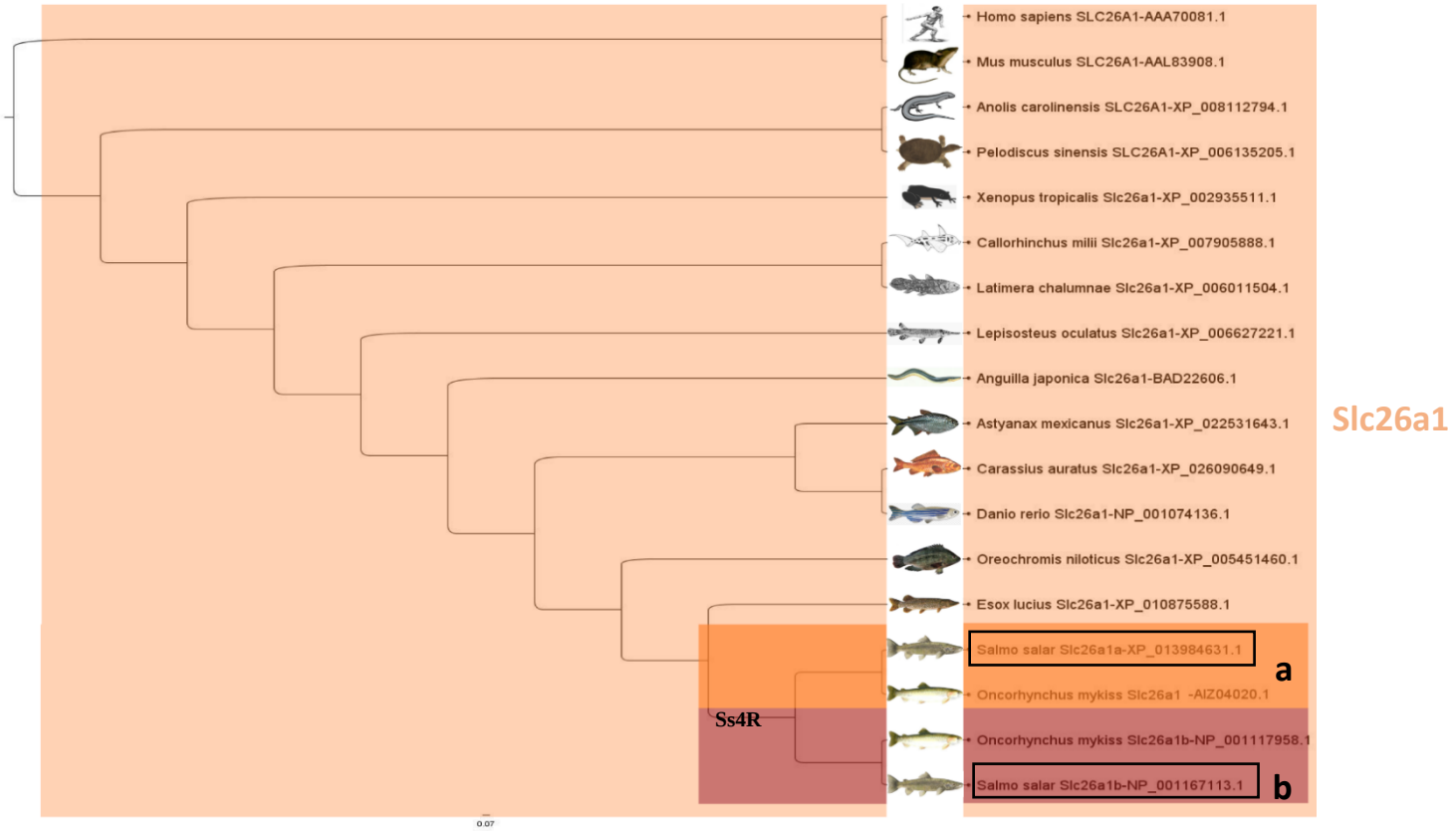

Figure 17: Phylogenetic analysis placing the two Atlantic salmon candidates for Slc26a1 homologues within the Slc26a1 family. *Phylogenetic tree presenting the relationship between the protein sequences of the two Slc26a1 of Atlantic salmon and a set of other vertebrate species, using Figtree as graphical viewer. The two sequences appeared to be paralogs and were named Slc26a1a (dark orange) and Slc26a1b (red). The salmon specific 4th round of whole genome duplication is marked (Ss4R). Protein sequences from fish species are written with first letter upper-case (Slc26a1), whereas mammals are written with all letters in upper case (SLC26A1). Pictures of all species are retrieved from public domain or fish base (www.fishbase.org).*

3.3.3 Phylogenetic tree for the Slc26a6a, b and c

The best candidates for salmon Solute carrier family 26 member 6 (Slc26a6) orthologues, identified by BLAST using the previously annotated Slc26a6a, b and c amino acid sequences in the Obscure Pufferfish (*Takifugu obscurus* - *Kato et al., 2009*), were aligned to a set of representative teleosts and vertebrates and submitted to a phylogenetic analysis. The resulting phylogenetic tree is presented on figure 18 (the corresponding alignment is presented on appendix 4). Four salmon genes grouped within the vertebrate Slc26a6 group, one in a subgroup containing *Takifugu obscurus* Slc26a6b, one in a subgroup containing *Takifugu obscurus* Slc26a6c and two in a subgroup containing *Takifugu obscurus* Slc26a6a. In the latter, the two salmon genes grouped with two sequences from rainbow trout and one from Northern pike, once again indicating a salmonid-specific duplication. The two Slc26a6a paralogues were thus named Slc26a6a1 and Slc26a6a2. Each pairing with a Rainbow trout (*Oncorhynchus mykiss)* gene, and the group containing these four sequences had as closest relative the single Northern pike (*Esox lucius*) Slc26a6a. This position was consistent with the evolutionary relationship between the Northern pike and indicated that the two salmon genes, thereafter, named Slc26a6a1 and Slc26a6a2, both are the result of a salmonid-specific duplication.

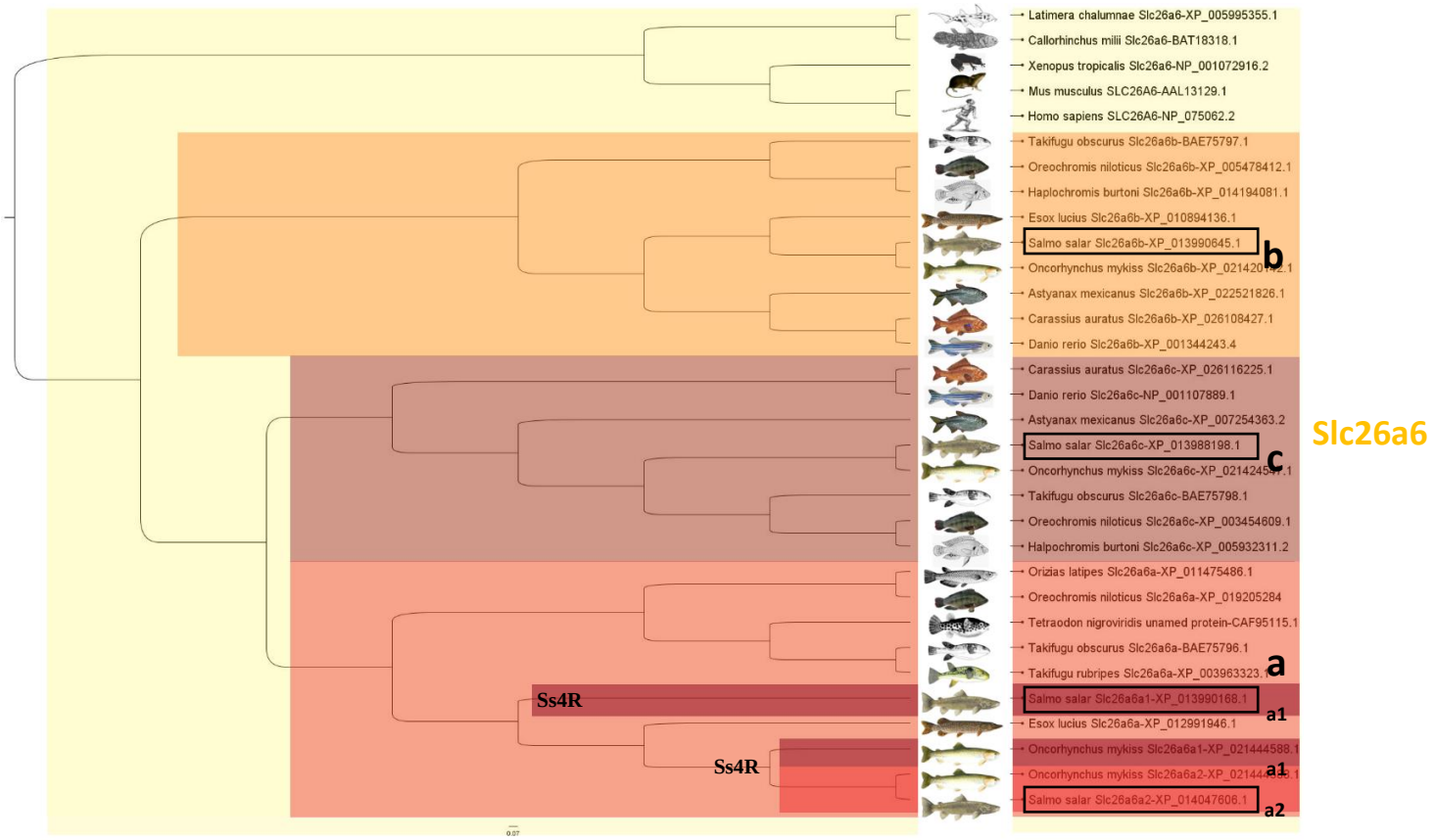

Figure 18: ***Phylogenetic analysis placing the two Atlantic salmon candidates for Slc26a6a, b and c homologues within the Slc26a6 family.*** *Phylogenetic tree presenting the relationship between the protein sequences of the four Slc26a6 of Atlantic salmon and a set of other vertebrate species, using Figtree as graphical viewer. The salmon specific 4th round of whole genome duplication is marked (Ss4R). Protein sequences from fish species are written with first letter upper-case (Slc26a6), whereas mammals are written with all letters in upper case (SLC26A6). Pictures of all species are retrieved from public domain or fish base (www.fishbase.org).*

3.4 Relative mRNA abundance of SO_4^{2-} transporters in kidney and gills

3.4.1 Relative mRNA abundance of *slc26a6a1* in the kidney

The relative mRNA abundance of *slc26a6a1* in the smolt group increased from 0,68±0,11 after 12 days (120 day degrees (d.d)) to 1,85±0,14 (p<0,0001) after 26 days (260 d.d) in FW phase (figure 19). The relative mRNA abundance of slc26a6a1 showed a rapid increase from 2,45±0,21 after 1 day in SW to 3,54±0,17 (p>0,05) after 2 days in SW, thus not significantly higher before reaching 4,24±0,24 (p<0,0123) after 38 days in SW (figure 19). In contrast, the relative mRNA abundance of slc26a6a1 remained low until a slight, albeit, significant increase from 0,18±0,019 to 0,39±0,077 between 45 days (450 d.d) and 83 days (830 d.d) during the FW phase (figure 19).

The smolt group displayed consistently higher relative mRNA abundance of *slc26a6a1* than those observed in the parr group (p<0,0001), hence slight lower significant at 120 d.d (p<0,05) (figure 19).

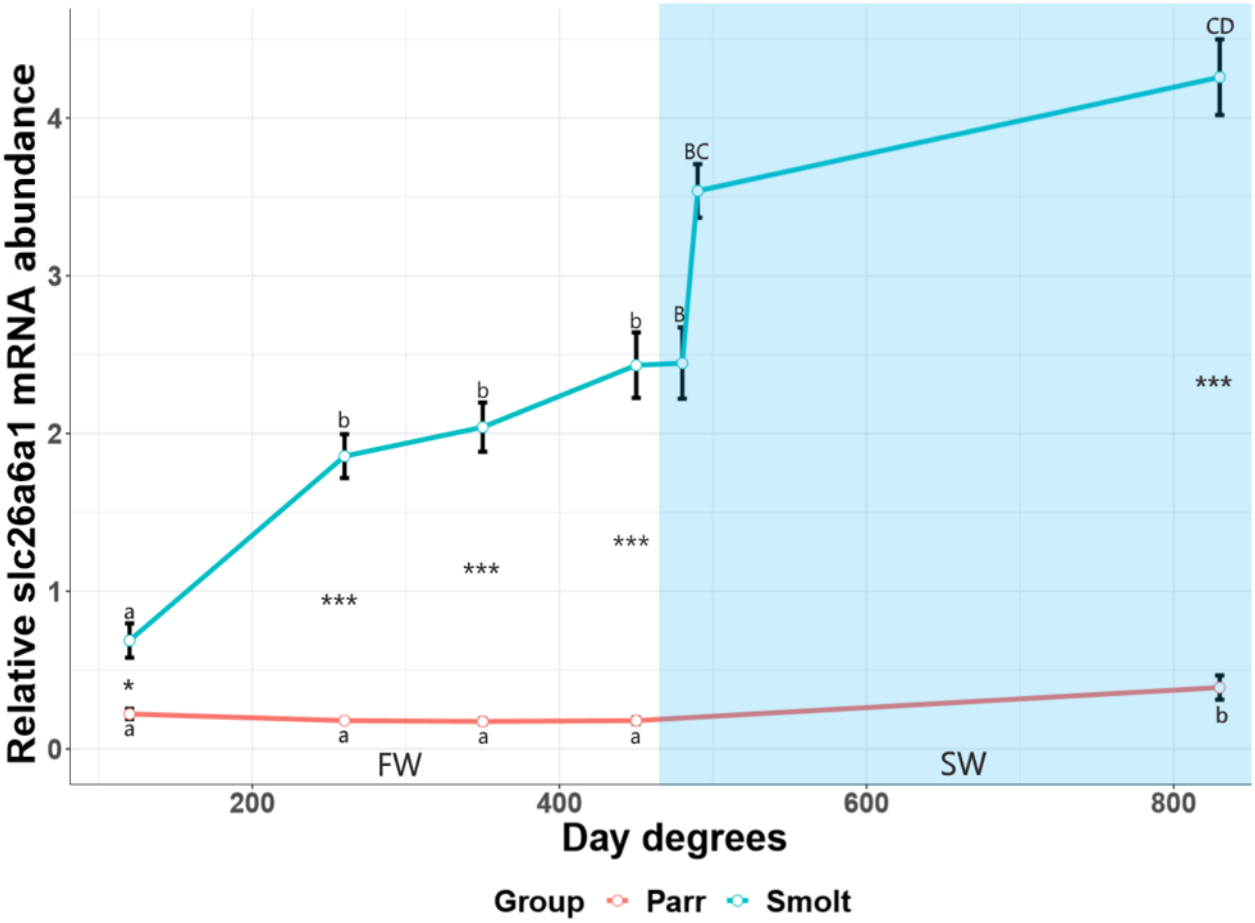

Figure 19: The mRNA abundance of *slc26a6a1* in the kidney of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * p<0,05; **p<0,01 and ***p<0,001 indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10.*

3.4.2 Relative mRNA abundance of *slc26a6b* and *slc26a6c* in the kidney (Not significant)

The relative abundance of both *slc26a6b* and *slc26a6c* were relatively equally expressed in the kidney for both the smolt and parr group, hence no significant difference was observed in either the smolt group or the parr group (figure 20). A small but significant difference was observed between groups in relative mRNA abundance of *slc26a6b* after 35 days (350 d.d) in FW (p<0,0214) (figure 20).

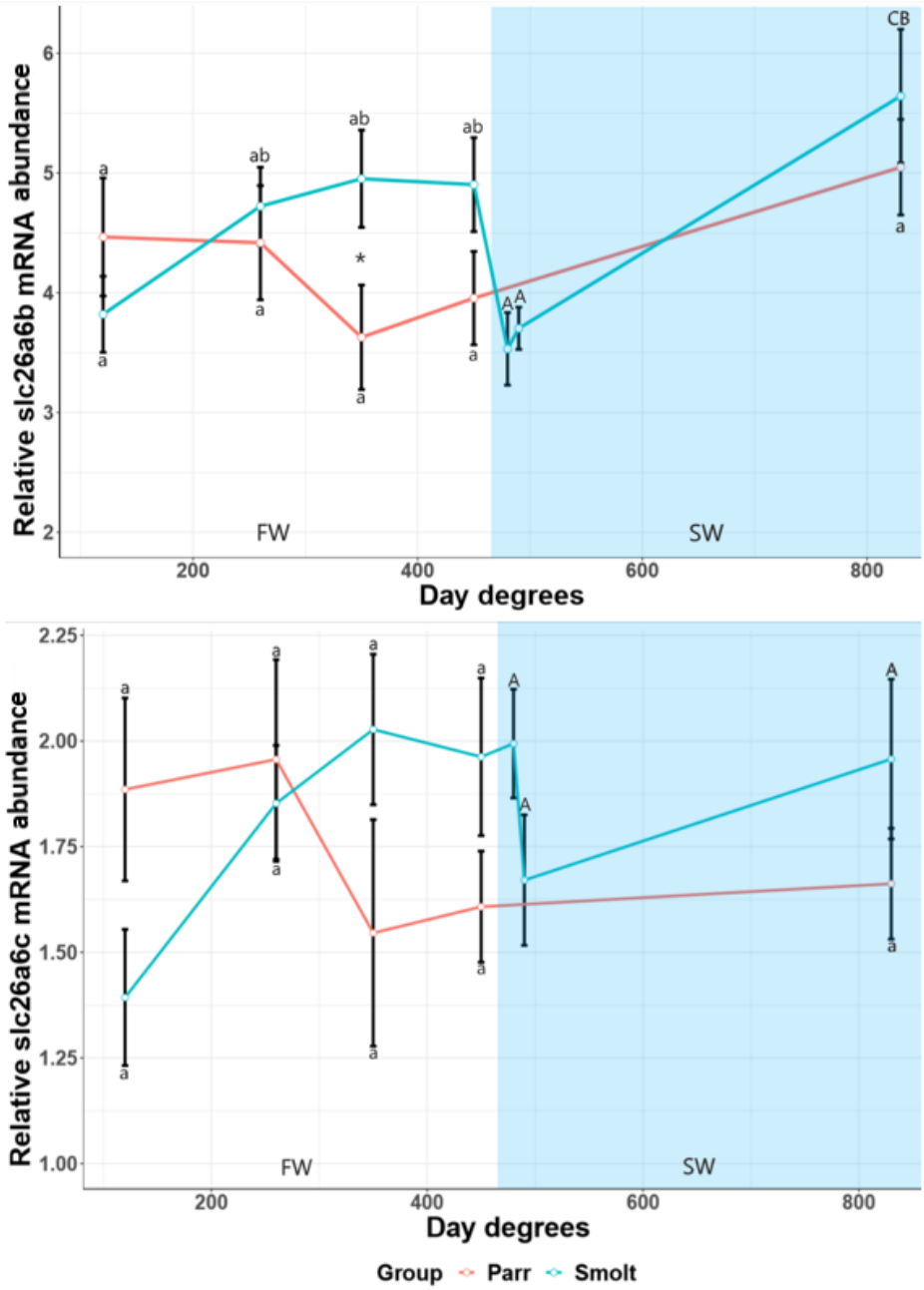

Figure 20: The relative mRNA abundance of *slc26a6b* and *slc26a6c* in the kidney of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * p<0,05; **p<0,01 and ***p<0,001 indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10.*

3.4.3 Relative mRNA abundance of *slc26a1a* in the kidney

The relative mRNA abundance of *slc26a1a* in the smolt group increased from 20,74±1,12 after 12 days (120 day degrees (d.d)) to 26,73±1,61 ($p<0,05$) after 26 days (260 d.d) in FW phase, marginally significant (figure 21). The relative mRNA abundance of *slc26a1a* significantly increased from 24,10±1,20 after 2 days in SW (480 d.d) to 27,89±2,26 ($p<0,0215$) after 38 days in SW (830 d.d) (figure 21). In contrast, the relative mRNA abundance of slc26a1a was not significantly different at any timepoint in the parr group (figure 21).

The smolt group displayed consistently higher relative mRNA abundance of *slc26a1a* than those observed in the parr group ($p<0,0001$), except after 12 days (120 d.d) (figure 21)

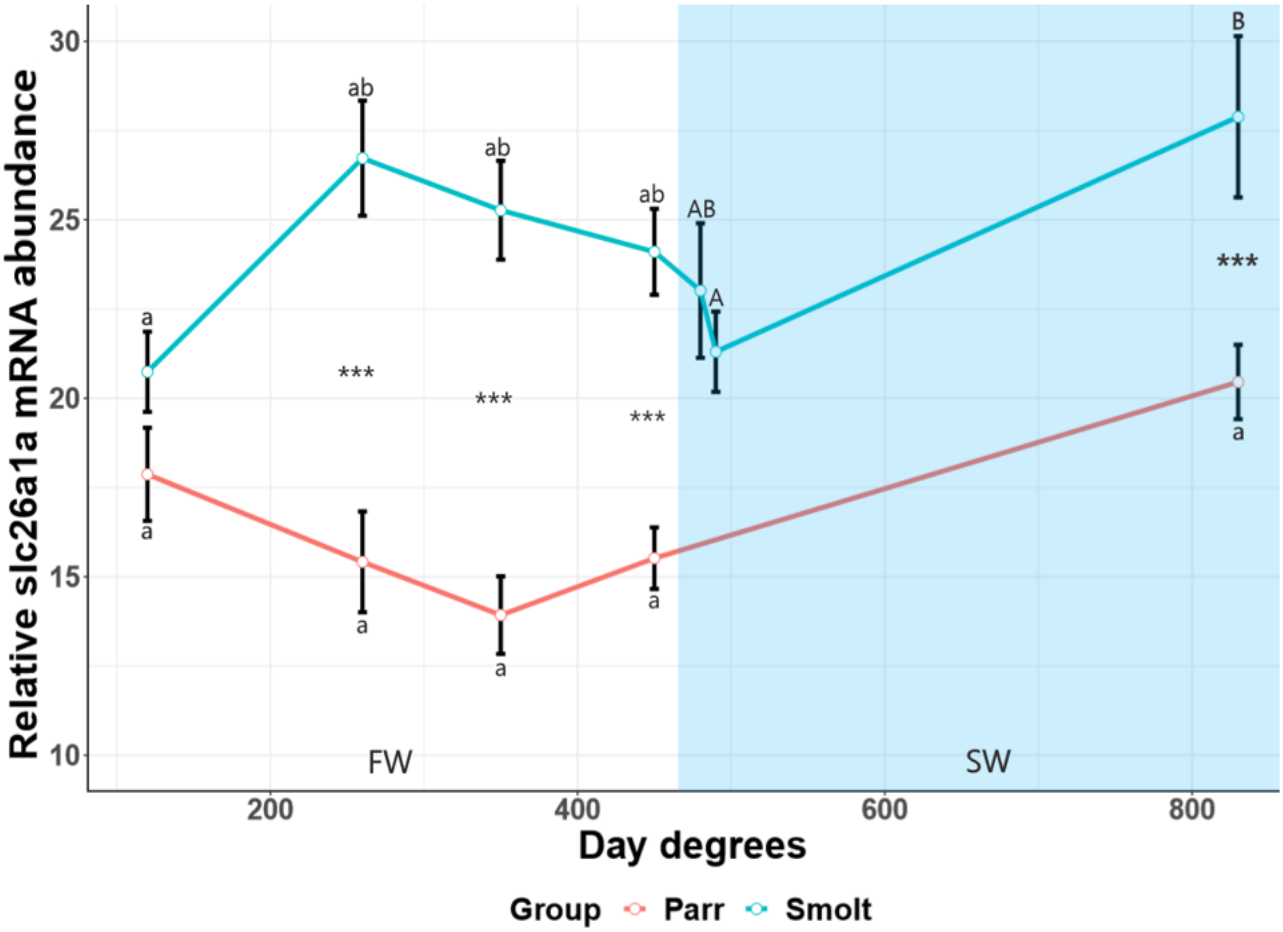

Figure 21: The mRNA abundance of *slc26a1a* in the kidney of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; ** $p<0,01$ and *** $p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10.*

3.4.4 Relative mRNA abundance of slc26a1bX1 and slc26a1bX3 in the kidney (Not significant)

The relative abundance of both *slc26a1bX1* (31,04-39,31) and *slc26a1bX3* (7,19-12,13) was high in the kidney for both the smolt and parr group, hence no significant difference was observed in either the smolt group or the parr group (figure 22). A small but significant difference was observed in relative mRNA abundance of *slc26a1bX3* after 35 days (350 d.d) in the groups ($p<0,0195$) in FW (figure 22).

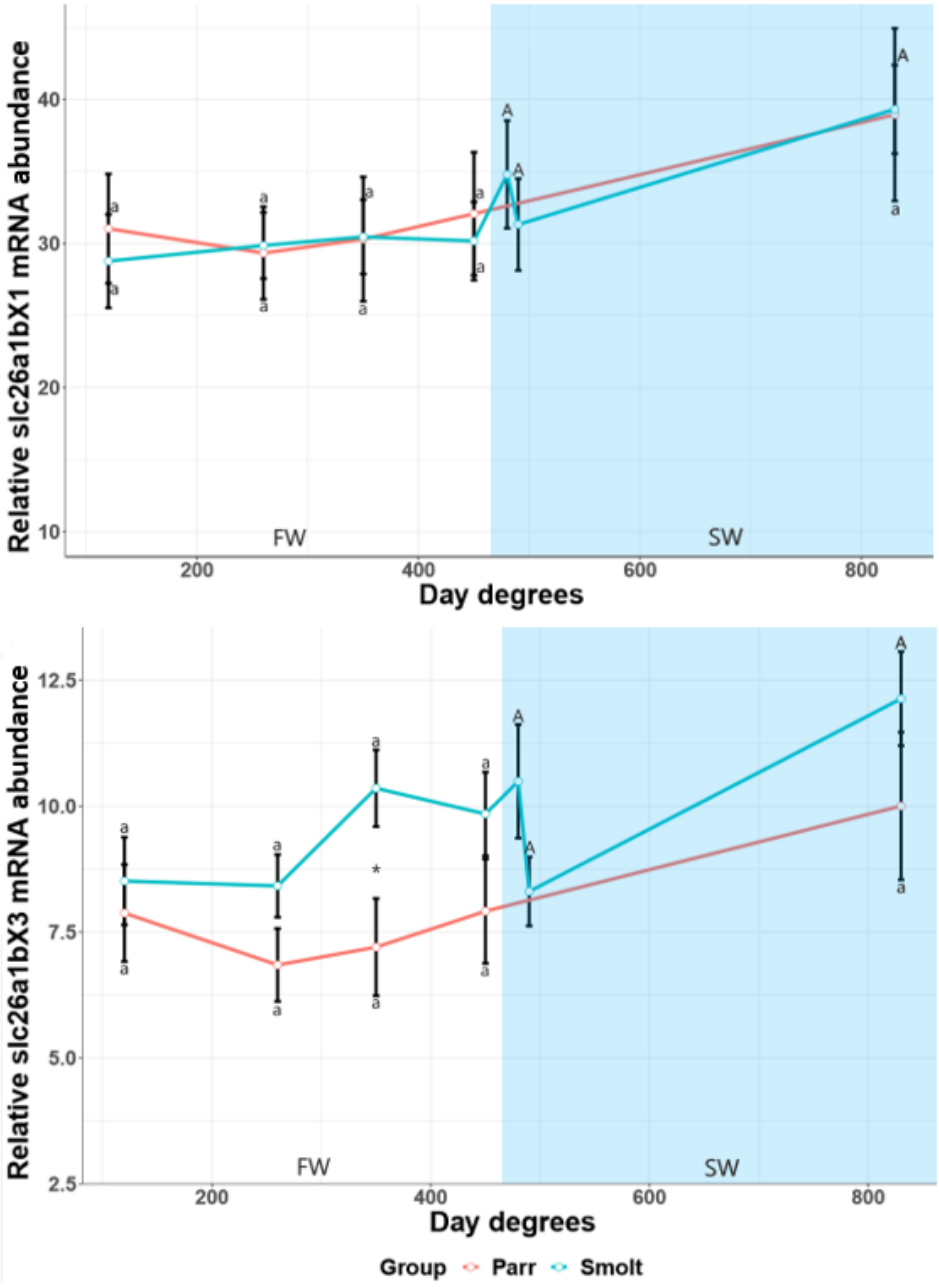

Figure 22: The relative mRNA abundance of *slc26a1bX1* and *slc26a1bX3* in the kidney of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; ** $p<0,01$ and *** $p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=10.*

3.4.5 Relative mRNA abundance of slc26a6a2 in gills

The relative mRNA abundance of *slc26a6a2* in gills for the smolt group decreased significantly from 154,2±33,66 after 12 days (120 day degrees (d.d)) to 59,04±6,32 ($p<0,0304$) after 26 days (260 d.d) in FW phase (figure 23). The relative mRNA abundance of *slc26a6a2* gradually decreased from 49,1±5,87 to 13,59±2,78 ($p<0,0001$) between last timepoint in FW after 45 days (450 d.d) and after 2 days in SW (490 d.d) reaching the lowest point after 38 days in SW (830 d.d) at 0,1±0,02 (figure 23). In contrast, the relative mRNA abundance of slc26a6a2 was not significantly different at any timepoint in the parr group (figure 23).

The smolt group displayed consistently higher relative mRNA abundance of *slc26a6a2* than those observed in the parr group ($p<0,0001$), except after 12 days (120 d.d) (figure 23).

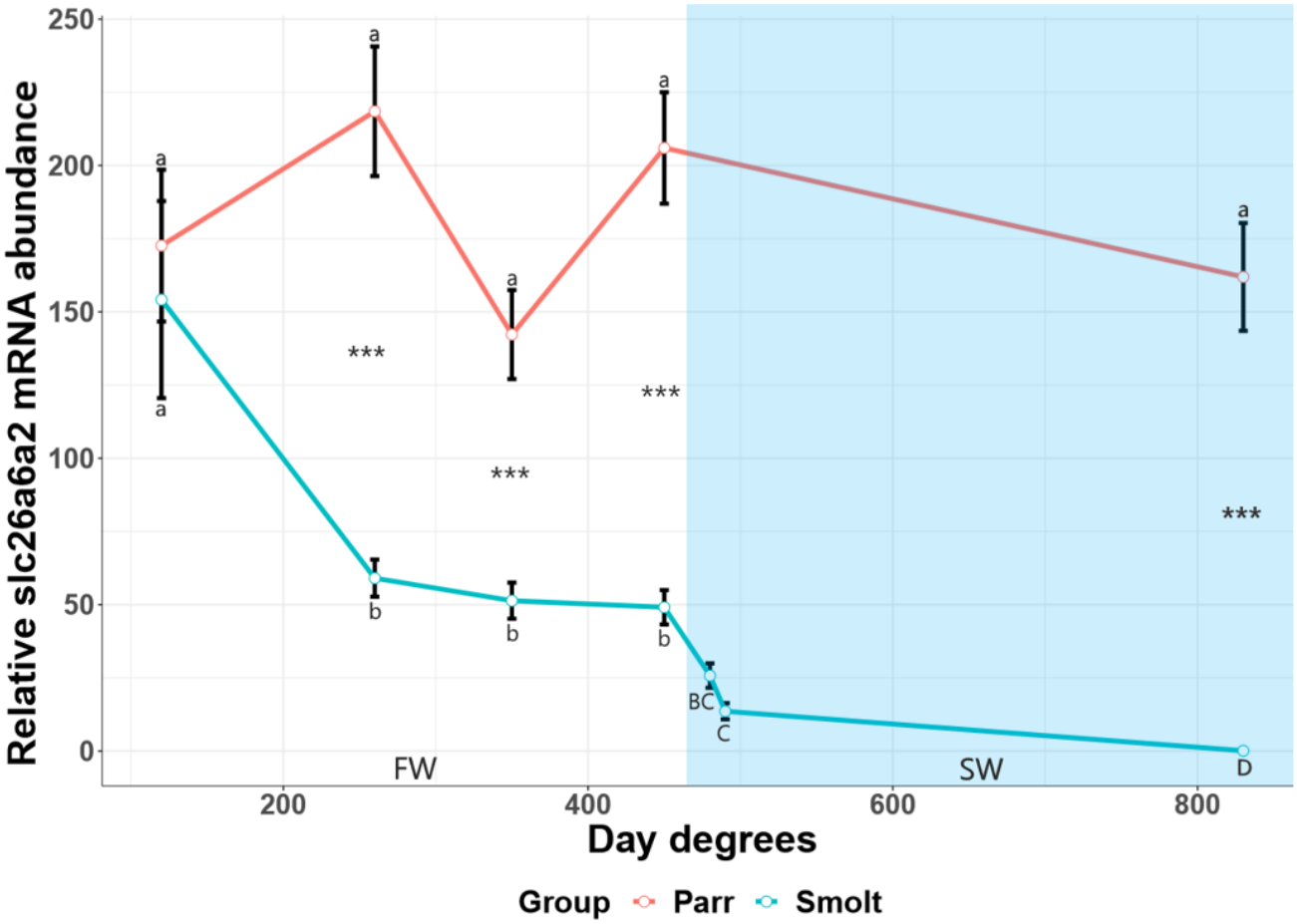

Figure 23: The mRNA abundance of *slc26a6a2* in the gills of juvenile Atlantic salmon parr and smolts in freshwater (FW) and smolts after seawater (SW) transfer. *Different small letters indicate significant differences between timepoints within the control group (parr) and experimental smolt group in FW (white area of graph), while capital letters indicate differences within each group in SW (blue area of graph). Note that significances following SW transfer is related to last timepoint in FW. Asterisk * $p<0,05$; **$p<0,01$ and ***$p<0,001$ indicate significant differences between groups at each timepoint in both FW and SW. The control group remained in FW during the entire experiment. Each data point is represented as mean ± Standard Error of Mean (SEM) and n=6-10.*

4.Discussion

4.1 Considerations material and methods

4.1.1 Experimental design

The study design and layout for the experiment used two $1m^3$ tanks, one tank per treatment group. Ideally one would try to control for potential tank effects by applying replicate tanks for each group. This is particularly important in growth and behaviour studies (Thorarensen, Kubiriza and Imsland, 2015; Johnsson and Näslund, 2018). However, several studies of the smoltification process in the ILAB facility using replicate tanks showed no tank effect and very little variation (Ebbesson *et al.*, 2003; Nilsen, Ebbesson and Stefansson, 2003; Stefansson *et al.*, 2007; Nilsen *et al.*, 2007; 2008; Sundh *et al.*, 2014). Hence, it is concluded that the use of one single tank per group was sufficient for this study.

In this experimental set up a traditional season independent photoperiod regime was applied, known to stimulate smoltification and facilitate a controlled production of smolts (Ebbesson *et al.*, 2007; Stefansson *et al.*, 2007; Strand, Hazlerigg and Jørgensen, 2018). Temperature appears to be more of a rate limiting factor (Sigholt, Åsgård and Staurnes, 1998; Handeland *et al.*, 2014), and low temperatures may lead to incomplete smolt development under short square wave regimes. Conversely, high temperatures may have adverse effects such as induction of early puberty (Fjelldal, Hansen and Huang, 2011; Good and Davidson, 2016). Thus, a 12D/12L regime was selected for the control group (parr) to reduce or at least minimize the chances that any smolting would transpire in the control group, while the experimental group (smolt) was given a 24 hours signal for smolting to transpire effectively (Kristensen *et al.*, 2012).

Low oxygen levels may cause stress that can impair growth or otherwise have a negative effect on the smoltification development (Thorarensen and Farrell, 2011; Olsvik *et al.*, 2013). Flow rate of 0.6 L/kg/fish/min or higher is recommended in the industry when stocking densities are above 50 kg m^{-3} (Calabrese *et al.*, 2017). In this study adequate flow rates of 0.6 L/kg/min and stocking density of maximum 100 kg/m^{-3} were used to ensure optimal rearing conditions. The oxygen saturation (FW/SW:<80%) and temperature (FW: 10±0.23 and SW: 9.2±0.30) were monitored by ILAB daily and was never lower than recommended values (pers communication, Sindre Grimmen, ILAB, see method 2.1.2). A commercial feeding regime (8.00-16.00) had been applied in similar experimental set up in the past (Nilsen, Ebbesson and Stefansson, 2003; Nilsen et al., 2007; 2008; Stefansson et al., 2007; Ebbesson et al., 2007). It can be concluded that the current experimental setup with respect to photoperiod, temperature, flow rate, water quality, stocking densities and feed regime were within the recommended levels and that no rearing artifacts occurred, at least not as far as could be detected.

Sampling procedure was completed according to standard sample protocols in similar smoltification experiments (Nilsen, Ebbesson and Stefansson, 2003; Nilsen et al., 2007; 2008), using a temperature-controlled room (between 5-10 degrees). Blood was withdrawn from all individuals within 6 minutes of disturbance, minimizing stress induced changes in hormones and ions (Mccormick *et al.*, 2003). After length and weight measurements were taken fish were always kept on ice until tissue was dissected out and frozen directly on dry ice to minimize tissue degradation. The only exception was gill and kidney samples for RNA isolation. These samples were put in RNAlater and kept in the fridge (4^0C) over night for the RNAlater to preserve RNA, before it was frozen on -80^0C. However, RNAlater is costly and once tissue is stored with this chemical alternative analysis such as enzyme activity and protein abundance may be challenging. Therefore, separate and additional tissue samples where obtained for enzyme activity and protein verification analysis (see method, 2.1.3).

4.1.2 Nka enzyme activity measurements kidney, gills and intestine

Gills, kidney and intestinal Nka enzyme activity was measured with the micro assay method of McCormick, (1993). The Nka activity is a representation of the hydrolysis rate of the adenosine triphosphate (ATP) to adensine diphosphate (ADP) and Phosphate (P). This reaction is highly sensitive to oubain and are enzymatically coupled to the oxidation of nicotinamide adenine dinuclotide (NADH), were it can be measured directly (real time) in a temperature controlled (25^0C) plate reader at 340nm.

The Nka enzymatic reactions consists of hydrolysis of adenosin triphosphate (ATP) to adenosine diphosphate (ADP) and phosphate (P), then pyruvate kinase converts phosphoenolpyruvate and ADP to Pyruvate and ATP. Lactate dehydrogenase reduces nicotinamide adeninie dinuclotide (NADH) to nicotiamide adenine dinuclutide (NAD^+) and converts pyruvate to lactate. The different subreactions are shown below:

$$ATP + H_2O \xrightarrow{Na^+/K^+\text{-}ATPase} ADP + Pi(I)$$

$$ADP + Phosphoenolpyruvate \xrightarrow{Pyruvate\ kinase} Pyruvate + ATP(II)$$

$$Pyruvate + NADH \xrightarrow{Lactate\ dehydrogenase} Lactate + NAD^+ (III)$$

At 340nm, NADH has a significantly higher absorbance than NAD^+ and as the NADH is converted to NAD^+ the absorbance gradually decreases. Hence, production of ADP in presence of Nka enzyme results in the disappearance of NADH presented in the above reactions. The assay performance was always validated by quality tests ensuring the range of the slope (range: 17-19 mOD/nmole ADP) of

the endpoint standard curve. Issues in quality performance was often related to components of the assay mixture, hence, new chemicals were used to ensure quality and hight reproducibilty. The McCormick method is the most widely used method to measure Nka activity and fold changes are similar to those measured using the method of Zaugg (1982). The McCormicks micro assay has the advantage of requireing only 4-5 filaments, facilitating a larger number of samples to be assayed within a reasonable time.

In our laboratory, the Nka enzyme activity assay has been used almost solely on gills. Hence optimizing the protocol prior to measuring enzyme activity in kidney and intestine was required. This entailed optimizing the amount of kidney tissue necessary for normal slopes (Appendix 3a) and determine the Nka activity from the anterior to posterior areas of the kidney (figure 25). Enzyme activity measured in five areas (A, B, C, D, E) in kidneys of SW adapted salmon (n=3), eight replicates per area, showed activity patterns that confirmed the assumption that higher Nka activity in the more posterior sections of the kidney (figure 25), would reflect the increased density of nephrons more posterior in the kidney than the anterior (figure 24).

The following optimization steps were implemented in the kidney Nka enzyme activity protocol:

- When sampling kidney tissue, the fish should be bled out immediately after a lethal dose of MS-222 has been given (this will decrease the amount of blood in the kidney).
- Just roughly measure the centre of the kidney (exclude head kidney), from there you can use the area of preference based on Nka activity samples (in this case D and E) however, be consistent and use the same area each sampling (figure 24 and 25).
- Use approximately 5-8 mg of tissue as the enzyme activity curve will flat out with 15-30 mg of tissue (Appendix 3a).
- Area A reveal almost exclusively hematopoietic tissue and red blood cells (RBC) in conjunction with almost undetectable Nka levels (figure 25), thus hemopoietic tissue has low interference with Nka activity (figure 24).
- The histology overview reveals higher density of nephrons from C-E compared with A and B, thus higher probabilities of attaining nephrons rather than hemopoietic tissue (figure 24).
- D and E area have very little hematopoietic tissue and a more homogenized distribution of nephrons (figure 24) and should be selected for sampling maximizing the probability that most of the tissue consists of nephrons (figure 26A and 26B).

Ultimately, these preparations have revealed that D and E are preferable sampling areas. This demonstrates that most of the tissue consists of nephrons and that hematopoietic tissue seems to have minor effects on Nka activity. This validates that Nka activity originates from the nephron and subsequently that all samples collected for the experiments almost exclusively contained urine producing tissue (nephron bundles).

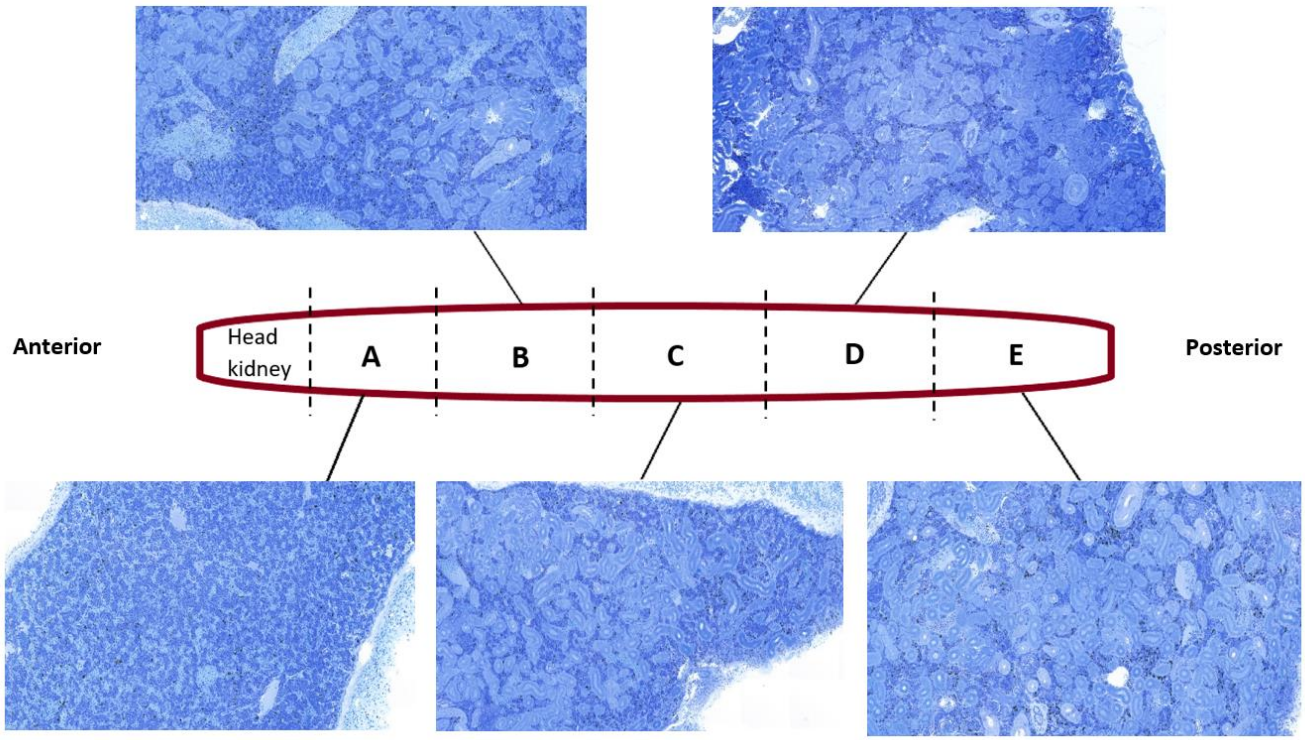

Figure 24. Overview showing the different sections of the kidney. *Kidney tissue sections stained with toluidine blue allowed visualisation of nephron density and hematopoietic tissue in the kidney (appendix 3). The head kidney was excluded for sampling and five equal sections was obtained from anterior to posterior (A to E) in the SW acclimated Atlantic salmon (n=3).*

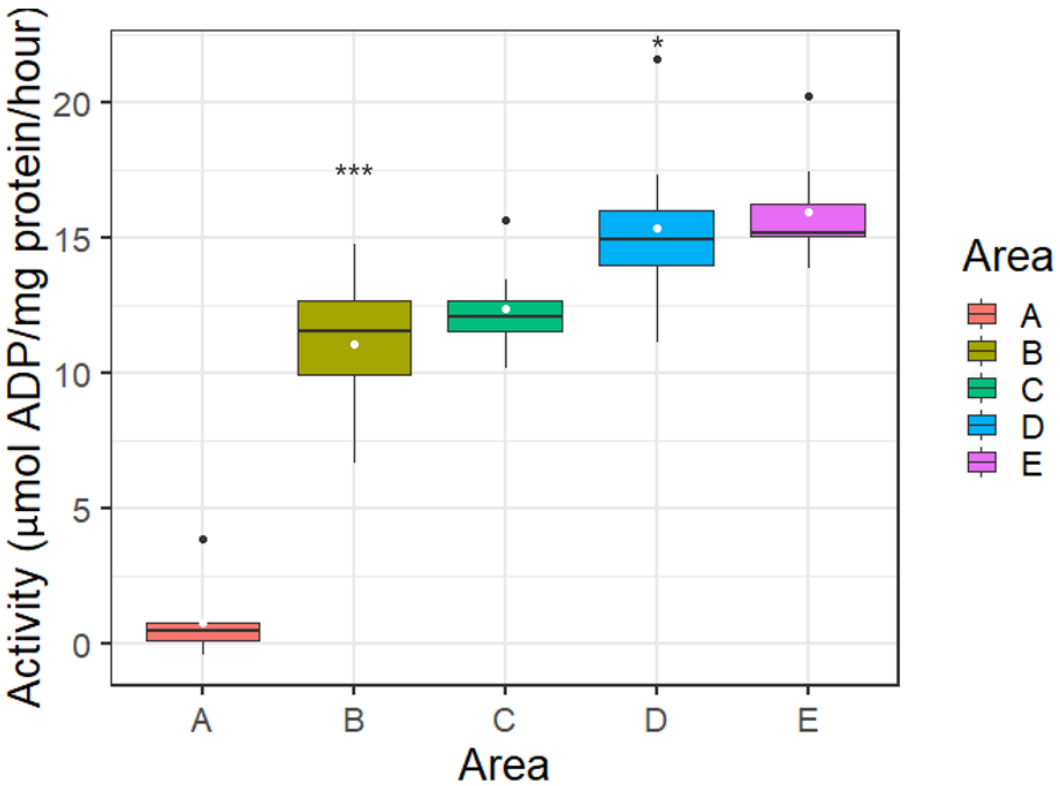

Figure 25: Distribution of Nka activity in five areas in the kidney of SW Atlantic salmon. Kidney Nka activity levels (µmoles ADP/mg protein/hour) of SW acclimated Atlantic salmon (n=3). Note that significances are related to last preceding datapoint from A to E. Asterix * p<0.05; **p<0.01 and ***p<0.001 indicates significant differences between areas in the kidney. Each datapoint is represented as mean ± standard error of mean (SEM) and n=24.

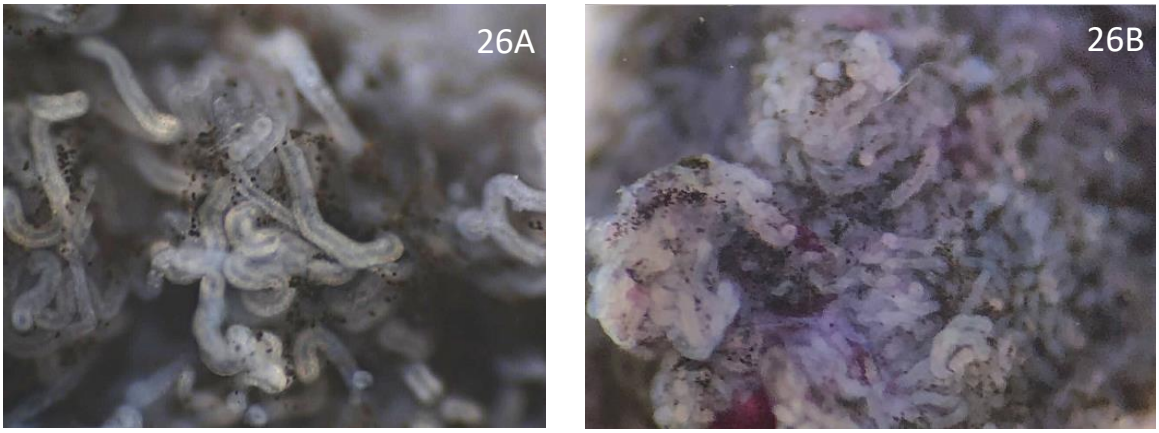

Figure 26: Nephron bundles in the kidney of SW acclimated Atlantic salmon between area D and E. Roughly 15 mg of *kidney tissue between area D and E was put in salt solution and observed in 100x and 40x magnification in the microscope, respectively.* ***26A:*** *show several nephrons almost transparent in close entanglement together with black pigmentations.* ***26B:*** *show the density and quantity of nephrons that are found in the kidney despite the very small surface area investigated. It was not possible to distinguish between parts or segments in the nephron from such observations.*

Intestinal tissue, as a result of many deteriorating enzymes, needed to be stored in a special buffer containing Complete^{TM} protease inhibitor cocktail tablet (04693124001 Roche diagnostic Oslo, Norway) and was run shortly after the tissue was sampled (see method 2.3.1). The buffer (containing inhibitory components) ensured that the deterioration process was postponed in the intestine long enough for Nka activity measurements to be performed. Most of the intestinal Nka activity was somewhat higher than previously presented in other studies (Sundell et al., 2003). The fish was non-fasted in the current experiment that may have interacted with the activity. Nevertheless, it is assumed that the high intestinal Nka activity predominantly was affiliated with changes during smoltification.

In general, gills, kidney and gut samples for Nka activity were assayed on either the sampling day or one day after sampling to minimize potential storing effect between tissues, which is of particular importance for intestinal tissue. It was reasoned best to run the assay for all tissues the same day using one large AM batch for each sampling. This allowed the smolt development, based on Nka enzyme activity in all three tissues, to be evaluated as the experiment progressed. Running everything at the end would still have resulted in the use of several AM batches as it would be difficult to run all 20 plates in 2-3 days (AM expires after 3 days).

4.1.3 Relative mRNA transcription (RNA isolation and gene expression)

The Qiasymphony (QIA) robot is a very accurate way to extract total RNA from tissue, yielding superior quality RNA with high precision and accuracy (Kruhøffer *et al.*, 2010). However, the Qiasymphony protocol does not handle extraction from all tissue types (Pers.com Associate professor Tom Ole Nilsen). Intestinal tissue (50-100 mg) was therefore isolated manually from fresh frozen tissue, including a precipitation step to ensure sufficient purity. Although, the QIA symphony robot

is extremely precise with a high output and quality for RNA extraction, it requires a much narrower range of tissue amount (20-25 mg) which can result in lower RNA yields compared to the TRI Reagent method. Unfortunately, despite the effort to obtain quality RNA from the intestine, the RNA integrity of intestinal tissue did not meet the criteria for sufficient RNA integrity, as judged by RNA Integrity Numbers (RIN).

High quality RNA is important for successful quantification of mRNA abundance in qPCR experiments (Bustin *et al.,* 2009). Hence, thorough evaluation of RNA quality, integrity and quantification was conducted to ensure accurate mRNA expression. The justification using the QuBit to determine RNA concentrations instead of the Nanodrop is that the QuBit provides very accurate quantifications of RNA. Even small RNA molecules can be detected very precisely without interference of minute levels of contaminating DNA. This is due to the dye used in the QuBit assay kit having an extremely low fluorescence until it binds to only the target RNA hence, making it more accurate than the nanodrop, spectrophotometry or PRO Nanoquant methods (Garcia-Elias *et al.*, 2017). The nanodrop, on the other hand, provides information about the potential contamination in the sample. Conversely, it is less accurate measuring RNA and unable to distinguish between RNA and DNA (Kapp *et al.*, 2015). Accurate input of RNA concentration in cDNA synthesis is crucial for precise qPCR analysis, thus, the potential inaccuracy of the NanoDrop justified using the QuBit. In the present experiment a total of 13 gill samples were excluded from the cDNA synthesis due to low RNA concentrations required to run qPCR on the sulfate transporters. These samples were spread out in different groups and did not have a substantial impact on the number of individuals (N) in each group except in the smolt group after four weeks/260 d.d (n=6).

In general, RNA is in a constant degradation process, thus the quality of RNA in each experiment can be variable and should be monitored. The Agilent 2100 bioanalyzer (RNA 6000 Nano LabChip kit) was an effective way to obtain accurate information about total RNA integrity in our samples. The Agilent provide superior results compared to use of traditional agarose gels (Kuschel Meike, 2000). It permits a large number of samples to be processed quickly, requires only small RNA volumes, and most importantly is far more independent from sample contaminants that other methods like ribogreen Analysis and UV spectrophotometry (Imbeaud, 2005; Lightfoot, 2016). The Agilent 2100 bioanalyzer provides the RNA integrity number (RIN) ranging from 1 to 10, with RIN value of 1 being low integrity and RIN value of 10 being the best integrity (Schroeder *et al.*, 2006). Consequently, samples having RIN values below 8.6 was excluded and not used for real-time qPCR. Previous experience in the lab have yielded RIN values above 8.6 for both gills and kidney. Testing 12 random samples (120 samples in total) for each tissue (gills and kidney) largely confirmed these notions as all 12 samples were well above the RIN values of 8.6. It is assumed that 12 samples are an adequate representative

and no more tests were performed. Conversely, 12 out of 240 random samples from the intestine had many RIN values below the threshold and needed more testing. After more extensive testing on the intestine samples (120/240) a substantial amount of the samples was still well below the threshold and further cDNA synthesis and qPCR analysis could not be performed. However, gills and kidney samples were used for further qPCR analysis.

There are several important aspects to consider before initiating qPCR experiments. Random or systemic error is predominantly the cause of variability and reproducibility pitfalls in qPCR experiments (Taylor *et al.*, 2019). The present study strived to follow as many criteria as possible from the MIQE Guidelines (Bustin *et al.*, 2009) ensuring the best possible consistent, accurate and reproducible qPCR results (Bustin *et al.*, 2013). To ensure and optimize the qPCR experiment several tests were performed to ensure primer quality and efficiency based on theoretical (1) and practical (2) tests before running the assay:

1. Theoretical criteria designing primers:
    - All of the criteria were thoroughly checked and tested theoretically to design quality primer sets (See method 2.3.2.2, Appendix 5a)
    - The design and theoretical testing were performed in collaboration with supervisors to validate and ensure all steps where conducted accordingly.

2. Practical tests performed verifying primer quality as follows:
    - a dilution curve was performed on all tissues to calculate each primers efficiency, both for target genes and reference genes (Appendix 5b). This was important for ensuring that primers had sufficient efficiency which was used in final calculations of mRNA abundance results.
    - The primers were validated by a tissue distribution, testing tissue specificity and ensuring that all primers were working optimally (Appendix 5c).
    - It was decided to analyse sulfate transporters displaying Ct values below 30, as genes expressed at Ct values higher than 30 are difficult to accurately measure with sufficient precision (Appendix 5c) (Forootan *et al.*, 2017).
    - Assay performance was considered adequate when efficiency was between 80 and 120 % and specificity assumed when a single melting temperature of PCR products for each primer set was obtained (Appendix 5b). All assays passed these quality criteria, except the *slc26a1bX3* in the kidney, which displayed a PCR efficiency of 79 %. The reasons for lower efficiency are difficult to identify but could be tissue specific differences in performance. The *slc26a1bX3* is a splice variant of the *slc26a1b* gene. It is widely accepted that accuracy of splice variant

sequences may be variable. One may speculate that small mismatches in the sequence could affect the primer binding properties, and thus efficiency.

- Three endogenous reference genes (housekeeping genes) were tested using several public available software programs found in RefFinder (Xie *et al.*, 2012): BestKeeper (Pfaffl *et al.*, 2004), Normfinder (Andersen, Jensen and Falck Ørntoft, 2004), Genorm (Vandesompele *et al.*, 2002), the comparative delta-Ct method (Silver *et al.*, 2006). These methods assessed the stability of each gene, identifying the best suitable candidate as an endogenous reference. Ef1a was selected as the best candidate based on all tests (Appendix 5d).

4.1.4 Statistical analysis

The experiment had properties were the use of a Two-way ANOVA analysis was reasoned to be the best fit for the dataset. Firstly, a continuous dependent variable could be measured on a scale that could be subdivided using increments. In addition, there were two categorical independent variables (predictor variable), in this case, group (parr and smolt) and samplings (1-7). Several dependent variables (response variable) such as length, weight, condition factor, Nka enzyme activity and mRNA abundance were analyzed for multiple samplings over time in both groups. In general, an ANOVA test permits comparing multiple groups following three main assumptions: 1) the distribution of the dependent variable should be continuous and approximately normal distributed, 2) independence of samples, and 3) homogeneity of variance. Different diagnostics were run to validate the models graphically to assess normality (q-q plots), influential outliers (Residuals vs Leverage with Cook`s distance) and non-linear patterns (Residuals vs fitted plots) or homogeneity of variance (scale-location plots). The homogeneity of variance may not always need to be met (p-value > 0.05) as the variance in many cases are affected by the model structure and overdispersion in the dataset (*6.1 - Introduction to Generalized Linear Models | STAT 504*). A generalized linear model (glm) for non-normal response variables was applied in cases were the data did not display a normal distribution. The data had a typical gamma or gaussian distribution, hence these families of glm were used in cases were the Two-Way ANOVA could not be applied.

In biological studies variability can be large and there are many different phenotypes controlling individual performance during smoltification and SW transfer. Nevertheless, the significant level of <0.05 has been used in many biological studies and provides an adequate method for distinguish significant differences between groups of interests. In the second sampling (260 d.d) only six fish (n=6, smolt group) could be used for relative mRNA abundance for gills, which statistically is considered to result in low statistical power. However, since timepoints before and after in the same group both had higher sampling numbers (7-10) and results (figure 23) did not show large variations or random fluctuations from expected trends it was considered as an adequate statistical result.

4.2 Discussion results

4.2.1 Weight, length, condition factor and smolt index

The overall findings of a steady increase in weight, length, smolt index and a clear decrease in condition factor in smolts is consistent with the common attributes of smolts, generally growing faster and developing a much slimmer silvery body compared to a parr (Stefansson *et al.*, 1991; Thorstad *et al.*, 2012). Not surprisingly, growth (judged as weight and length gain) was lower in parr than in smolts, despite rearing conditions such as temperature, fish density and feeding regime being kept similar for both groups. The higher growth in the smolt group is probably due to the well-established growth promoting effects of constant light in salmonids (Sigholt, Åsgård and Staurnes, 1998; Duncan, Mitchell and Bromage, 1999; Stefansson *et al.*, 2008). The dramatic larger growth in post-smolts during the seawater phase was probably further enhanced by the classic increased scope for growth among salmon in seawater (Mccormick *et al.*, 1998; Handeland *et al.*, 2003).

The smolt index is, to some extent, used by the industry as an indicator of changes in morphological characteristics (see method 2.2.2) of smolts. In the present study, the smolt index gradually increased during the smoltification, reaching a max score of 5 at peak smoltification, 45 days post initiation of continuous light in the smolt group (figure 11). Our results largely coincide with the typical and commonly known morphological traits of Atlantic salmon parr and smolt (Hoar, 1988; Høgåsen, 1998; Thorstad *et al.*, 2012). Despite not being observed in our study, it should be mentioned that modern production strategies ((i.e.) growing ever larger smolts) have questioned the validity of applying smolt index as a reliable smolt criteria. Increasing reports from producers suggest that juveniles develops increased silvering independent of photoperiod stimulated smoltification. This is particularly evident when juveniles above 100 grams are reared under constant light and high temperatures, 12-16°C (Pers. communication, Tom Ole Nilsen). Hence, use of smolt index should be used with caution in large smolt production. In summary, all morphological parameters largely reflected a classic overall condition and development of Atlantic salmon smolt.

4.2.2 Nka enzyme activity in the gills, intestine and kidney

The Na^+/K^+- ATPase (NKA) pump is not only crucial in regulation of intracellular ionic gradients used for cellular homeostasis, but also as a secondary transport of many compounds (Skou and Esmann, 1992). In the teleost gills, intestine and kidney the Nka enzyme generates electrochemical gradients, enabling the reciprocal switch of ion and water transport during migrations between FW and SW (Marshall, 2002; Dantzler, 2003; Grosell, 2010). In the current study Nka activity levels were monitored during smoltification and subsequently in SW for gills, intestine and kidney. Before discussing current findings remember that the Nka enzyme activity observed are from in vitro

measurements of the enzyme potential. This potential can effectively be measured during smolt development, though the Nka is not fully operational before SW. If the enzyme potential would be activated in FW it would result in major difficulties in maintaining overall homeostasis.

Gills have been extensively studied and are the key organ to monitor through smolt development in determining Atlantic salmons physiological capacity to remove salts in SW (Björnsson and Bradley, 2007; Nilsen *et al.*, 2007; Hiroi and McCormick, 2012; McCormick *et al.*, 2013). Similarly, in the intestine the absorption of water and monovalent ions through smoltification and SW transfer have been well studied (Veillette, Sundell and Specker, 1995; Sundell *et al.*, 2003; Sundell and Sundh, 2012; Sundh *et al.*, 2014). The Nka activity in the kidney and other important transport pathways have been studied to some extent in euryhaline species (Madsen *et al.*, 1994; Perry *et al.*, 2003; Lin, Tsai and Lee, 2004; Nebel *et al.*, 2005; Tang, Lai and Lee, 2012), though almost none in Atlantic salmon (McCartney, 1976; McCormick, Moyes and Ballantyne, 1989).

4.2.2.1 Nka activity in gills, intestine and kidney during smoltification (FW)

Surprisingly, few studies have addressed the concurrent changes in the gills, intestine and kidney. The present study demonstrated increased Nka enzyme activity in gills, intestine and kidney during smoltification, consistent with the notion of preparatory change to secure sufficient osmoregulation when entering the marine environment (Mccormick *et al.*, 1998). Due to its key role in development of SW tolerance, gill Nka activity or gene expression of central subunit isoforms are used by industry to determine smolt quality, yet there is increasing concern that the traditional use of the gills as reporter organ for smolt development is becoming less predictable as a biomarker of seawater tolerance. Gill enzyme activity measurements in large industry smolts often display elevated activity levels, despite fish being produced under light conditions that should not induce smoltification. Such de-coupled changes in smolt related traits occurred in the control (parr) group kept in a 12D/12L light condition, with increased gill Nka activity in the last sampling of the study (figure 12), while no obvious morphological changes were observed (figure 11). This stresses the importance of more comprehensive knowledge of smolt development in intensive production, including all three organs gills, intestine and kidney.

The elevated Nka activity levels in the current study corresponded with development in the peak smolt window in line with previous findings (McCormick, Moyes and Ballantyne, 1989; Nilsen, Ebbesson and Stefansson, 2003; Nilsen *et al.*, 2007; McCormick *et al.*, 2013). The increasing gill Nka activity were found to be satisfactory already after 26 days (figure 12), while the intestine and kidney reach acceptable levels first after 35 days (figure 13, 14 and 15). Assuming that intestinal and kidney Nka activity reaches sufficient activity levels later than in gills, one may infer that to transfer smolts to

SW based on the initial increase in gill enzyme activity is premature, thus being a contributing factor to suboptimal SW performance.

The peak Nka activity in both proximal and distal segments of the intestinal tract in the present study resonates well with previous findings were a similar increase was observed during smoltification. Further, argued to be vital for ion-coupled water transport and fluid absorption thus, preparing smolts for the marine environment (Veillette, Sundell and Specker, 1995; Sundell *et al.*, 2003; Sundell and Sundh, 2012). The current study reports relatively high Nka enzyme activity levels compared to other comparable studies. Since higher Nka levels also were observed in the parr group compared to that reported by others it is likely a consequence of using non-fasting fish in the present study. It is commonly known that nutrient uptake from the formulated feed (contains salts) can interfere and stimulate transport activity in the intestine (Bucking and Wood, 2006, 2007; Bucking, Wood and Grosell, 2013). The high intestinal enzyme activity observed for this study compared to other studies using fasted fish are therefore not surprising and whether to withheld feed or not prior to sampling needs more focus in future studies.

The present study is one of only two studies that have addressed preparatory changes in kidney Nka activity during smoltification. An increase in kidney Nka enzyme activity was observed during smolting in peak smolts (McCartney, 1976), similar to the present study. The peak smolt increase in kidney Nka activity likely reflects a preparation to meet enhanced requirements for active ion reabsorption. Elevated Nka activity is argued to promote and enable reabsorption of roughly 95 % NaCl, minimizing salt loss in FW fish (Perry *et al.*, 2003; Tang *et al.*, 2010). In contrast to other FW species, Atlantic salmon may even require more reabsorption of salts to support the increased energy demand during the peak smolt window which partly can explain the increase. The high reabsorption of NaCl is only possible as distal and collecting ducts have a very low permeability, which enables concurrent salt reabsorption and obstruction of water, ultimately producing the dilute urine (Hickman and Trump, 1969; Marshall and Grosell, 2006). In the current study urine collection was not possible and since almost no studies address kidney function during smoltification, the following conjectures are partly stipulated and based on studies addressing urine composition, UFR and GFR as well as measurements of reabsorption or secretion of ions in FW environments (Hickman and Trump, 1969; Eddy and Talbot, 1985; Talbot *et al.*, 1989; Curtis James and Wood, 1991; Talbot, Stagg and Eddy, 1992; Wood and Patrick, 1994; Burgess *et al.*, 2000). These studies largely conclude that FW fish generally have much higher GFR and UFR compared to SW fish, producing very dilute urine with only low concentrations of Na^+ and Cl^- (10-20 mM). It is interesting to note that a previous study on Atlantic salmon demonstrates an increase in UFR during the peak smolt period (Eddy and Talbot, 1985), indicating an overall increased UFR and urine production in smolts. Combined with the

elevated Nka activity in the current study, this suggests a predominant requirement for smolt to increase overall activity in the nephron tubules during the demanding smolt period. The parr group that was in FW through the entire experiment showed substantially lower Nka activity when compared to smolts. Despite showing lower Nka enzyme activity then the smolt, the parr group still had relatively high Nka activity in the kidney. This points towards the importance of the Nka enzyme as an indirect driver of reabsorbing ions such as Na^+ and Cl^- in FW acclimated parr. In addition, it has been reported that migrating adult Atlantic salmon substantially increase UFR and GFR from SW to FW, reabsorbing about 80 % of Na^+ in FW compared to only 23 % in SW (Talbot *et al.*, 1989). This indicates a general requirement for Atlantic salmon to have a relatively high tubular activity in FW parr and adults alike, that may partly reflect the relatively high Nka enzyme levels observed in the current study. Nonetheless, it is important to highlight the delayed and different onset of the kidney compared to the gills in increasing the Nka activity in smolts. This suggests that the increased use of intensive smolt protocols and abrupt transfer to SW can be detrimental to the overall osmoregulatory capacity if Atlantic salmon prematurely are transferred to SW.

4.2.2.2 Nka enzyme activity in gills, intestine and kidney in juvenile Atlantic salmon after SW transfer

Before discussing the role osmoregulatory organs have in SW, it is important to point out that the gills, intestine and kidney generally have higher Nka activity in SW environments than FW environments to counteract high salinity and to prevent dehydration (Evans, Piermarini and Potts, 1999; Perry *et al.*, 2003; Beyenbach, 2004; Grosell, 2010).

The present study supports the large body of evidence demonstrating increased gill Nka activity following SW transfer in Atlantic salmon, ensuring efficient active secretion of excess Na^+ and Cl^- in SW (McCormick, Moyes and Ballantyne, 1989; Pelis, Zydlewski and McCormick, 2001; Nilsen *et al.*, 2007; Stefansson *et al.*, 2008; Hiroi and McCormick, 2012; McCormick *et al.*, 2013; Einarsdóttir *et al.*, 2014). The elevated intestinal Nka activity in smolts supports the importance of the basolateral Nka pump to drive the ion-coupled water uptake to counter water loss once they enter SW (Sundell *et al.*, 2003; Grosell, 2010; Sundell and Sundh, 2012). Nka activity remained high in both proximal and distal intestine of the smolt group after 1 day in SW (figure 13 and 14) and probably reflects the necessity for active ion and water absorption in the short-term SW phase. This is to be expected, considering fish often start drinking relatively fast after SW transfer to replace water (Whittamore, 2012). Additionally, the use of the Ussing chamber techniques have enabled direct real time measurements of paracellular and transcellular transport by measuring electrical characteristics of the epithelium (TER) (Sundell *et al.*, 2003). These studies have elucidated the paracellular permeability features of the intestinal tract, were a reduced paracellular permeability (increased TER) in both the proximal and distal segment was observed in Atlantic salmon acclimation to SW (Sundell and Sundh,

2012). It appears that a high Nka enzyme activity, together with a higher TER (reduced paracellular permeability) are vital for SW acclimation and ion-coupled water absorption in the intestine. These aspects are a challenging endeavor, involving a range of transporter pathways and are explained in detail in the seminal review of Sundell and Sundh (2012). This was further elucidated during smoltification and after SW transfer by the fellow master student Sigval Myren.

Many studies have provided evidence of the importance of the role of the Nka enzyme on transport routes in gills and intestine (Marshall, 2002; Marshall and Grosell, 2006; Nilsen *et al.*, 2007; Cutler and Cramb, 2008; Grosell, 2010; Hwang, Lee and Lin, 2011; Whittamore, 2012; Hiroi and McCormick, 2012; Sundell and Sundh, 2012; McCormick *et al.*, 2013; Sundh *et al.*, 2014; Esbaugh and Cutler, 2016). Though it is intriguing that a different Nka activity pattern was observed in the kidney, particularly the substantially rapid decrease following the two first days in SW (figure 15). Previously, very few studies have looked at kidney Nka activity of euryhaline fish (compared to intestine and gills). Even fewer studies have investigated this during smoltification in Atlantic salmon and to the extent that has been done for this study, with multiple samplings at several representative timepoints in the smoltification and after SW transfer. Therefore, a more comprehensive section has been dedicated to these findings in the kidney.

4.2.2.3 Decreased NKA activity in the kidney and its relevance to ion and water transport in short-term SW transfer

Interestingly, a substantial short-term decrease in kidney Nka activity in smolts transferred to full strength SW resonates well with previous predictions, hypothesizing a decrease in Nka activity as response to SW (McCartney, 1976). Conversely, no change in Nka activity was observed in Atlantic salmon gradually exposed to salinities of 10 ppt or 30 ppt over the course of two weeks (McCormick, Moyes and Ballantyne, 1989). However, kidney Nka enzyme activity have been shown to be sensitive and responsive to changes in environmental salinity, with increased Nka activity being linked to a heightened requirement for secretion in SW (Madsen *et al.*, 1994; Herrera *et al.*, 2009). Other reports show no change in Nka activity after SW transfer (Sangiao-Alvarellos *et al.*, 2005; Arjona *et al.*, 2007), suggesting that, at least some species, do not display altered activity in the kidney upon SW transfer. The current findings contradict the Nka activity patterns presented in response to salinity changes in other species. The substantial decrease in kidney Nka activity in Atlantic salmon may be a result of the need to reduce water loss through the kidney in short-term SW. The teleost kidney handles NaCl by secretion in the proximal tubules and reabsorption in the distal tubule and collecting duct in fish (Nishimura, Imai and Ogawa, 1983; Beyenbach, 1986, 1995; Kato *et al.*, 2011), yet it was not possible to determine if reduction in Nka activity originated from one or more segments of the

nephron in present study. Since very few studies have addressed these aspects in Atlantic salmon the following conjectures are partly based on other euryhaline species.

To prevent water loss and dehydration SW fish rapidly shut down the activity in the nephrons during the acute SW phase (Hickman, 1968b; Brown *et al.*, 1978). Moreover, it has been suggested that GFR significantly reduces and only a limited portion (~5%) of nephron tubules filter in SW (Schmidt-Nielsen and Renfro, 1975; Brown *et al.*, 1978). Maybe the decrease in GFR and UFR are not rapid or adequate enough for the acute SW challenge, or able to deal with sudden dehydration and major water loss. The reduction in filtering nephrons commonly found in previous studies could be correlated with a decrease in tubular Nka activity observed in this study. However, it has been demonstrated that GFR reaches an all-time low much later (10 days after SW) in Rainbow trout (Holmes, 1963) and similarly low GFR and UFR have been shown in Atlantic salmon (Talbot *et al.*, 1989; Talbot, Stagg and Eddy, 1992). The dramatic transient short-term drop in Nka activity in the current study indicates a certain requirement for reducing the enzyme activity in nephron tubules, perhaps coupled with a reduction in GFR and UFR. It could be suggested that Nka activity, at least in the kidney, requires a longer acclimation period in SW to regain high levels observed in the peak smolt. These observations indicate a substantial physiological modulation of Nka activity within a couple of days. A decrease in such Nka enzyme activity may be linked to FXYD protein families and phosphorylation properties, as emerging evidence have demonstrated the role these may have in modulating Nka activity towards SW acclimation (Tang, Lai and Lee, 2012; Yang *et al.*, 2016). These aspects should be addressed in future studies of Atlantic salmon that hopefully can explain the rapid modulation and reduction of Nka enzyme activity observed in the current study.

It can also be hypothesised that gills are the chief regulator for salts and intestine for ion-coupled water absorption reflected by the Nka enzyme activity. Fish unwillingly lose water through the gills and must drink, whereas the kidney attempts to retain as much water as possible by reducing overall kidney activity reflected in reduced GFR and UFR (Talbot *et al.*, 1989; Talbot, Stagg and Eddy, 1992). Since both gills and intestine display elevated Nka enzyme activity after SW transfer (figure 12, 13 and 14) one could argue that this should be sufficient to handle removal of monovalent ions (gills), and active absorption of water (intestine). However, the kidney also contributes to avoid dehydration, were a reduction in tubular activity clearly is necessary, reflected by a reduction in UFR and GFR (previous findings) and the reduction of Nka enzyme activity (current findings). In fact, the need for conserving water in the kidney might be greater than the need for secreting monovalent ions while in short-term SW, requiring lower Nka activity and overall transport activity. This argument is further based on the notion that the predominant role of the kidney is to remove divalent ions in SW (Kato *et al.*, 2009; Islam *et al.*, 2011, 2013) while removal of monovalent ions is secondary (approx.

5 % NaCl, Teranishi and Kaneko, 2010). In addition, divalent ions quite rapidly can become toxic if not removed efficiently and the kidney almost exclusively handles these ions (see figure 4B, introduction). Given these assumptions it would be surprising if the Nka enzyme activity reduction derives from divalent ion transport which are vital to be secrete into the tubule lumen and subsequently be excreted through the urine in SW.

These assumptions are also based on the apparent impermeable features of the distal and collecting tubule of FW Atlantic salmon (Madsen, Engelund and Cutler, 2015). Such permeability features are not necessarily rapid enough during abrupt transfer to SW were these segments largely should be permeable to reduce water loss. However, to produce an isotonic urine, fish need to control water movement and increase water permeability in the distal segment and collecting duct, which is in turn essential to produce minute volumes of concentrated urine rich in SO_4^{2-} and Mg^{2+} in SW (Hickman and Trump, 1969; Beyenbach, 2004; Watanabe and Takei, 2011a; Islam *et al.*, 2013). The reduction in Nka activity in the nephron may either be a response to reducing secretion of monovalent ions in proximal segment I, or inadequate permeability features in the distal segment and collecting tubule that would force an overall reduced tubular activity. This would ultimately lower the loss of water through the kidney since less plasma is filtrated (lower GFR), producing less urine (lower UFR) and less secretion of monovalent ions would reduce the potential water that automatically would follow (see figure 4B, introduction). Lastly, inadequate permeability features in distal and collecting segments would infer transient transport of Na^+ and Cl^- without precious water following (see figure 4B, introduction). Therefore, a reduction in Nka activity here would at least minimize the extra reabsorption of salts since water at the time are unable to follow. Also, Nka activity has largely been linked to transport of SO_4^{2-} (discussed in further detail in 4.2.3). Without further investigation it is not possible to give any certain answers on where specifically in the nephron this Nka enzyme activity reduction is required or the overall effect, if any, on the osmoregulatory capacity of the fish.

4.2.2.4 Future perspective on NKA transport and related transport pathways in the Atlantic salmon kidney

The role and regulation of Na^+, K^+ - ATPase (NKA) transporter in euryhaline species is poorly understood and even more so during smoltification. How Atlantic salmon effectively alter water permeability in conjunction with effective reabsorption of NaCl and secretion of divalent ions to produce minute volumes of an isotonic urine rich in Mg^{2+}, SO_4^{2-} and Ca^{2+}, remains elusive. Further evidence can be drawn from emerging knowledge from other euryhaline species. Localization of Nka isoforms have been found mostly in proximal and distal segments of the nephron and linked to essentially all types of transport through the nephron tubules. This includes; transcellular transport of water (Engelund and Madsen, 2011, 2015), monovalent ions (Na^+, Cl^-) (Lin, Tsai and Lee, 2004; Cutler and Cramb, 2008; Katoh *et al.*, 2008; Teranishi and Kaneko, 2010; Kato *et al.*, 2011) and

divalent ions (SO_4^{2-}, Mg^{2+} and Ca^{2+}) (Kato *et al.*, 2009; Islam *et al.*, 2011, 2013, 2014; Watanabe and Takei, 2011a, 2011b; Kato and Watanabe, 2016). It is rather strange that studies on NKA, NKCC or CFTR transporters are almost completely absent in the kidney of Atlantic salmon, considering the substantial research conducted on these transporters in the gills and intestine. Furthermore, tight junctions (claudins) and AQP related to permeability features and divalent ion transporters for SO_4^{2-} ions (addressed in the current study), Mg^{2+} ions (CNNM, SLC41, TRPM) and Ca^{2+} ions (NCX, TRPM, PMCA) are also vital aspects to address in the kidney. Contemplating the very intrinsic and complicated aspects of renal regulation in FW and SW acclimation, future studies in Atlantic salmon should focus on further identification of all the above transport pathways. At the center of all this transport is the NKA transporter and the current study highlights its importance in future research when investigating a new transport pathway. Linking the kidney`s role in osmoregulation with the gills and intestine during smolt development and in SW tolerance will be crucial for increasing our overall understanding of Atlantic salmon`s osmoregulation capabilities.

4.2.3 SO_4^{2-} transport in Atlantic salmon

Seawater (SW) environments have high concentrations of SO_4^{2-} that marine teleost needs to excrete from the kidney to prevent hypersulfatemia. This is especially demanding since the plasma levels in teleosts generally are strictly regulated within 0.2-1 mM and SO_4^{2-} concentrations are 30-50 fold higher than plasma levels in SW (Edwards and Marshall, 2012). In fact, as much as 97 % of SO_4^{2-} excretion is accredited to the kidney (Watanabe and Takei, 2012). To better understand SO_4^{2-} regulation in Atlantic salmon its transporters were first discovered and characterized in the salmon genome by theoretical phylogenetic analysis and a tissue distribution determining mRNA expression. Thereafter, mRNA abundance measurements were determined during smoltification and SW transfer by mRNA abundance measurements.

4.2.3.1 Discovering and characterizing salmon specific SO_4^{2-} transporters in Atlantic salmon

In the current study, seven putative SO_4^{2-} transporters were identified in Atlantic salmon using BLAST search, multiple sequence alignment tools (Clustal) and phylogenetic tree building (PhyML) for putative protein sequences. Annotation was supported by synteny analysis using Ensembl genome browser annotations, via the Genomicus platform (Nguyen *et al.*, 2018), complemented with salmon sequences from the NCBI Genbank when not available in Ensembl. The Solute Carrier Family 13 Member 1 (Slc13a1) was already annotated in the salmon genome and additional searches using putative nucleotide and protein sequences from both salmon and eel (Nakada *et al.*, 2005) did not reveal any other salmon-specific paralogues (figure 16). It is possible that the salmon rediploidization process currently occurring in salmonids (Lien *et al.*, 2016) may have led to a diploid state for the

Slc13a1. One cannot rule out the possibility that database searches missed a potential Slc13a1 paralogue in salmon, though approximately 10-20 % of the salmon genome still retain residual tetrasomy (Allendorf *et al.*, 2015; Lien *et al.*, 2016). A key feature of rediploidization in salmonids suggests retention of about half of the duplicated gene pairs from the salmonid-specific 4RWGD (Lien et al., 2016), leading to a wider repertoire of gene families than other teleosts. Our results indicate that the salmon may have retained novel paralogues for both the Solute Carrier Family 26 Member 6 (Slc26a6a) and Member 1 (Slc26a1). Each of the salmon Slc26a1 paralogues, annotated and termed Slc26a1a and Slc26a1b, grouped closely with their Rainbow trout counterparts and the single Northern pike (*Esox lucius*) Slc26a1 (figure 17). The salmon Slc26a1b sequence was originally annotated as Slc26a2 in the salmon genome database. Synteny analysis using Genomicus platform (Nguyenet al., 2018), supports that our re-annotation of the Slc26a1b is correct. The Solute Carrier Family 26 Member 6 orthologue has several teleost specific paralogues, annotated as Slc26a6a, Slc26a6b and Slc26a6c (Kato *et al.*, 2009). We also identified a single salmon Slc26a6b and Slc26a6c sequences and two salmon Slc26a6a paralogues, annotated as Slc26a6a1 and Slc26a6a2 (figure 18). Even though the phylogenetic and synteny approach applied in the present study makes us confident that the sequences are correctly annotated, we acknowledge that bioinformatic sequence assembly is challenging (Houston and Macqueen, 2019). Genomic duplication events are postulated to be an important mechanism generating phenotypic diversity (Kondrashov *et al.*, 2002; Kellogg, 2003). There are currently three main theories that exists concerning the fate of paralogous genes; 1) the dosage balance model, 2) sub-functionalization, and 3) neo-functionalization (Warren *et al.*, 2014). During diploidisation non-functional gene duplicates are often lost. According to Lien and co-authors (2016) Atlantic salmon display more instances of neo-functionalization than sub-functionalization. However, discussions of SO_4^{2-} transporters sequences in an evolutionary context are beyond the scope of this thesis, thus no more conjectures will be formulated.

All putative SO_4^{2-} transporters identified in the present study were expressed as mRNAs in FW and SW acclimated salmon (Appendix 5c). The *slc13a1* was only expressed in intestine in the current study more elevated in FW compared to SW (Appendix 5c). Conversely the *slc13a1* has only been found in the kidney of FW eel (Nakada et al., 2005). However, the higher expression in FW intestine may suggest a role of this transporter in the intestine of FW acclimated Atlantic salmon. The *slc26a6a1* was highly expressed in kidney of SW acclimated salmon, while in the intestine it was found to be relatively equally expressed in both FW and SW (Appendix 5c). Expression profiles in both kidney and intestine agree with that found in pufferfish and eel (Kato *et al.*, 2009; Watanabe and Takei, 2011b). Though, only determined in the intestinal tract of eel, demonstrating equal expression in the rectum and a more prevalent expression in SW compared to FW in the proximal and distal

intestine. In the current study no differentiation was made between proximal and distal intestine and they were pooled for the tissue distribution. In contrast, the *slc26a6a2* was only found in gills in FW fish (Appendix 5c), also the first potential SO_4^{2-} transporter discovered in the fish gill. The *slc26a6b* seems to be relatively equally expressed in both FW and SW kidney but seems to be higher expressed in FW in the intestine (Appendix 5c). However, the slc26a6c was only detected in the kidney equally expressed in both FW and SW. Furthermore, the *slc26a1a* and *slc26a1b* paralogues were found to be relatively equally expressed in both FW and SW acclimated salmon (Appendix 5c). The expression pattern in *slc26a6b, slc26a6c* and *slc26a1* seem to be similar to what has previously been reported in eel and pufferfish (Nakada *et al.*, 2005; Kato *et al.*, 2009; Watanabe and Takei, 2011a). However, it should be highlighted that the *slc26a1a* paralog seems to show a trend towards a higher expression in SW compared to FW, which is not observed in the *slc26a1b* (Appendix 5c).

Several predicted splice variants were identified in the NCBI gene models, and their expression patterns were examined in the present study. Alternative splicing, or differential splicing, is a critical regulatory process that permits a single gene to code for multiple proteins in biological systems (Kim, Goren and Ast, 2008; Wang *et al.*, 2015). Alternative splicing variants have been found in fish species like fugu (*Takifugu rubripes*), medaka (*Oryzias latipes*) and zebra fish (*Danio rerio*) and proposed to be important for the understanding of the functional and evolutionary mechanism of the genomes in teleost fish (Lu *et al.*, 2010). It has been proposed that alternative splice variants may also be activated in the process of adapting to altered salinities or otherwise events that can be stressful for the fish (Kijewska, Malachowicz and Wenne, 2018). However, the many different types of splicing are extremely complex; hence, discussion of splice mechanisms are considered beyond the scope of this study. The following discussion will pertain more to tissue expression patterns of SO_4^{2-} transporter splice variants in salmon. One of the two identified *slc26a1b* splice variants displayed tissue specific expression, with *slc26a1bX3* only being expressed in the kidney, while the splice variant *slc26a1bX1* was present in both kidney and intestine (Appendix 5c). Both splice variants *slc26a1bX1* and *slc26a1bX3* showed similar expression patterns in FW and SW acclimated salmon (Appendix 5c). However, the *slc26a1bX2* showed no expression for either of the tissues selected in the current study (not included in the Appendix). There was also splice variants for the slc26a6a2 (*slc26a6a2X1* and *X2*) in which only the *X1* was expressed (gills) while the other *X2* was not expressed in either of the tissues for the tissue distribution (not included in Appendix). It was therefore reasoned to use the general *slc26a6a2* primer set (Appendix 5a) during the remainder of the experiment as it showed no difference in expression pattern then the *slc26a6a2X1* variant. However, one cannot rule out that the splice variant *slc26a6a2X2* and *slc26a1bX2* is specific to other tissues not investigated in the current study. Investigations of splice variants are a complicated endeavour addressed in genetic studies

which are considered a different field of biology. Therefore, no further conjecture is formulated as it goes beyond the scope of the study. However, putative splice variants were discovered in the current study for both *slc26a6a2* (*slc26a6a2X1* and *slc26a6a2X2*) and *slc26a1b* (*slc26a1bX1*, *slc26a1bX2*, *slc26a1bX3*) which interestingly are both putative paralogs. These observations only begin to explain the very complicated regulation and plasticity of the Atlantic salmon on genomic level. Further study should try to further analysis and better understand these aspects.

4.2.3.2 Cellular transport pathways of SO_4^{2-} in the kidney of teleost fish related to relative mRNA abundance of SO_4^{2-} transporters in Atlantic salmon

Salmon specific Slc26a6 transporters in the kidney (Slc26a6a, Slc26a6b and slc26a6c)

In mammals the SLC26A6 transporter has been localized to apical membranes of proximal tubules in the kidney and proposed to exchange numerous anions: oxalate/SO_4^{2-}, Cl^-/formate, Cl^-/oxalate, oxalate/formate, oxalate/oxalate, Cl^-/$HCO3^-$ and Cl^-/OH^- (Markovich, 2001). In the fish kidney, the prevailing hypothesis has largely been its apparent ability of SO_4^{2-} transport to be directed via a Cl^- gradient (Renfro and Pritchard, 1983; Renfro *et al.*, 1999), which does not occur in the mammalian kidney (Burckhardt and Burckhardt, 2003).

Electrophysical studies of teleost sequences in Xenopus oocytes revealed a 50-200 fold higher electrogenic transport by the Slc26a6a than by the Slc26a6b paralogue, with Slc26a6a displaying the highest SO_4^{2-} transport activity among the Slc26a6 family (Kato *et al.*, 2009; Watanabe and Takei, 2011b). These electrophysical studies largely suggest that a negative cytosolic charge powered by Nka enzyme yields low cytoplasmic Cl^- concentrations via chloride channels, aided by the basolateral SO_4^{2-}/$HCO3^-$ exchanger Slc26a1 (see also Slc26a1 discussion). This allows sufficient build-up of high cytoplasmic concentrations of SO_4^{2-} driving apical secretion of SO_4^{2-} by Slc26a6a in SW pufferfish and eel (Kato *et al.*, 2009; Watanabe and Takei, 2011b, 2011a). The upregulation during smoltification, the rapid increase after 2 days in SW and the further increase after 38 days in SW (figure 19) suggest an important role of the *slc26a6a1* for secreting excess SO_4^{2-} in SW. The *slc26a6a1* mRNA abundance was barely detectable in the parr group (FW) while increased *slc26a6a1* levels in FW smolts suggest that this transporter not only is activated by salinity, as previously suggested in eel and pufferfish (Kato et al 2009; Watanabe and Takei, 2011a), but also activated by the smoltification process.

The current upregulation after short and long-term SW exposure in smolts infers an important role of *slc26a6a1* in SW, similar to what has been demonstrated for other species upon SW transfer (Kato et al 2009; Watanabe and Takei, 2011). There is also a common consensus that most SO_4^{2-} is actively secreted from the renal proximal tubules of marine teleost and euryhaline species in SW to maintain plasma SO_4^{2-} levels within 0.2-1 mM, and that the urine is rich in SO_4^{2-} ions (roughly 45-50 mM)

(Hickman and Trump, 1969; Renfro, 1999; Beyenbach, 2004; Marshall and Grosell, 2006; Watanabe and Takei, 2011b, 2012). Furthermore, under controlled conditions, SO_4^{2-} secretion rates average around 25 µmol/kg/h with a clearance ratio around 12 µmol/kg/h in the glomerulus (Hickman and Trump). Thus, indicates that excess SO_4^{2-} are secreted through the nephron tubule. The plasma levels of SO_4^{2-} in salmonids have been found around 0,1-0,3 mM in FW and 0,8-1,2 mM in SW (Katoh *et al.*, 2006; Watanabe and Takei, 2012). Similar plasma SO_4^{2-} levels are observed in Atlantic salmon FW smolts (0,2-0,5 mM) and 3 days in post transfer to SW (0,8-1,1 mM) (Unpublished data, associate professor Tom Ole Nilsen) reiterate the significant upregulation of the *slc26a6a1* isoform in the current study. In the FW acclimated Rainbow trout SO_4^{2-} plasma levels of 0,45 mM (base level), was substantially elevated to approx. 2,25 mM after administration of SO_4^{2-} followed by a subsequent rapid return to roughly 0,6 mM, reflecting efficient regulation of plasma SO_4^{2-} concentrations, mainly removed by the kidney (Katoh *et al.*, 2006). Unfortunately, these authors only addressed the Slc26a1 transporter that commonly was thought to be involved in secretion at the basolateral side of proximal tubule cells (Watanabe and Takei, 2011a). Though Katoh and authors (2006) only address the basolateral transporter Slc26a1, it is tempting to suggest a similar mechanism in Atlantic salmon, were the apical Slc26a6a1 secretes and removes excess SO_4^{2-} after SW transfer in conjunction with the basolateral Slc26a1 family (see discussion slc26a1 family).

Predictions about the location of the salmon Slc26a6a1 transporter may be inferred based on findings from eel and pufferfish, locating the Slc26a6a on the apical membrane of cells in the proximal segment I (Kato et al 2009; Watanabe and Takei, 2011). Assuming the SO_4^{2-} transporters identified in this study are translated into proteins, and similar regulation pattern of the *slc26a6a* in eel and pufferfish to the *slc26a6a1* in SW acclimated salmon, one may argue a similar regulation and location exists in the salmon kidney. Ultimately, the regulative pattern in this study and conjectures from previous studies suggest strong evidence that the slc26a6a family is the most likely candidate for SO_4^{2-} secretion in the kidney of teleost fish. Nevertheless, before protein and electrophysical analysis has been conducted for salmon Slc26a6a1, predictions can only be inferred based on mRNA abundance and comparative studies in other species. Verification at the protein level has proved particularly difficult in this study as the protein sequence of Slc26a6a1 and Slc26a6a2 paralogues are highly similar. Still, mRNA abundance for these two paralogues revealed completely different regulation patterns for *slc26a6a1* (Kidney; SW) and *slc26a6a2* (Gills; FW, see discussion on gills) and it will be intriguing to address the cellular location once antibodies are validated.

The *slc26a6b* and *slc26a6c* paralogues in teleost fish are upregulated in both FW and SW and also linked to apical transport of SO_4^{2-} (Kato et al., 2009; Watanabe and Takei., 2011). In the current study, the salmon *slc26a6b* and *slc26a6c* mRNA levels was relatively equally expressed in parr and smolt,

suggesting that both paralogues are indeed active in both FW and SW (figure 20). In eel, both Slc26a6b and Slc26a6c have been located on the apical side of the renal proximal tubule (Watanabe and Takei, 2011b). Electrophysical analysis using Xenopus oocytes showed that Slc26a6b exhibits considerably lower activity than Slc26a6a (Kato *et al.*, 2009) and Slc26a6b and Slc26a6c are proposed to be located apically in the renal proximal segment II and I, respectively (Watanabe and Takei, 2011b). Slc26a6b is suggested to be a Cl^-/SO_4^{2-} anion exchanger, similar to the Slc26a6a. However, electrophysical studies of the pufferfish Slc26a6c in oocytes suggest that this transporter is not an anion exchanger (Kato *et al.*, 2009). Contemplating the apparent gene expression of both *slc26a6b* and *slc26a6c* in both FW and SW, it can be argued that these transporters have dual roles, reabsorbing in FW and secreting in SW, maybe on the apical side.

Accumulated evidence suggests that *slc26a6a1* predominantly works in SW environments, which is further substantiated in the current study. In contrast the *slc26a6b* and *slc26a6c* active in both FW and SW require more examinations to find the potential role they may have in both environments. The *slc26a6b* and *slc26a6c* paralogues are only found in teleost fish and only a single gene, *slc26a6*, is found in mammals and elasmobranchs (Markovich, 2001; Hasegawa *et al.*, 2016). Future studies on all three paralogues (Slc26a6a, Slc26a6b and Slc26a6c) are recommended to fully elucidate the regulation and physiological properties of these transporters in Atlantic salmon.

Salmon specific SO_4^{2-} transport Slc26a1 family (Slc26a1, Slc26a1bX1, Slc26a1bX3)

The *slc26a1a* mRNA was abundantly expressed in both parr and smolts, though only smolts showed significantly elevated mRNA levels during smoltification (FW) and after SW transfer (figure 21). Both *slc26a1bX1* and *slc26a1bX3* was also abundantly expressed in both parr and smolts (figure 22), yet no significant changes occurred during smoltification and given the uncertainty about sequences validity of splice variants in the databases they will not be further discussed.

SLC26A1 (SAT-1) is a SO_4^{2-}/anion exchanger, mediating SO_4^{2-} efflux across the basolateral membrane in exchange of HCO_3^- in mammals (Karniski *et al.*, 1998). In teleosts, Slc26a1 exhibit dual roles, being active in both FW and SW environments, being linked to absorption of SO_4^{2-} in eel together with the Slc13a1 (Nakada *et al.*, 2005) and secretion of SW eels and puffer fish in conjunction with Slc26a6a (Watanabe and Takei, 2011). In the current study, *slc13a1* was not expressed in the kidney, only the intestine (Appendix 5c). Since the Slc13a1 is largely responsible for SO_4^{2-} absorption in FW eel a different mechanism must be present in salmon. The SO_4^{2-} concentration in SW requires basolateral transport of SO_4^{2-} from the plasma into the cell and elevated *slc26a1a* levels in smolts provide a plausible basolateral candidate. Similarly, abundant expression of salmon *slc26a1a* and *slc26a1b* (*slc26a1bX1* and *slc26a1bX3*) in FW do not preclude a potential basolateral role for all of them. Secretion apically into the tubule lumen could be facilitated by *slc26a6a1*, which is elevated in

SW smolts (see discussion Slc26a6a1). Interestingly, it has been proposed that the basolateral Slc26a1 can switch transport direction of SO_4^{2-}, depending on the requirements in FW or SW (Nakada, 2005; Watanabe and Takei, 2011a). However, given the elevated mRNA levels found during smoltification and SW for *slc26a1a* it may have a more predominant role in SW acclimated salmon. Interestingly, the *slc26a1* in Rainbow trout was found to be highly upregulated after SO_4^{2-} administration that points towards a more prominent role in SO_4^{2-} secretion (Katoh *et al.*, 2006). Although they did not differentiate between putative paralogs in the preceding study one may argue that the slc26a1a upregulated in SW Atlantic salmon in the current study serve as the best potential candidate for SO_4^{2-} secretion. Together with the apparent basolateral location of the Slc26a1 in Rainbow trout (Katoh et al., 2006) it is likely to find a similar location in Atlantic salmon. However, one cannot rule out a potential role also in FW since the mRNA abundance are relatively high also here. Furthermore, the *slc26a1b* (X1 and X3) regulation pattern equally expressed in both FW and SW suggest a dual role in both environments. Before more thorough investigations have been conducted, verifying and locating protein in the nephron tubules, these salmon specific SO_4^{2-} transport remain in the more preliminary phase. However, based on the mRNA abundance results in the present study and the more comprehensive investigations conducted in eel, pufferfish and trout it is likely that these salmon specific sulfate transports (Slc26a1a, Slc26a1bX1 and Slc26a1bX3) are also located on the basolateral membrane involved in both absorption and secretion mechanisms. Before similar properties and location examination are conducted in Atlantic salmon, no certain conclusions can be made in the current study.

Final remarks and formulated hypothesis for SO_4^{2-} transport in the kidney of Atlantic salmon

In summary, the Slc26a6a1 transporter is the most likely candidate for apical SO_4^{2-} secretion in conjunction with the basolateral Slc26a1a in SW. These claims are substantiated by the apparent upregulation of both transporters during smolt development and after SW transfer in the current study. Since we do not have salmon specific antibodies validated yet, these assumptions are partly based upon findings in SW acclimated eel and pufferfish. Final confirmation will be completed when the salmon antibodies are ready. Slc26a6b has been found, similarly to Slc26a6a, to exchange Cl^- with SO_4^{2-} but Slc26a6c specific function is yet to be determined (Kato *et al.*, 2009; Watanabe and Takei, 2011b). Surprisingly, expression of *slc13a1* was not detected in the salmon kidney, which is at odds with the proposed model of SO_4^{2-} absorption postulated based on findings in FW acclimated eel. Therefore, an alternative hypothesis can be formulated related to SO_4^{2} absorption in Atlantic salmon; An apical Slc26a6b can change the direction of SO_4^{2-} transport, thus absorbing SO_4^{2-} in FW and secreting in SW. This transport is possible through the apical Slc26a6b (maybe Slc26a6c) and

basolateral Slc26a1b (and Slc26a1a) in renal proximal tubules (figure 27). These claims are formulated based on four main assumptions:

1) In this study, *slc26a6b* and *slc26a1b* are equally expressed in FW and SW, designating dual roles in both environments.
2) Earlier work using electrophysiological data revealed that a low cytoplasmic concentration of Cl^- (chloride channels), high cytoplasmic concentrations of SO_4^{2-} and a negative membrane potential by the NKA transporter, are found to be sufficient for apical secretion of SO_4^{2-} (Slc26a6a) in SW pufferfish (Kato et al., 2009). If this transport mechanism is able to switch in the opposite direction, by apical absorption through Slc26a6b (maybe also Slc26a6c) and basolateral transport by Slc26a1b (possibly also Slc26a1a) absorption in FW might be possible assuming: high cytoplasmic concentration of Cl^- (chloride channels), low cytoplasmic concentration of SO_4^{2-} and a positive membrane potential by the NKA transporter.
3) Nka enzyme activity in the kidney was observed in the current study that potentially can power this transport in FW acclimated Atlantic salmon.
4) The basolateral Slc26a1 has also been shown to be active in both FW and SW, that have been demonstrated to enable both absorption and secretion in eel, pufferfish and trout, having dual roles in SO_4^{2-} transport (Nakada, 2005; Katoh et al, 2006; Watanabe and Takei, 2011). Assuming similar functions in Atlantic salmon, this could be possible using Slc26a1a and Slc26a1b transport family.

In general, recent comprehensive studies exist on SO_4^{2-} transport in pufferfish (Kato *et al.*, 2009), eels (Kato and Watanabe, 2016), mammals (Markovich, 2001) and to some extent elasmobranchs (Hasegawa *et al.*, 2016) and trout (Katoh et al., 2006). Although the present study is the first to address SO_4^{2-} transporters in Atlantic salmon, the current model of SO_4^{2-} transport in the Atlantic salmon kidney is premature. More comprehensive studies are required to understand the transport mechanisms, particularly with respect to cellular localization and co-transport with other ion transporters.

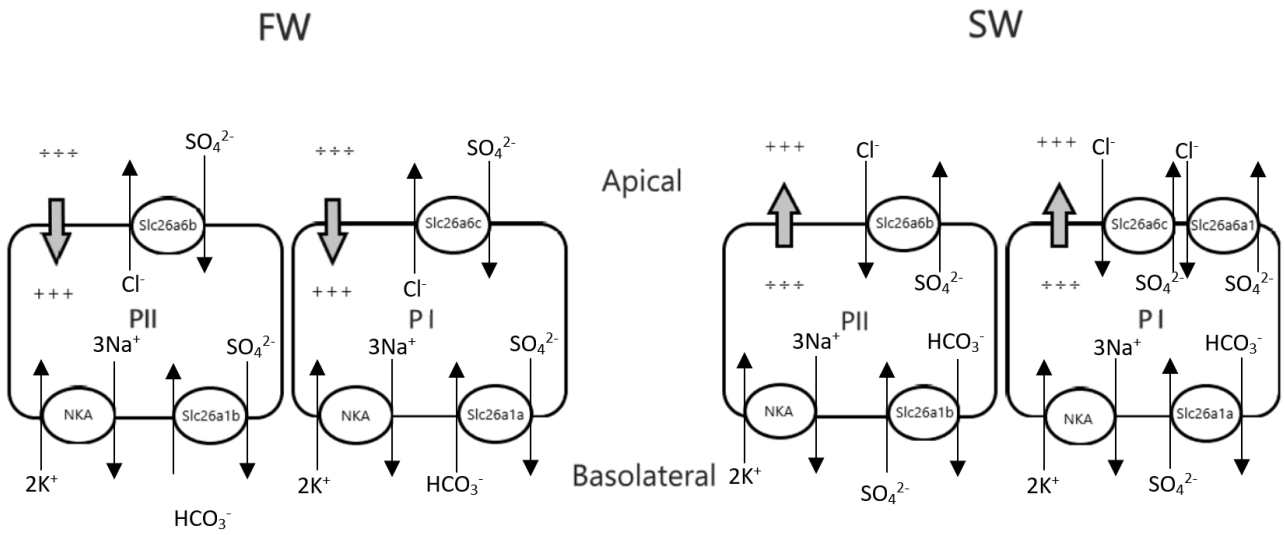

Figure 27: Hypothetical model for SO_4^{2-} epithelial absorption and secretion system in proximal tubules of Atlantic salmon. ***FW:*** *Slc26a6b possibly localized apical in proximal tubule II (PII) acts as an electrogenic Cl^-/SO_4^{2-} exchanger where a positive membrane potential (NKA) may be the driving force for SO_4^{2-} absorption. This may also be possible through the apical Slc26a6c proximal tubule I (PI) (electrophysical analysis still uncertain). A potential basolateral located Slc26a1a and b (Slc26a1 family) may act as an electroneutral exchanger of HCO_3^{2-}/SO_4^{2-} that may further transport the SO_4^{2-} to the blood.* ***SW:*** *Slc26a6a1 (highly active in SW) act as an electrogenic Cl^-/SO_4^{2-} and a negative membrane potential (NKA) may drive SO_4^{2-} secretion. A potential basolateral located Slc26a1a and b may act as electroneutral exchanger of HCO_3^{2-}/SO_4^{2-} transporting SO_4^{2-} from blood to the cell. This model is still very premature and largely based on the papers of Katoh et al., 2006, Kato et al., 2009 and Watanabe and Takei, 2011a; 2011b in conjunction with mRNA expression found in the current study.*

4.2.3.3 The Slc26a6a2 paralog in Atlantic salmon is highly expressed in FW gills and suggest for the first time a transcellular pathway in the fish gill

Currently no transporters have been reported in the gills. The present study is the first to present a potential candidate for SO_4^{2-} transport in salmon. The putative *slc26a6a2* paralogue was by far the most abundant transporter expressed in FW acclimated salmon. Notably, a clear decrease was exhibited during smoltification, with a 1600-fold decrease following long term SW exposure, suggesting a role in SO_4^{2-} uptake across the gills in FW acclimated fish (figure 23).

It has been suggested that the teleost gill may be a major site for SO_4^{2-} absorption in FW acclimated fish. Further a low influx of SO_4^{2-} from the medium to the body (0.09 µmol/kg/h) has been observed in FW teleosts (low permeability obstruction of a paracellular pathway), thus plasma concentrations are about 20-fold that of the surrounding environment suggesting that gills might absorb SO_4^{2-} into the blood (Watanabe and Takei, 2012). Here it is suggested that transcellular transport against an electrochemical gradient is possible through the Slc26a6a2, probably driven by the NKA pump (figure 28). In addition, it is widely accepted that in hypoosmotic environments, such as FW, tight junctions prevent paracellular ion loss across the gill epithelia, enabling active transcellular transport instead of

paracellular pathways (Chasiotis, Kolosov and Kelly, 2012). Interestingly, expression of *slc26a6a2* disappear rapidly in SW acclimated salmon, suggesting a minimal role of *slc26a6a2* in SW. Therefore, a transcellular pathway are less likely in the SW environment containing high SO_4^{2-} concentrations (30-50 fold higher then plasma) (Edwards and Marshall, 2012). Further, tight junctions are leaky, favouring Na^+ secretion in the gills (Chasiotis *et al.*, 2012), while also facilitating a compulsory paracellular transport of other ions, including SO_4^{2-} which are highly concentrated in SW (Watanabe and Takei, 2012). Thus, the high compulsory influx of SO_4^{2-} (85 %) through gills are in large removed by the kidney as discussed in the previous section. Again, it must be highlighted that this requires verification by electrophysical methods, localization, and protein verification. While it is of interest to determine the cellular location of salmon specific Slc26a6a2, a basolateral transporter is also required for transport from the cell to the blood, prompting the question are the putative gill Slc26a6a2 localized to ionocytes?

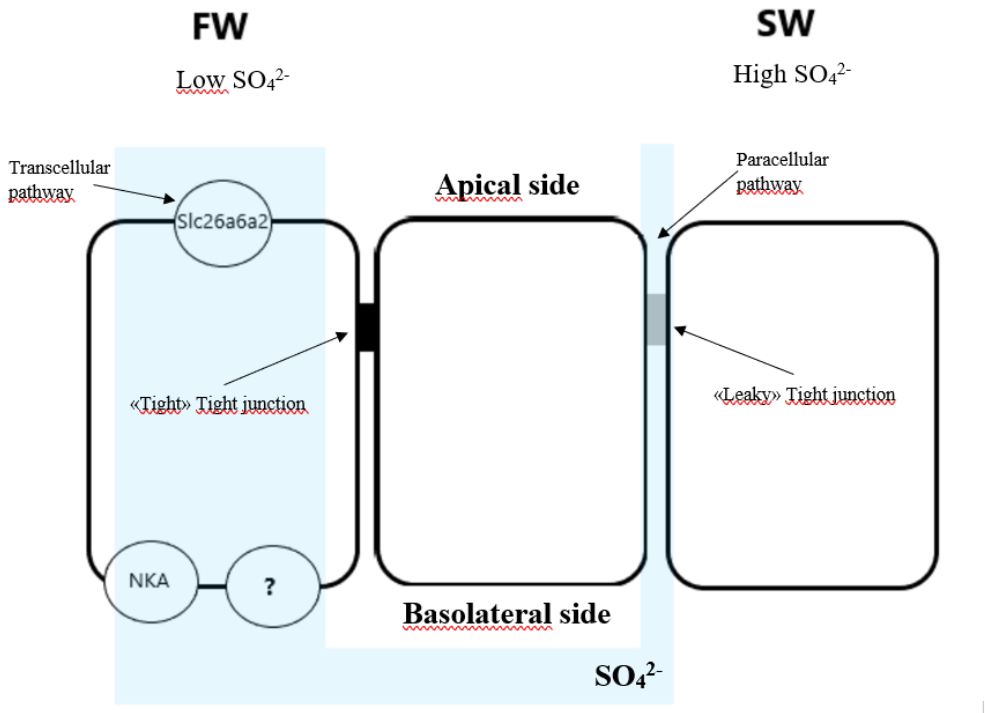

Figure 28: Hypothetical model for transport through gill in FW and SW environments. ***FW:*** *tight junctions (claudins) prevent paracellular transport where a potential apical Slc26a6a2 (highly active in FW) transporter can absorb* SO_4^{2-} *possibly driven by the NKA pump where* SO_4^{2-} *are low in the environment. An unknown basolateral transporter is also required.* ***SW:*** *tight junctions (claudins) are "leaky" likely transporting* SO_4^{2-} *(and other ions) through a paracellular pathway where* SO_4^{2-} *concentrations are high in the environment. The undetectable mRNA levels of Slc26a6a2 suggest a minimal role in SW acclimated Atlantic salmon. Model based on Watanabe and Takei, 2012 and mRNA expression levels in current study.*

4.2.3.4 Five SO_4^{2-} transporters detected in intestine of Atlantic salmon (Ct values) possible role in SO_4^{2-} homeostasis in fish

Intestine contribute less to the overall SO_4^{2-} budget in SW teleosts, with roughly 15 % uptake through the intestinal tract (Watanabe and Takei, 2012). Furthermore, up to 85 % of SO_4^{2-} uptake originates from gills/skin and are almost exclusively secreted through the kidney (97 %) in SW (see discussion 4.2.3.2). The intestinal fluid is rich in SO_4^{2-} and the intestine of marine teleosts are believed to be almost impermeable to SO_4^{2-} (Hickman, 1968a; Marshall and Grosell, 2006). This is somewhat contradictory since both Cl^- and HCO_3^- appear to influence SO_4^{2-} transport in the intestine of marine teleosts (Pelis and Renfro, 2003; Grosell, 2010), and several intestinal SO_4^{2-} transporters have been detected in eels (Watanabe and Takei, 2011b). Thus, the transport activity of SO_4^{2-} is generally low in the intestine and reflects the high concentrations of SO_4^{2-} in the intestinal fluids in marine fish (Hickman, 1968a; Marshall and Grosell, 2006; Grosell, 2010). Secretion of SO_4^{2-} has been found to be an electroneutral exchange, and marine teleost intestine are suggested to be site for absorption of SO_4^{2-} but are actively secreted in exchange for luminal Cl^- depending on the environmental conditions in the intestinal lumen (Pelis and Renfro, 2003). Though the Slc26a6 family has been largely proposed to exchange HCO_3^- for Cl^- in the intestine of marine teleosts and SW acclimated euryhaline teleost (Sundell and Sundh, 2012). In the present study, tissue distributions revealed the following intestinal SO_4^{2-} transporters in salmon; *slc13a1*, *slc26a6a1*, *slc26a6b*, *slc26a1a* and *slc26a1bX1* (Appendix 5c). Further, due to degradation, and thus inadequate RNA integrity from intestinal tissue sampled during the smoltification experiment, only data from the initial tissue distribution is included and discussed here. The current discovery of transporters Slc26a1, Slc26a6 and Slc13a1 may have a role in SO_4^{2-} or HCO_3^- transport in the intestine of Atlantic salmon. However, the conflicting observations related to SO_4^{2-} transport in the intestine of teleost fish pointed out and given the very limited data available in the current study one can only speculate conclusions at this time. More comprehensive investigation should be conducted in the future to oversee the regulation of these transporters in the intestine through smoltification and after SW transfer. This should also include protein verification and location in the intestine of Atlantic salmon.

4.2.4 Application for aquaculture and future perspective

The delayed onset and increase in Nka enzyme activity in both the intestine and kidney compared to gills warrants more research attention in the future. The present findings might be of practical use since the Nka enzyme activity is used as a key indicator of smolt development in the industry. Under modern intensive production protocols, the present findings indicate that at the time of smolt transfer, according to the initial increase in gill Nka activity, the intestine and kidney have not reached their osmoregulatory peak yet, probably leading to transfer of smolts with suboptimal osmoregulatory

capacity. This can have detrimental consequences for marine survival. One should perhaps focus more on the preparatory changes of all three organs to ensure improved and optimal smolt quality. The discovery and characterization of key SO_4^{2-} transporters in Atlantic salmon gills, intestine and kidney further substantiates the importance of addressing all three major organs regulating the water and ion balance. The industry has experienced several acute episodes with high mortality in land-based RAS facilities, and in some instances, this has been attributed to hydrogen sulfide. This has prompted industry to test membrane technology to filter out sulfate from the water. This will affect the ion composition of the water and potentially compromise osmoregulation, particularly with respect to divalent ions. Hence, knowledge of how salmon regulate divalent ions will be important in understanding adverse physiological consequences and prevent adverse effects of altered ion content in the water. Therefore, the current study addressing SO_4^{2-} is one small step in the direction of understanding divalent ion regulation in Atlantic salmon. This may give clues and increase our understanding of reduced smolt quality and SW performance aspects in Norwegian salmon farming. Ultimately, all three organs; gills, intestine and kidney should be examined collectively in future studies, as the current study reflects the inherent and cooperative work in preparation during smolt development and subsequently in SW tolerance.

5. Conclusion

It has previously been determined in the current study that the gill Nka enzyme activity alone in surveillance of smolt development is currently an insufficient assessment tool in predicting overall osmoregulatory capacity and optimal SW performance in Atlantic salmon. Thus, intestine and kidney measurements should be included in smolt quality assessment. A transient decrease in kidney Nka activity may reflect a considerable need for short time reduction of water loss via the kidney after abrupt SW exposure. These Nka activity levels return to initial peak smolt levels after 38 days in SW, thus indicating that the kidney may need more time adjusting to the marine environment. Searches in the salmon genome and phylogenetic analysis revealed annotated and non-annotated sequences of solute carrier family 13 (Slc13) and 26 (Slc26), including: Slc13a1 (intestine), Slc26a6a (gills, intestine, kidney), Slc26a6b (intestine, kidney), Slc26a6c (kidney) and Slc26a1 (intestine, kidney). An additional repertoire of Slc26a6a (Slc26a6a1 and Slc26a6a2) and Slc26a1 (Slc26a1a and Slc26a1b) paralogues appear to be present after the salmonid-specific fourth vertebrate whole-genome duplication. The preparatory increase in kidney specific *slc26a6a1* and *slc26a1a* mRNA levels, in addition to the gill specific decrease of *slc26a6a2* mRNA levels during smoltification and SW transfer suggests an important role of these sulfate transporters in the regulatory shift from absorption to secretion moving from FW to SW. The mRNA abundance of *slc26a6b*, *slc26a6c* and

slc26a1b remained stable, with no significant differences over time and between parr and smolts that may suggest dual roles, thus active in both environments.

As such, given the findings of the project I would like to briefly highlight the outcomes of the objectives set forth in the introduction of this report.

Objective 1. Determine the Nka enzyme activity in the three osmoregulatory organs during smolt development

A collective increase of the Nka enzyme activity in all three osmoregulatory organs during smolt development was confirmed in the current study and corresponds well with previous work. However, it appears that the Nka enzyme activity patterns are different in the three osmoregulatory organs gills, intestine and kidney, that was not initially predicted.

Objective 2. Determine the Nka enzyme activity in the three osmoregulatory organs after SW transfer

Levels of Nka enzyme activity either increased or remain high in the gills and intestine after abrupt SW transfer was corresponding well with previous work in Atlantic salmon. The kidney decreases overall tubular activity in SW, reflected by overall reduction in Nka enzyme activity. This was not confirmed in long-term SW, indicating a longer adaptation period in SW phase that deviates from initial predictions.

Objective 3. Identification and characterization of key SO_4^{2-} transporters in Atlantic salmon

SO_4^{2-} transporters are present in the gills, intestine and kidney but predominantly found in the kidney, with seven potential SO_4^{2-} transporter candidates being discovered. Given the Ss4R event in salmonids species there several paralog genes present in Atlantic salmon were confirmed. This was only discovered in the Slc26a1 and Slc26a6a family and was not confirmed in the Slc13a1, Slc26a6b and slc26a6c family.

Objective 4. Gene expression patterns of SO_4^{2-} transporters during smoltification and after SW transfer

Both *slc26a6a1* and *slc26a1a* was upregulated during smolt development and confirms the preparatory change often observed in Atlantic salmon. The upregulation of *slc26a6a1* resonates with previous findings in SW fish and is substantiated by the unchanged regulation in the control group. This was only partly confirmed for the *slc26a1a* isoform in Atlantic salmon since the control group also showed relatively high regulation of the gene. Surprisingly, a completely different and reciprocal

regulation was observed for *slc26a6a2* in the gills compared to the *slc26a6a1* in the kidney, suggesting a different mode of SO_4^{2-} regulation in Atlantic salmon.

6. References

6.1 - Introduction to Generalized Linear Models | STAT 504. Available at: https://online.stat.psu.edu/stat504/node/216/ (Accessed: 19 February 2020).

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7. Appendix

Appendix 1. Morphological smolt characteristics

1a. Weight measurement overview

Table 1. *Wet weight (gram, g), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean (g)	SEM (g)	Min (g)	Max (g)	P-value
1 (120)	Parr (12)	32.9	0.90	29.9	36	0.7814
	Smolt (12)	32.3	0.51	29.2	35.4	
2 (260)	Parr (12)	35.6	0.86	30.01	38.97	0.1710
	Smolt (12)	38.6	1.11	33.57	47.1	
3 (350)	Parr (12)	37.1	0.96	30	40	0.0062
	Smolt (10)	43.5	1.64	36.9	51.4	
4 (450)	Parr (12)	42.8	1.33	38	54.9	
	Smolt (11)	51.5	1.49	44.8	60.2	0.0002
5 (480)	Smolt (12)	51.1	1.20	44.6	59.1	x
6 (490)	Smolt (12)	52.8	1.55	45.08	45.08	x
7 (830)	Parr (12)	58.4	1.83	48.3	67	<.0001
	Smolt (12)	78.2	3.38	56.4	95.5	

Sampling	Parr	Smolt
1-2	0.8805	0.0669
1-3	0.4653	0.0001
1-4	0.0003	<.0001
1-5	x	<.0001
1-6	x	<.0001
1-7	<.0001	<.0001
2-3	0.9926	0.3404
2-4	0.0215	<.0001
2-5	x	<.0001
2-6	x	<.0001
2-7	<.0001	<.0001
3-4	0.1371	0.0147
3-5	x	0.0181
3-6	x	0.0015
3-7	<.0001	<.0001
4-5	x	1.0000
4-6	x	0.9962
4-7	<.0001	<.0001
5-6	x	0.9871
5-7	x	<.0001
6-7	x	<.0001

1b. Length measurement overview

Table 2. *Fork length (centimeters, cm), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean (cm)	SEM (cm)	Min (cm)	Max (cm)	P-value
1 (120)	Parr (12)	13.91	0.27	11.1	14.5	0.9565
	Smolt (12)	13.9	0.07	13.4	14.5	
2 (260)	Parr (12)	14.09	0.10	13.4	14.5	0.4137
	Smolt (12)	13.90	0.07	13.4	14.5	
3 (350)	Parr (12)	14.42	0.13	13.5	14.95	<.0001
	Smolt (10)	15.68	0.16	15	16.4	
4 (450)	Parr (12)	15.18	0.18	14.4	16.5	<.0001
	Smolt (11)	16.54	0.14	15.8	17.2	
5 (480)	Smolt (12)	16.57	0.15	15.6	17.3	x
6 (490)	Smolt (12)	16.94	0.17	15.9	17.8	x
7 (830)	Parr (12)	16.98	0.20	15.9	18.8	<.0001
	Smolt (12)	19.30	0.18	18.4	20.4	

Sampling	Parr	Smolt
1-2	0.9878	1.0000
1-3	0.3000	<.0001
1-4	<.0001	<.0001
1-5	x	<.0001
1-6	x	<.0001
1-7	<.0001	<.0001
2-3	0.7792	<.0001
2-4	0.0001	<.0001
2-5	x	<.0001
2-6	x	<.0001
2-7	<.0001	<.0001
3-4	0.0198	0.0112
3-5	x	0.0052
3-6	x	<.0001
3-7	<.0001	<.0001
4-5	x	1.0000
4-6	x	0.6078
4-7	<.0001	<.0001
5-6	x	0.6917
5-7	x	<.0001
6-7	x	<.0001

1c. Condition factor calculations

Table 3. *Condition factor (CF=Wet weight (W)/Fork length (L)^3), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean	SEM	Min	Max	P-value
1 (120)	**Parr (12)**	**1.18**	**0.017**	**1.09**	**1.29**	**0.4399**
	Smolt (12)	**1.20**	**0.011**	**1.14**	**1.26**	
2 (260)	**Parr (12)**	**1.27**	**0.010**	**1.22**	**1.34**	**0.0013**
	Smolt (12)	**1.20**	**0.011**	**1.13**	**1.28**	
3 (350)	**Parr (12)**	**1.25**	**0.011**	**1.17**	**1.32**	**<.0001**
	Smolt (10)	**1.13**	**0.010**	**1.08**	**1.18**	
4 (450)	**Parr (12)**	**1.23**	**0.014**	**1.17**	**1.32**	**<.0001**
	Smolt (11)	**1.15**	**0.015**	**1.08**	**1.23**	
5 (480)	**Smolt (12)**	**1.14**	**0.15**	**1.07**	**1.21**	**X**
6 (490)	**Smolt (12)**	**1.10**	**0.012**	**1.04**	**1.21**	**X**
7 (830)	**Parr (12)**	**1.20**	**0.015**	**1.13**	**1.32**	**<.0001**
	Smolt (12)	**1.09**	**0.027**	**0.91**	**1.20**	

Sampling	Parr	Smolt
1-2	0.0016	1.0000
1-3	0.0410	0.0439
1-4	0.2408	0.1472
1-5	x	0.0396
1-6	x	0.0001
1-7	0.9710	<.0001
2-3	0.9536	0.0432
2-4	0.5897	0.1453
2-5	x	0.0390
2-6	x	0.0001
2-7	0.0316	<.0001
3-4	0.9895	0.9982
3-5	x	1.0000
3-6	x	0.6742
3-7	0.3242	0.5087
4-5	x	0.9993
4-6	x	0.2945
4-7	0.7916	0.1784
5-6	x	0.5479
5-7	x	0.3793
6-7	x	1.0000

1d. Smolt index

Table 4. *Smolt index, mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean	SEM	Min	Max	P-value
1 (120)	Parr (11)	1.01	0.011	1	1.125	<0.0001
	Smolt (11)	1.489	0.057	1.25	1.75	
2 (260)	Parr (10)	1.038	0.019	1	1.125	<0.0001
	Smolt (12)	3.115	0.116	2.5	3.75	
3 (350)	Parr (12)	1.229	0.053	1	1.625	<0.0001
	Smolt (12)	4.271	0.125	3.63	4.785	
4 (450)	Parr (11)	1.159	0.018	1.125	1.25	<0.0001
	Smolt (11)	4.931	0.031	4.75	5	

Sampling	Parr P-value	Smolt P-value
1-2	0.9946	<.0001
1-3	0.1313	<.0001
1-4	0.4732	<.0001
2-3	0.2443	<.0001
2-4	0.6534	<.0001
3-4	0.8977	<.0001

Smolt index evaluation criteria

The smolt index was evaluated based on all the criteria described in method section (method 2.3.2) for each individual fish sampled. Below is an example of one fish evaluated to have a smolt index of 1 (parr) and one fish evaluated to have a smolt index of 5 (complete smolt):

Smolt index of 1:

Smolt index of 5:

Appendix 2. Nka activity

2a. Refining Nka activity method for the kidney

Table 5. *Nka enzyme activity in different areas of the kidney significant levels (p-values), mean, Standard error of mean (SEM) and number of samples (N) for each area.*

Area (N)	Mean	SEM
A (24)	0.780	1.316
B (24)	11.11	2.604
C (24)	12.38	1.612
D (24)	15.39	3.090
E (24)	15.97	2.003

Area	p-value
A-B	5.45^{-11} (***)
A-C	2.71^{-12} (***)
A-D	4.19^{-15} (***)
A-E	1.31^{-15} (***)
B-C	0.26
B-D	0.0005 (***)
B-E	0.0001 (***)
C-D	0.01 (*)
C-E	0.003 (**)
D-E	0.60

The initial testing found the high Nka enzyme activity in kidney outperformed the chemical reaction. The kinetic activity flattened out prior to the last pre-set reaction cycles in the Spark multimode microplate reader (Tecan, Mannedorf, Switzerland). Reducing tissue amount with approximately 1/3 resulted in only a few reactions still outperforming the kinetic assay before the reactions last cycle. Table 6 show the amount of tissue tested in different kidney areas and whether the kinetic reaction was acceptable or flattened out prior to pre-set reaction cycles in the Nka assay.

Table 6. *Overview of slope activity in response to the amount (milligram, mg) of kidney tissue used in different areas of the kidney.*

Area B	Reaction	Area C	Reaction	Area D	Reaction	Area E	Reaction
7 mg	Normal	5 mg	Normal	6 mg	Normal	8 mg	Normal
10 mg	Normal	12 mg	Normal	9 mg	Normal	8 mg	Normal
10 mg	Normal	15 mg	Normal	17 mg	Flats out	15 mg	Normal
10 mg	Normal	16 mg	Normal	20 mg	Flats out	18 mg	Flats out
14mg	Normal	24 mg	Normal	27 mg	Flats out	27 mg	Flats out
24 mg	Normal	27 mg	Normal	30 mg	Flats out	28 mg	Flats out

Conclusion: The slope flats out using ≥17 mg of tissue from the preferred areas, D and E. Hence, 5-8 mg of tissue was sampled between area D and E to prevent flattening the slope when analysis kidney Nka enzyme activity.

Appendix 2b. Nka activity in the gills, intestine and kidney

Table 7. *Gill Nka activity (µmoles ADP/mg protein/hour), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean (activity)	SEM (activity)	Min (activity)	Max (activity)	P-value
1 (120)	Parr (12)	3.47	0.38	0.58	5.38	0.0075
	Smolt (12)	5.93	0.68	1.89	9.21	
2 (260)	Parr (12)	2.78	0.54	0.19	6.58	<.0001
	Smolt (12)	12.98	0.75	9.23	18.97	
3 (350)	Parr (12)	3.70	0.30	2.18	5.19	<.0001
	Smolt (10)	15.31	0.90	9.10	18.18	
4 (450)	Parr (12)	4.08	0.33	2.24	5.77	
	Smolt (11)	17.19	0.76	12.96	20.20	<.0001
5 (480)	Smolt (12)	18.53	0.59	15.50	23.15	x
6 (490)	Smolt (12)	18.08	0.47	16.28	21.53	x
7 (830)	Parr (12)	7.99	1	3.37	13.03	<.0001
	Smolt (12)	20.17	0.73	16.61	24.35	

Sampling	Parr	Smolt
1-2	0.9886	<.0001
1-3	1.0000	<.0001
1-4	0.9938	<.0001
1-5	x	<.0001
1-6	x	<.0001
1-7	<.0001	<.0001
2-3	0.9498	0.1898
2-4	0.7847	0.0003
2-5	x	<.0001
2-6	x	<.0001
2-7	<.0001	<.0001
3-4	0.9996	0.4564
3-5	x	0.0160
3-6	x	0.0609
3-7	0.0001	<.0001
4-5	x	0.7793
4-6	x	0.9608
4-7	0.0006	0.0274
5-6	x	0.9990
5-7	x	0.5450
6-7	x	0.2545

Table 8. *Proximal intestine Nka activity (µmoles ADP/mg protein/hour), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean	SEM	Min	Max	P-value
1 (120)	Parr (12)	4.33	0.5	1.43	6.66	0.0126
	Smolt (12)	6.48	0.78	3.21	13	
2 (260)	Smolt (11)	14.4	1.2	1.96	20.9	x
3 (350)	Smolt (12)	22.8	1.81	12.5	36.1	x
4 (450)	Parr (12)	2.54	0.30	0.40	4.79	<.0001
	Smolt (11)	14.5	2.16	5.52	27.4	
5 (480)	Smolt (12)	23.1	3.1	8.06	43.1	x
6 (490)	Smolt (11)	20.4	1.29	15.4	27.1	x
7 (830)	Parr (12)	18.6	1.55	10.6	29.5	0.0189
	Smolt (9)	27.7	3.89	5.56	40.5	

Sampling	Parr	Smolt
1-2	x	0.0357
1-3	x	<.0001
1-4	0.0212	0.0001
1-5	x	<.0001
1-6	x	<.0001
1-7	<.0001	<.0001
2-3	x	0.0007
2-4	x	0.5602
2-5	x	0.0005
2-6	x	0.0061
2-7	x	<.0001
3-4	x	0.1050
3-5	x	1
3-6	x	0.9930
3-7	x	0.9096
4-5	x	0.0856
4-6	x	0.4017
4-7	0.0013	0.0070
5-6	x	0.9865
5-7	x	0.9367
6-7	x	0.5688

Table 9. *Distal intestine Nka activity (µmoles ADP/mg protein/hour), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean	SEM	Min	Max	P-value
1 (120)	Parr (11)	1.89	0.55	0.25	5.34	0.5690
	Smolt (12)	2.15	0.32	0.17	4.06	
2 (260)	Smolt (12)	4.39	0.82	1.03	11.47	x
3 (350)	Smolt (12)	10.7	1.18	6.3	18.8	x
4 (450)	Parr (12)	1.30	0.18	0.27	2.25	<.0001
	Smolt (11)	7.53	1.01	2.46	13.8	
5 (480)	Smolt (12)	14.5	1.82	6.44	29	x
6 (490)	Smolt (12)	9.27	0.54	5.25	11	x
7 (830)	Parr (11)	4.14	0.68	1.29	8.53	0.0006
	Smolt (10)	10.2	0.74	5.25	13.7	

Sampling	Parr P-value	Smolt P-value
1-2	x	0.0394
1-3	x	<.0001
1-4	0.6428	0.0001
1-5	x	<.0001
1-6	x	<.0001
1-7	0.0304	<.0001
2-3	x	0.006
2-4	x	0.2203
2-5	x	0.0002
2-6	x	0.0283
2-7	x	0.0121
3-4	x	0.7164
3-5	x	0.8003
3-6	x	0.9945
3-7	x	1
4-5	x	0.0923
4-6	x	0.9671
4-7	0.0004	0.8505
5-6	x	0.4062
5-7	x	0.7375
6-7	x	0.9996

Table 10. *Kidney Nka activity (µmoles ADP/mg protein/hour), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean (activity)	SEM (activity)	Min (activity)	Max (activity)	P-value
1 (120)	Parr (12)	14.35	0.79	7.58	16.81	0.6961
	Smolt (12)	14.74	0.53	11.30	17.71	
2 (260)	Parr (12)	14.47	0.70	10.30	17.99	0.9138
	Smolt (12)	14.36	0.85	9.72	18.21	
3 (350)	Parr (12)	16.28	0.57	12.85	18.58	0.0051
	Smolt (10)	19.30	0.88	13.68	22.71	
4 (450)	Parr (12)	15.88	0.38	13.20	17.98	0.0001
	Smolt (11)	20.03	0.66	17.61	23.03	
5 (480)	Smolt (12)	13.98	0.70	10.88	18.74	x
6 (490)	Smolt (12)	14.29	0.47	12.08	16.92	x
7 (830)	Parr (12)	11.17	1.04	6.06	20.37	<.0001
	Smolt (12)	19.78	0.83	11.90	23.03	

Sampling	Parr	Smolt
1-2	1.0000	0.9998
1-3	0.4769	0.0007
1-4	0.7334	<.0001
1-5	x	0.9882
1-6	x	0.9994
1-7	0.0327	<.0001
2-3	0.5606	0.0002
2-4	0.8050	<.0001
2-5	x	0,9997
2-6	x	1.0000
2-7	0.0226	<.0001
3-4	0.9997	0.9933
3-5	x	<.0001
3-6	x	0.0001
3-7	<.0001	0.9993
4-5	x	<.0001
4-6	x	<.0001
4-7	0.0002	1.0000
5-6	x	0.9999
5-7	x	<.0001
6-7	x	<.0001

Appendix 3. Histology

Kidney samples from different areas (A-E; see discussion 4.1.2) was fixed in 4% paraformaldehyde (PF) in standard phosphate buffer (28 mM NaH_2PO_4, 71mM Na_2HPO_4, pH 7.2) over night. The tissue was then dehydrated in 50% and 70% ethanol, each step for 30 minutes. From each area of the kidney three sepeatre blocks of tissue was embededd in a solid plastic solution (poleamyrase) according to manufaturers instructions. The block where then sectioned on a Scanscope microtome and sections (2 µm) transferred to standard glass slides. Tissue was stained with hematoxolin (toluidine blue) giving a dark blue coulour and subsequantly dried. Sections were analysed using a Whole Slide Scanner (confocal florucent slide scanner) aquring high resolution images of the entire slide that subsequnatly could be further viewed with the Carl Zeiss (Zen blue Edtion 2.6) program.

Appendix 4. Protein sequences and alignments for sulfate transporters

Table 11. *Overview of Solute Carrier Family Members (Slc13a1, Slc26a6 and Slc26a1 paralogues) protein sequences in Japanese eel, Obscure pufferfish and Atlantic salmon. The table includes protein reference number, gene identification when available, putative subcellular location, locus reference from the NCBI database and under what environmental conditions each transporter is expressed.*

Ion	Transporter name	Literature					Atlantic salmon			
		Species	Protein reference	Tissue localisation	Subcellular localization	Water conditions	Name	Protein ref	Locus ref	Verification
sulphate	slc13a1	**Anguilla japonica**	BAD22605.1	?	apical	**FW**	slc13a1	XP_014025461.1	slc13a1 (Gene ID: 106584558)	confirmed by synteny
	slc26a6a	**Takifugu obscurus**	BAE75796.1	prox tub 2	apical	**SW**	**slc26a6a1**	XP_013990168.1	LOC106566566	confirmed by synteny
							slc26a6a2?	XP_014047606.1	LOC106600630	
	slc26a6b	**Takifugu obscurus**	BAE75797.1	prox tub 2	apical	FW + SW	slc26a6b	XP_013990645.1	LOC106566781	
	slc26a6c	**Takifugu obscurus**	BAE75798.1	prox tub 1	apical	FW + SW	slc26a6c	XP_013988198.1	LOC106565505	
	slc26a1	**Anguilla japonica**	BAD22606.1	prox tub 2	**basolateral**	FW + SW	**slc26a1a**	XP_013984631.1	LOC106563502	confirmed by synteny
							slc26a1b	NP_001167113.1	slc26a2 (Gene ID: 100380357) *wrong annotation*	

Alignment Slc13a1 family:

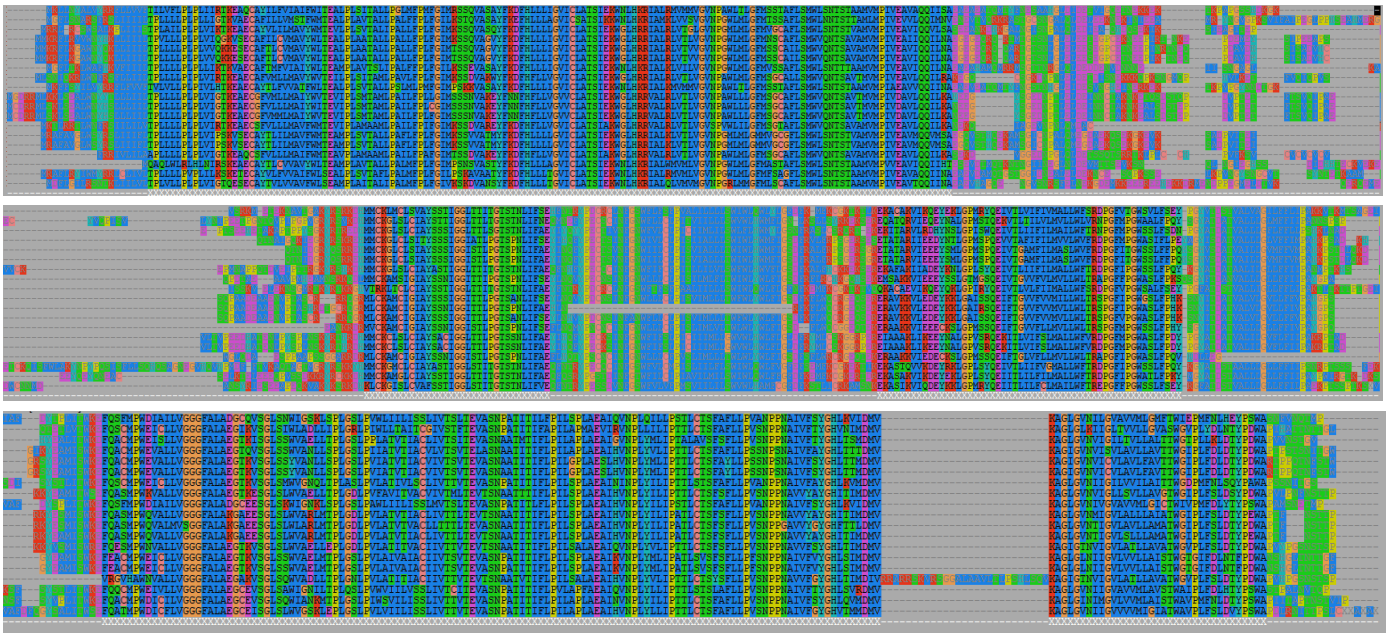

Figure 1: Putative protein sequences, Solute Carrier Family 13 Member 1, Slc13a1. *The sequences were retrieved from the NCBI database and aligned using Seaview. The GBLOCK function has removed non-conserved positions (faded in color), leaving conserved domains (not faded) to build a phylogenetic tree. The complete sequence is read from left to right for each block (1-3) and the following 19 species are located from top to bottom within each block: Mus musculus, Anguilla japonica, Danio rerio, Salmo salar (1), Salmo salar (2), Salmo salar (3), Lepistoutus oculuctus, Orizias latipes , Homo sapiens, Oreochromis niloticus (1), Oreochromis niloticus (2), Oreochromis niloticus (3), Tetraodon nigroviridis, Latimera chalumnae, Xenopus tropicalis and Callorhinchus milli. Identical sequences were removed leaving 16 out of 19 potential protein sequences for the construction of the phylogentic tree (see figure 16, main text).*

Alignment Slc26a1 family:

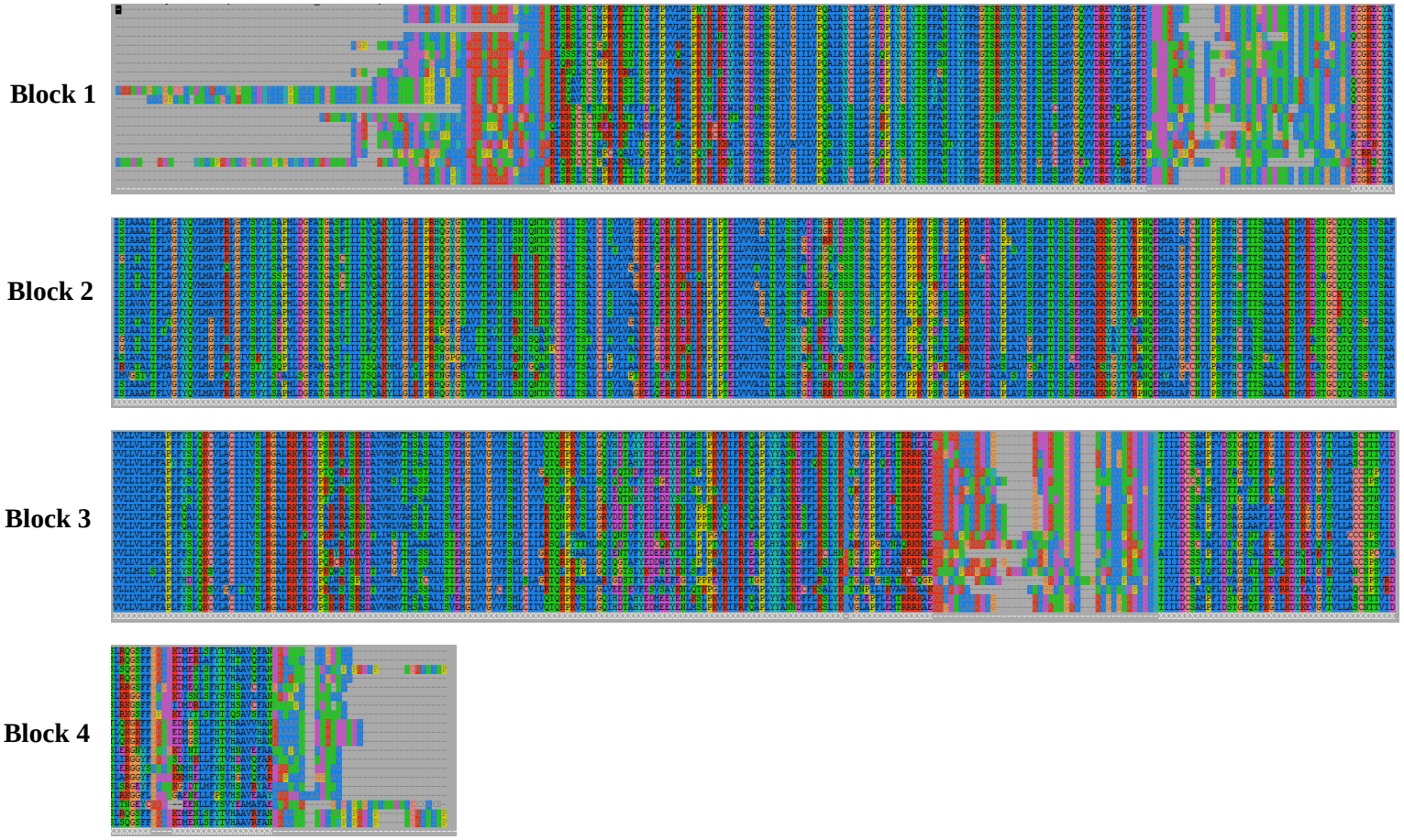

Figure 2: Putative protein sequences, Solute Carrier Family 26 member 1, Slc26a1. *The sequences were retrieved from the NCBI database and aligned using Seaview. The GBLOCK function has removed non-conserved positions (faded in color), leaving conserved domains (not faded) to build a phylogenetic tree. The complete sequence is read from left to right for each block (1-4) and the following 20 species are located from top to bottom within each block: Salmo salar (1), Oncorhynchus mykiss (1), Anguilla japonica, Esox lucius, Carassius auratus, Lepistoutus oculuctus, Danio rerio, Astyanax mexicanus, Oreochromis niloticus (1), Oreochromis niloticus (2), Oreochromis niloticus (3), Latimera chalumnae, Xenopus tropicalis, Anolis carolinensis, Pelodiscus sinensis, Callorhinchus milli, Mus musculus, Homo sapiens, Salmo salar (2) and Oncorhynchus mykiss (2). Identical sequences were removed leaving 18 out of 20 potential protein sequences for construction of the phylogentic tree (see figure 17, main text).*

Alignment Slc26a6 family:

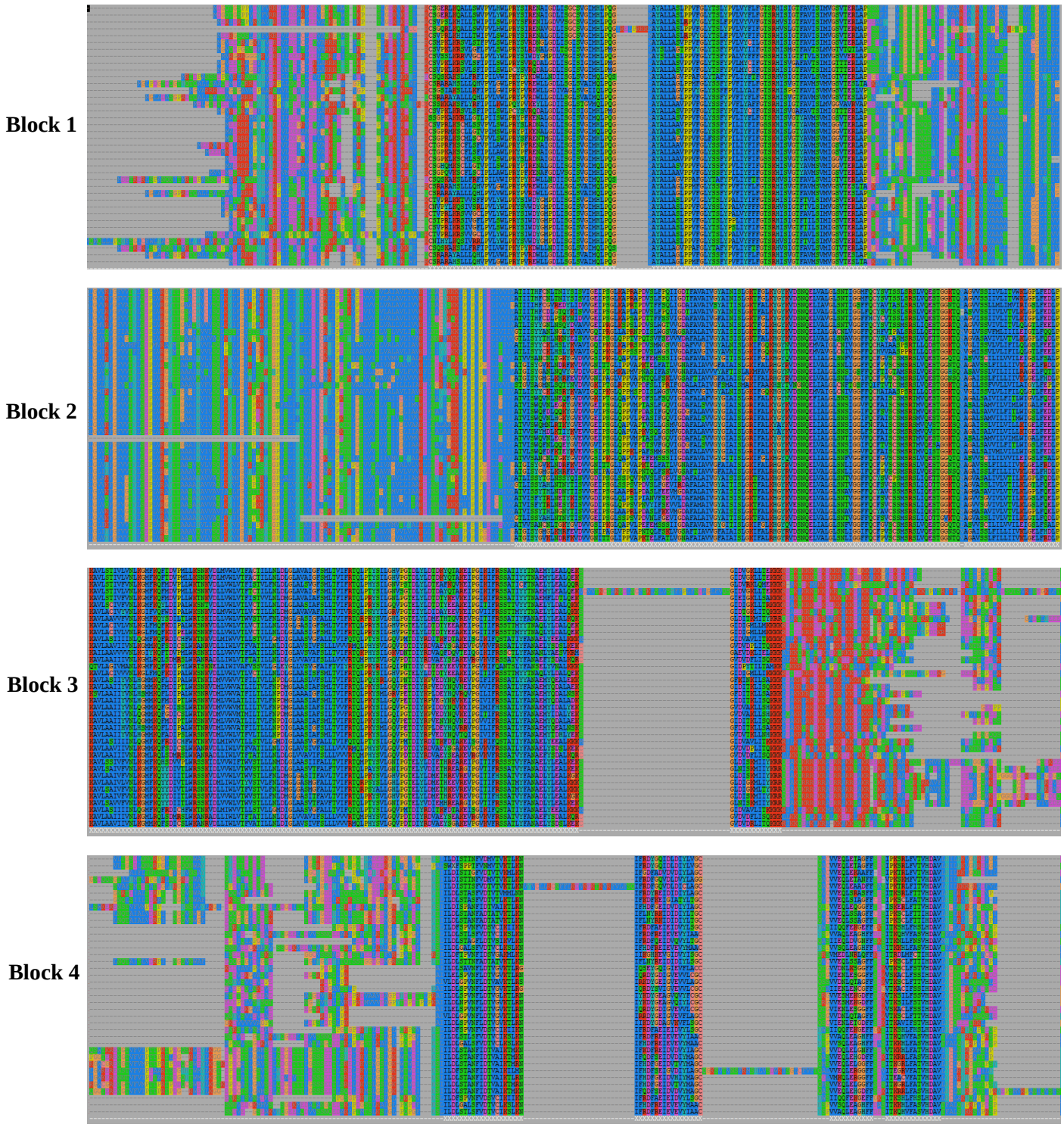

Figure 3: Putative protein sequences, Solute Carrier Family 26 member 6, Slc26a6. *The sequences were retrieved from the NCBI database and aligned using Seaview. The GBLOCK function has removed non-conserved positions (faded in color), leaving conserved domains (not faded) to build a phylogenetic tree. The complete sequence is read from left to right for each block (1-4) and the following 38 species are located from top to bottom within each block: Takifugu obscurus (1), Takifugu rubripes, Orizias latipes, Tetraodon nigrovirid, Oreochromis niloticus, Salmo salar (1), Esox Lucius,* Astyanax mexicanus, *Oncorhynchus mykiss (1), Oncorhynchus mykiss (2),* Callorhinchus milli , *Mus musculus,* Xenopus tropicalis, *Homo sapiens*, Latimera chalumnae, *Salmo salar (1), Takifugu obscurus (2), Salmo salar (2), Oreochromis niloticus, Esox lucius (2), Carassius auratus, Danio rerio, Oncorhynchus mykiss (3), Haplochromis burtoni, Astyanax mexicanus, Takifugu obscurus (3), Oreochromis niloticus (2), Salmo salar (3), Carassius auratus (2), Danio rerio (2), Oncorhynchus mykiss (4), Haplochromis burtoni (2). Identical sequences were removed leaving 32 out of 38 potential protein sequences for construction of the phylogentic tree (see figure 18, main text).*

Gene identity confirmation for Slc13a1, Slc26a1 and Slc26a6 protein family:

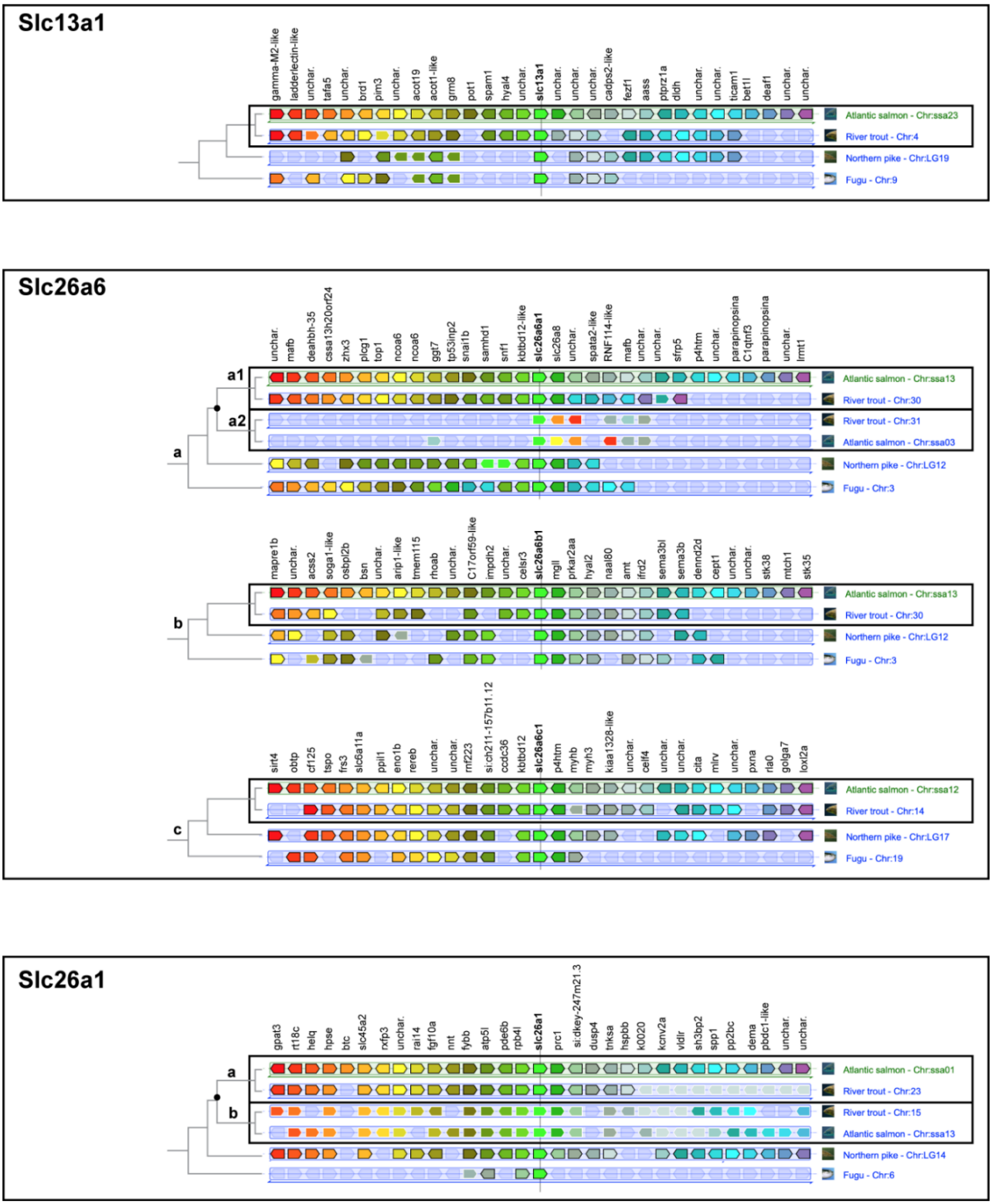

Figure 4. Gene identity confirmation for Slc13a1, Slc26a1 and Slc26a6 protein family. *A synteny analysis was performed, comparing the chromosomal arrangement of genes surrounding the Slc13a1, Slc26a1 and Slc26a6 genes. The gene environment for each transporter was retrieved using- Ensembl genome browser annotations via the Genomicus platform (Nguyenet al., 2018), complemented with salmon sequences from the NCBI Genbank when not available in Ensemble. Genomicus and the genes around the salmon transporters was manually analyzed on the genome browser NCBI and, subsequently visualized with InkScape. The synteny results concluded that Atlantic salmon underwent a fourth round of genome duplication not observed in the closely related Northern pike (Esox lucius). Paralogs was detected for slc26a1 (slc26a1a and slc26a1b) and slc26a6a (slc26a6a1 and slc26a6a2) in the Atlantic salmon genome.*

Appendix 5. Gene expression information including primer design, reference genes, melt curves, tissue distribution, mRNA abundance

Appendix 5a. Primer design sulfate transporters and stability of reference genes

Table 12: *Overview of all forward (F) and reverse (R) primers designed for putative solute carrier family 26 member 1 (slc26a1a, slc26a1b) and member 6 (slc26a6a1, slc26a6a2, slc26a6b, slc26a6c) and family 13 member 1 (slc13a1) in the Atlantic salmon. The table includes gene name, primers sets, sequence (5` to 3` direction), Amplicon length (nucleotides), position (exon (ex) junctions (jct)), primer length (nucleotides), GC content (%), melting temperature (Tm; basic, salt adjusted, nearest neighbor), primer dimer formation (Hairpin, Complementarity, Self-annealing) and Ta (Annealing temperature).*

Salmon sulfate transporter primers

Gene	Primer	Sequence	Amplicon length	Position	Primer length	GC %	*Oligo calc*						*Thermo Fisher*
							Tm basic	Tm salt adjusted	Tm nearest neighbor	Hairpin	Complemen-tarity	Self-annealing	Ta
Slc26a6a1	Slc26a6a1F	CTCATCTCCTACTACGGCAACCTG	83	Ex 6	24	54	59.1	66.9	58.53	no	no	no	66.9
	Slc26a6a1R	CTGGGAGACTTCAGCCCTCTG		Ex 6-7 jct	21	62	58.3	65.3	57.24	no	no	no	
Slc26a6a2	Slc26a6a2F	GACCTGAAATTGAACCAGACGGCC	200	Ex 17	24	54	59.1	66.9	57.73	no	no	no	66.4
	Slc26a6a2R	GTGTGTGTCGTTGACGGAGTTC		Ex 18	22	55	56.7	64.2	54.99	no	no	no	
Slc26a6a2 isoform X1	Slc26a6a2X1F	CACCAGACCTCTCCTGAAGGAACG	152	Ex 3	26	58	62.7	71.1	61.65	no	no	no	69
	Slc26a6a2X1R	GAGGTCTCCCAGTGCGTCTTGTC		Ex 4	23	61	60.6	68.3	58.81	no	no	no	
Slc26a6a2 isoform X2	Slc26a6a2X2F	CATGGTGAAAATGAGGCGC	150	Ex 2-6 jct	19	53	51.1	57.5	51.98	no	no	no	62.7
	Slc26a6a2X2R	GTACTCTGAAGGCATCACGG		Ex 6	20	55	53.8	60.5	52.88	no	no	no	
Slc26a6b	Slc26a6bF	ACAGAGAGGTGCTGGATGAGGG	107	Ex 1	22	59	58.6	65.8	57.98	no	no	no	67.4
	Slc26a6bR	GGGGACAGAACACCTCACTGAC		Ex 1-2 jct	22	59	58.6	65.8	55	no	no	no	
Slc26a6c	Slc26a6cF	GTACTGGATGAGCAGAGACTGGAGG	112	Ex 1	25	56	61	69.1	59.73	no	no	no	68.5
	Slc26a6cR	GCCTGGGTACAGTACATCTGAAGGACTC		Ex 1-2 jct	28	54	62.9	71.8	60.73	no	no	no	
Slc13a1	Slc13a1F	ACCCTCTCAGACCAATGCGATTGG	132	Ex 6-7 jct	24	54	59.1	66.9	59.32	no	no	no	69.5
	Slc13a1R	GGAAGGGTGGCAATCCCTCCTATAGAG		Ex 7	27	56	62.8	71.5	60.74	no	no	no	
Slc26a1a	Slc26a1aF	GTAGAGCGAGTTGGTTGTGAGG	146	Ex 1	22	55	56.7	64.2	55.86	no	no	no	65.6
	Slc26a1aR	GCTGTGCTCCCACACTTCG		Ex 1-2 jct	19	63	55.4	61.6	55.25	no	no	no	
Slc26a1b	Slc26a1bF	GGTTGGGATTTACCAGGTGTTGATGG	190	Ex 2-3 jct	26	50	59.5	67.9	56.96	no	no	no	67.8
	Slc26a1bR	GATCCAGGTGACCACCACTGTTC		Ex 3	23	57	58.8	66.6	55.52	no	no	no	
Slc26a1b isoform X1	Slc26a1bX1F	GTTGGCTGTAAGTGTGAGGGAC	188	Ex 1	22	55	56.7	64.2	54.3	no	no	no	64.7
	Slc26a1bX12R	CCTCTGGAAGTGGTAGGCTG		Ex 2	20	60	55.9	62.5	54.3	no	no	no	
Slc26a1b isoform X2	Slc26a1bX2F	AGAAGAGGAGGCATGGCTAC	113	Ex 1	20	55	53.8	60.5	53.9	no	no	no	64.3
	Slc26a1bX12R	*CCTCTGGAAGTGGTAGGCTG*		*Ex 2*	*20*	*60*	*55.9*	*62.5*	*54.3*	*no*	*no*	*no*	
Slc26a1b isoform X3	Slc26a1bX3F	GTGACACATGTTGGCTGAGCAC	161	Ex 1-2 jct	22	55	56.7	64.2	55.89	no	no	no	66
	Slc26a1bX3R	GCTTCGTCTTCAGGATGGCC		Ex 2	20	60	55.9	62.5	55.42	no	no	no	

Appendix 5b. Dilution curves, Melt curves and Primer efficiency

Realtime qPCR treshold cyles against cDNA dilutions: (1:10, 1:20, 1:40, 1:80, 1:160) from Atlantic salmon kidney (figure 5). X-axis is log transformed. Similar dilution series done for sulfate transporters and refrence genes expressed in intestine and gills, but results not included here. The primer efficiency E was determined based on the slope of the regression line generated from the dilution curve was all above 80 % (see discussion 4.1.3). Linear regression results are also shown (Figure 5). Representative melt curves for all target genes kidney shows one singel peak, suggesting only one spefic PCR product being amplified (figure 6).

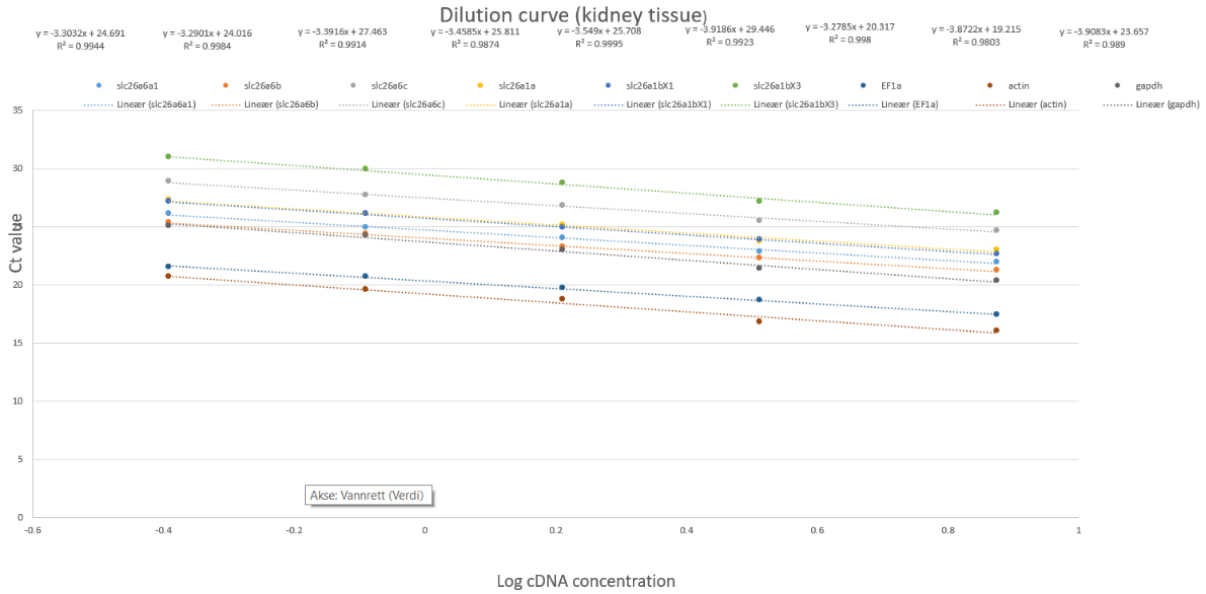

Figure 5: *Dilution curve for target genes and reference genes. Ct values was investigated in a dilution series of all target genes (slc26a6a1, slc26a6b, slc26a6c, slc26a1a, slc26a1bX1 and slc26a1bX3) and reference genes (ef1a, b-actin and gapdh). Each dilution was performed in triplicates (n=3) for each individual gene.*

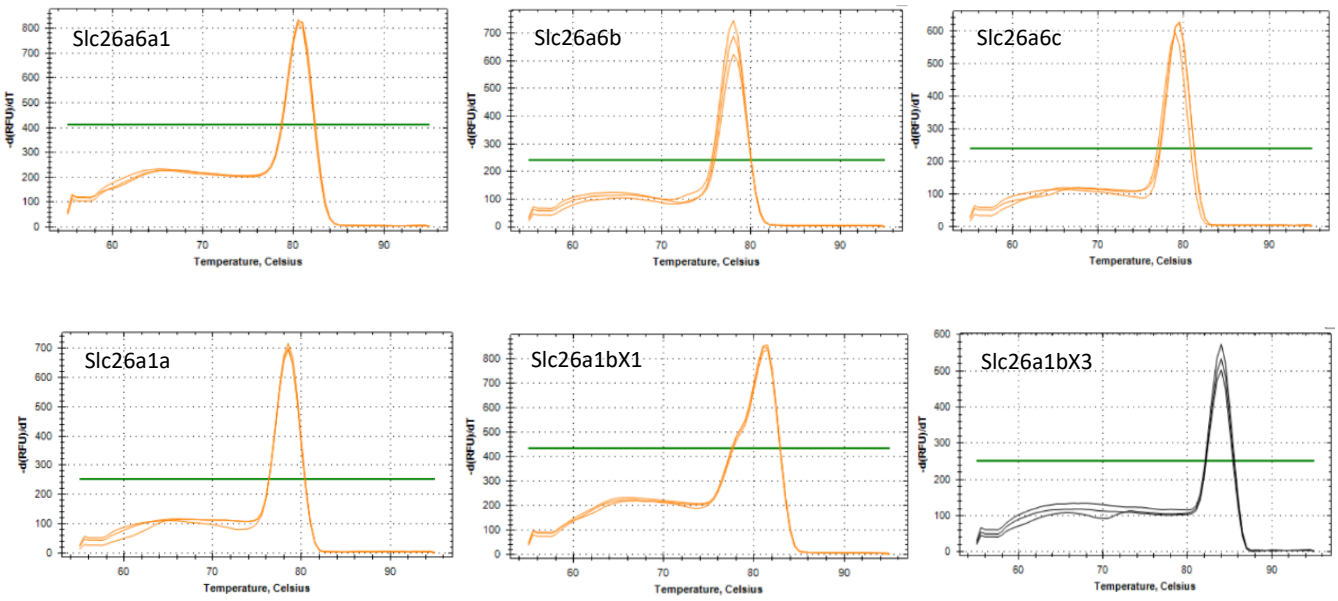

Figure 6: Overview melt curves for all target primers *slc26a6a1, slc26a6b, slc26a6c, slc26a1a, slc26a1bX1, slc26a1bX3* in the kidney. *All genes display one single peak demonstrating that there is only one specific PCR product being amplified.*

Appendix 5c: Tissue distribution of the sulfate transporters

Threshold levels (Ct values) indicate tissue specific distribution and mRNA abundance in gills, intestine, kidney, liver and the urinary bladder of both FW and SW acclimated Atlantic salmon (figure 7 and 8).

Tissue distribution of the slc13a1, slc26a1a, slc26a1bX1 and slc26a1bX3:

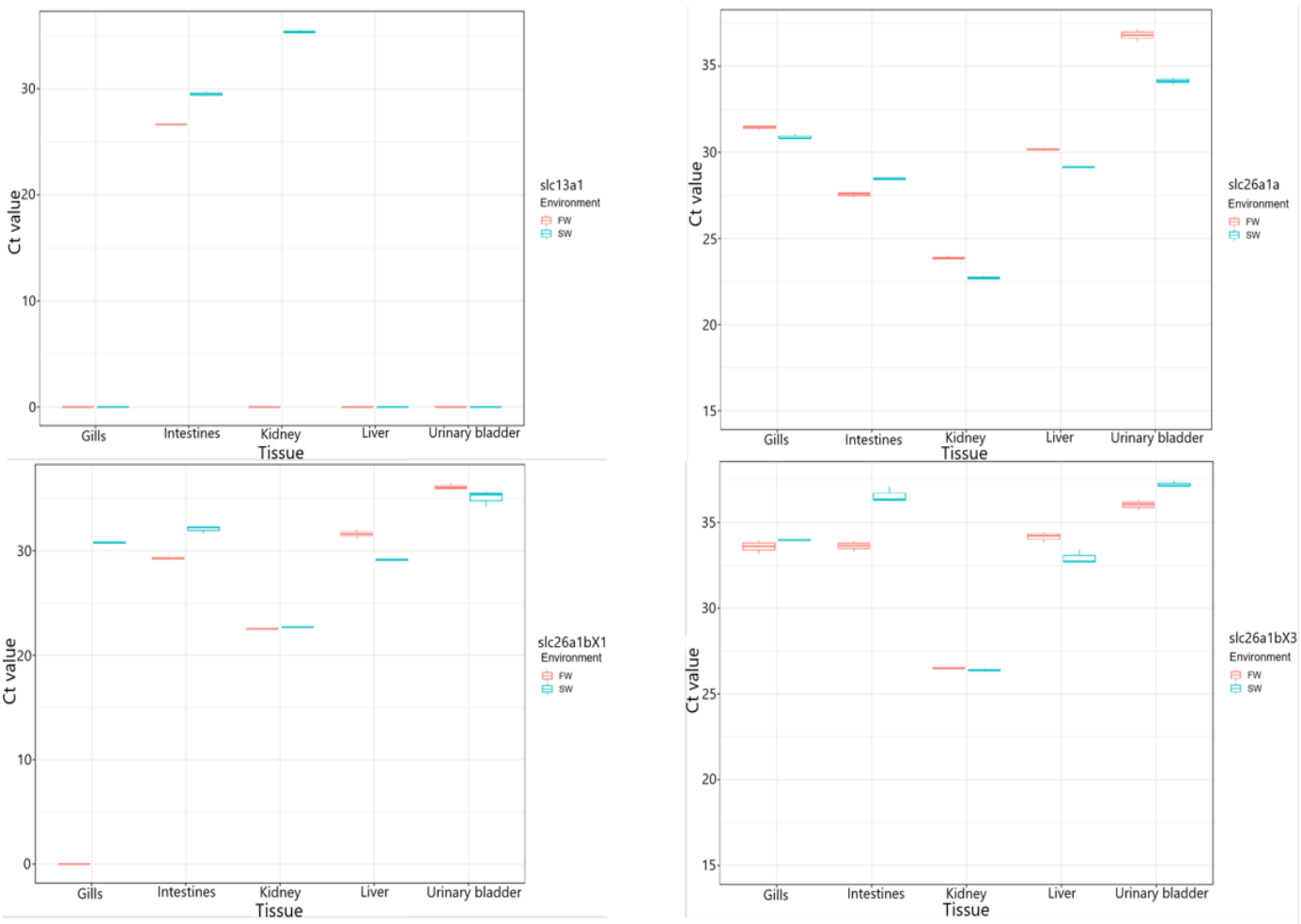

Figure 7: Tissue distribution of the slc13a1, slc26a1, slc26a1bX1 and slc26a1bX3. *Threshold cycles (Ct) in intestine, kidney, liver and urinary bladder indicate tissue specific distribution and mRNA abundance putative sulfate transporters distribution in FW (red) and SW (blue) acclimated Atlantic salmon. Each individual tissue is based on Ct values from triplicate reactions from cDNA pool of three individual salmon (N=3) in FW and SW.*

Tissue distribution of the slc26a6a1, slc26a6a2, slc26a6b and slc26a6c:

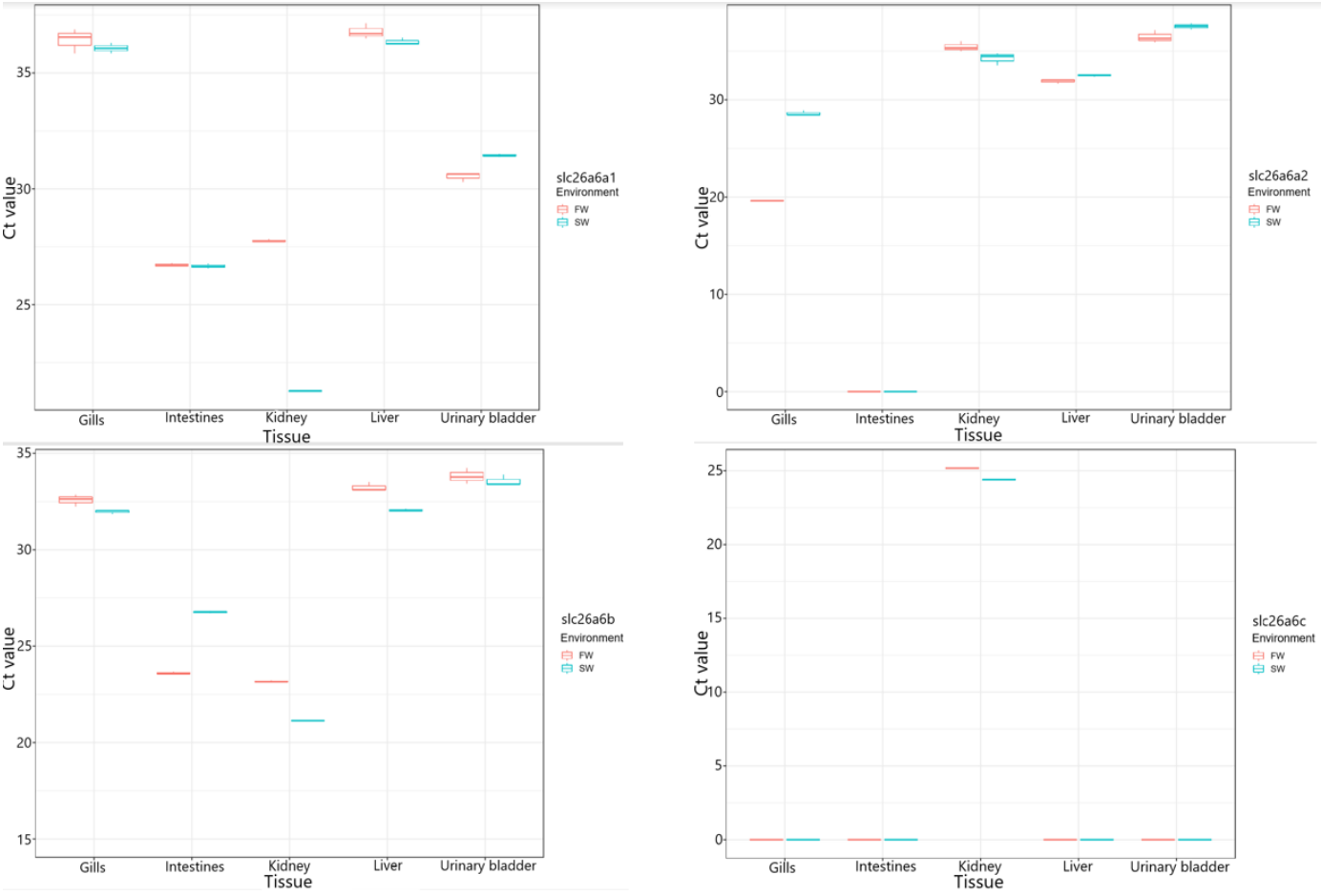

Figure 8: Tissue distribution of the slc26a6a1, slc26a6a2, slc26a6b and slc26a6c. *Threshold cycles (Ct) in intestine, kidney, liver and urinary bladder indicate tissue specific distribution and mRNA abundance putative sulfate transporters distribution in FW (red) and SW (blue) acclimated Atlantic salmon. Each individual tissue is based on Ct values from triplicate reactions from cDNA pool of three individual salmon (N=3) in FW and SW.*

Appendix 5d. Reference gene stability

Gene stability of the three reference genes in the kidney. All three reference genes displayed relatively equal Ct values during the entire experiment with no significant difference between datapoints within actin, gapdh and ef1a group (figure 9). Thus, there was less variation in the *ef1a* after running more comprehensive tests (figure 10). All fish (parr, smolt) have been pooled together for each sampling point to find the reference gene with highest stability. These results were similar in gills, thus *ef1a* was selected for this tissue as well.

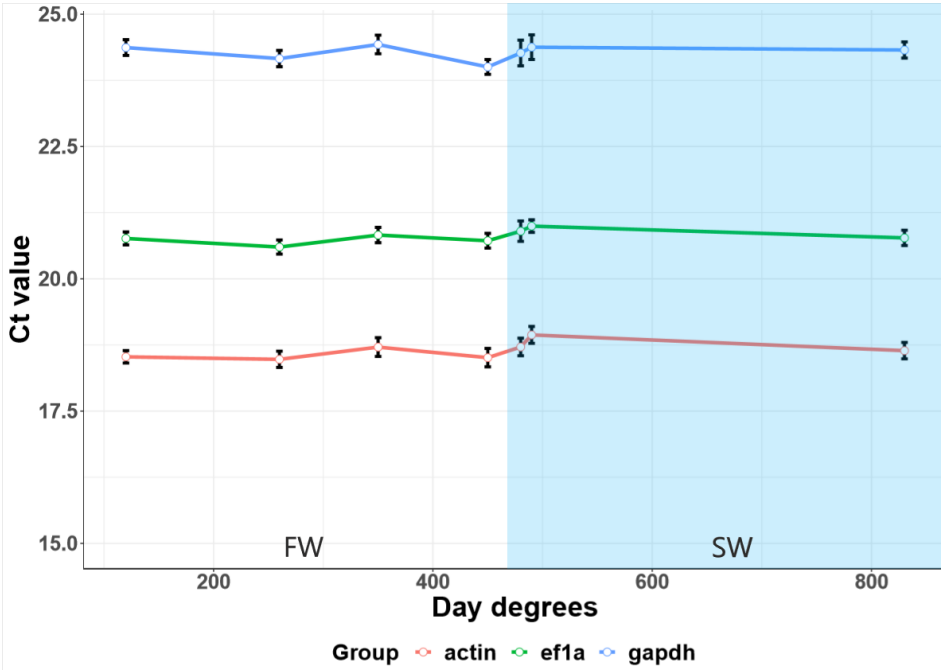

Figure 9: Reference gene overview during the experiment for actin, gapdh and ef1a in the kidney. The stability of all three reference genes was relatively equal during the experiment. No significant difference was found between timepoints (n=20) or in any of the selected reference genes in the kidney cDNA samples (n=140).

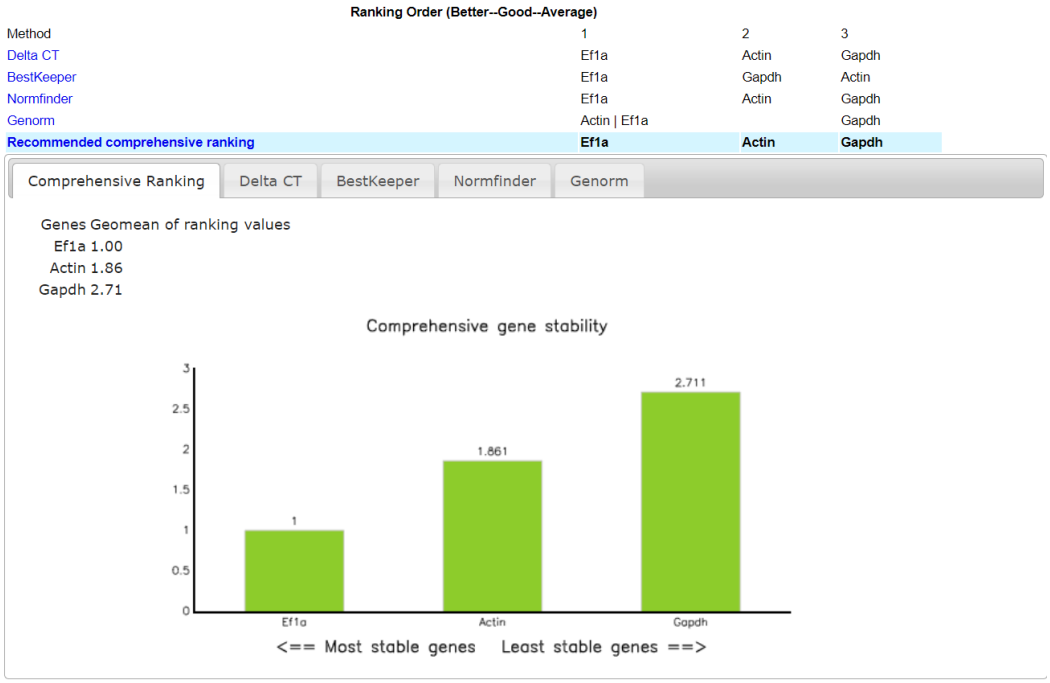

Figure 10. Comprehensive analysis of reference gene stability. *The most stable reference gene was selected based on several tests ranking reference genes from most stable to least stable. The ef1a was found to be the most stable gene in the kidney based on the criteria incorporated in RefFinder (Xie et al., 2012)*

Appendix 5d. Statistical analysis relative mRNA abundance of SO_4^{2-} transporters

Slc26a6a1:

Table 13. *Slc26a6a1 mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (10)	0.22	0.032	0.03	0.42	<.0001
	Smolt (10)	0.68	0.11	0.30	1.16	
2 (260)	Parr (10)	0.18	0.01	0.13	0.25	<.0001
	Smolt (10)	1.85	0.14	1.20	2.49	
3 (350)	Parr (10)	0.17	0.017	0.08	0.28	<.0001
	Smolt (10)	2.04	0.16	1.33	3.01	
4 (450)	Parr (10)	0.18	0.019	0.11	0.30	
	Smolt (10)	2.43	0.21	1.54	3.49	<.0001
5 (480)	Smolt (10)	2.45	0.23	1.64	3.76	x
6 (490)	Smolt (10)	3.54	0.17	2.89	4.32	x
7 (830)	Parr (10)	0.39	0.077	0.16	0.81	<.0001
	Smolt (10)	4.26	0.24	3.06	5.69	

Sampling	Parr	Smolt
1-2	0.8271	<.0001
1-3	0.7214	<.0001
1-4	0.8267	<.0001
1-5	x	<.0001
1-6	x	<.0001
1-7	0.0104	<.0001
2-3	1.0000	0.9964
2-4	1.0000	0.5943
2-5	x	0.5720
2-6	x	0.0021
2-7	0.0001	<.0001
3-4	1.0000	0.9167
3-5	x	0.9052
3-6	x	0.0133
3-7	0.0001	0.0003
4-5	x	1.0000
4-6	x	0.2138
4-7	0.0001	0.0112
5-6	x	0.2282
5-7	x	0.0123
6-7	x	0.8956

Slc26a1a:

Table 14. *Slc26a1a mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (10)	17.87	1.30	12.95	27.80	0.1524
	Smolt (10)	20.74	1.12	14.59	26.54	
2 (260)	Parr (10)	15.42	1.41	11.72	22.35	<.0001
	Smolt (10)	26.73	1.61	19.28	33.06	
3 (350)	Parr (10)	13.92	1.09	7.25	18.17	<.0001
	Smolt (10)	25.27	1.38	20.31	31.53	
4 (450)	Parr (10)	15.52	0.86	12.52	20.31	
	Smolt (10)	24.10	1.20	18.12	32.11	<.0001
5 (480)	Smolt (10)	23.02	1.88	14.95	32.20	x
6 (490)	Smolt (10)	21.30	1.13	17.70	27.30	x
7 (830)	Parr (10)	20.46	1.05	16.05	26.83	0.0003
	Smolt (10)	27.89	2.26	18.41	41.23	

Sampling	Parr	Smolt
1-2	0.8807	0.0504
1-3	0.4341	0.2682
1-4	0.9006	0.6267
1-5	x	0.9137
1-6	x	1.0000
1-7	0.8517	0.0090
2-3	0.9891	0.3404
2-4	1.0000	0.8433
2-5	x	0.5109
2-6	x	0.1030
2-7	0.1597	0.9972
3-4	0.9815	0.9972
3-5	x	0.9179
3-6	x	0.4277
3-7	0.0232	0.8444
4-5	x	0.9981
4-6	x	0.7981
4-7	0.1783	0.4867
5-6	x	0.9776
5-7	x	0.1917
6-7	x	0.0215

Slc26a1bX1:

Table 15. *Mean Slc26a1bX1 mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included.*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (10)	31.04	3.79	4.27	43.45	0.6633
	Smolt (10)	28.78	3.24	9.40	47.35	
2 (260)	Parr (10)	29.34	3.22	25.49	45.76	0.9190
	Smolt (10)	29.86	2.29	15.35	44.23	
3 (350)	Parr (10)	30.30	4.31	5.72	51.39	0.9756
	Smolt (10)	30.46	2.57	19.01	41.15	
4 (450)	Parr (10)	32.07	4.26	6.30	53.03	
	Smolt (10)	30.18	2.71	16.47	41.24	0.7158
5 (480)	Smolt (10)	34.80	3.73	15.92	59.17	x
6 (490)	Smolt (10)	31.32	3.18	16.03	47.99	x
7 (830)	Parr (10)	38.94	5.99	16.97	80.38	94.29
	Smolt (10)	39.31	3.08	28.40	55.67	

Sampling	Parr	Smolt
1-2	0.9999	1.0000
1-3	1.0000	0.9999
1-4	1.0000	1.0000
1-5	x	0.9062
1-6	x	0.9989
1-7	0.7273	0.3981
2-3	1.0000	1.0000
2-4	0.9984	1.0000
2-5	x	0.9627
2-6	x	1.0000
2-7	0.5133	0.5334
3-4	0.9999	1.0000
3-5	x	0.9804
3-6	x	1.0000
3-7	0.6374	0.6102
4-5	x	0.9730
4-6	x	1.0000
4-7	0.8368	0.5737
5-6	x	0.9939
5-7	x	0.9758
6-7	x	0.7169

Slc26a1bX3:

Table 16. *Mean Slc26a1bX3 mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (10)	7.88	0.96	0.75	10.46	0.6337
	Smolt (10)	8.51	0.86	3.20	13.86	
2 (260)	Parr (10)	6.85	0.72	5.38	10.11	0.2407
	Smolt (10)	8.42	0.62	5.08	12.29	
3 (350)	Parr (10)	7.20	0.97	1.30	11.76	0.0195
	Smolt (10)	10.36	0.76	6.61	13.01	
4 (450)	Parr (10)	7.91	1.03	1.43	12.69	
	Smolt (10)	9.85	0.83	5.25	13.35	0.1494
5 (480)	Smolt (10)	10.49	1.13	5.17	18.22	X
6 (490)	Smolt (10)	8.31	0.69	5.34	11.94	X
7 (830)	Parr (10)	10	1.46	5.64	20.08	0.1125
	Smolt (10)	12.13	0.93	9.38	17.53	

Sampling	Parr	Smolt
1-2	0.9870	1.0000
1-3	0.9987	0.8087
1-4	1.0000	0.9526
1-5	x	0.7515
1-6	x	1.0000
1-7	0.6840	0.1031
2-3	1.0000	0.7691
2-4	0.9844	0.9344
2-5	x	0.7077
2-6	x	1.0000
2-7	0.2207	0.0865
3-4	0.9982	0.9997
3-5	x	1.0000
3-6	x	0.7198
3-7	0.3561	0.8338
4-5	x	0.9990
4-6	x	0.9084
4-7	0.7012	0.6052
5-6	x	0.6547
5-7	x	0.8799
6-7	x	0.0701

Slc26a6b:

Table 17. *Slc26a6b mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (10)	4.46	0.49	0.46	6.01	0.2575
	Smolt (10)	3.82	0.32	2.40	5.62	
2 (260)	Parr (10)	4.42	0.48	3.02	6.63	0.5925
	Smolt (10)	4.72	0.33	3.71	7.20	
3 (350)	Parr (10)	3.63	0.44	1.08	5.53	0.0214
	Smolt (10)	4.95	0.41	3.28	6.76	
4 (450)	Parr (10)	3.96	0.39	0.95	5.56	
	Smolt (10)	4.90	0.39	3.03	7.05	0.0976
5 (480)	Smolt (10)	3.53	0.30	1.92	5.69	x
6 (490)	Smolt (10)	3.70	0.17	3.18	4.89	x
7 (830)	Parr (10)	5.05	0.40	2.45	6.81	0.2974
	Smolt (10)	5.64	0.56	3.32	9.10	

Sampling	Parr	Smolt
1-2	1.0000	0.6887
1-3	0.7580	0.4234
1-4	0.9718	0.4788
1-5	x	0.9987
1-6	x	1.0000
1-7	0.9467	0.0280
2-3	0.8050	0.9996
2-4	0.9829	0.9999
2-5	x	0.3606
2-6	x	0.5536
2-7	0.9233	0.6686
3-4	0.9974	1.0000
3-5	x	0.1680
3-6	x	0.3034
3-7	0.1684	0.8863
4-5	x	0.2012
4-6	x	0.3515
4-7	0.4673	0.8492
5-6	x	0.9999
5-7	x	0.0057
6-7	x	0.0151

Slc26a6c:

Table 18. *Mean Slc26a6c mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (10)	1.89	0.22	0.24	2.64	0.0581
	Smolt (10)	1.39	0.16	0.71	2.42	
2 (260)	Parr (10)	1.96	0.24	0.98	2.87	0.6862
	Smolt (10)	1.85	0.14	1.12	2.59	
3 (350)	Parr (10)	1.55	0.27	0.29	3.42	0.0637
	Smolt (10)	2.03	0.18	1.43	3.34	
4 (450)	Parr (10)	1.61	0.13	0.53	1.95	
	Smolt (10)	1.96	0.19	1.02	2.81	0.1704
5 (480)	Smolt (10)	1.99	0.13	1.34	2.40	x
6 (490)	Smolt (10)	1.67	0.15	0.97	2.48	x
7 (830)	Parr (10)	1.66	0.13	1.20	2.32	0.2535
	Smolt (10)	1.96	0.19	1.31	3.18	

Sampling	Parr	Smolt
1-2	1.0000	0.5594
1-3	0.8406	0.1815
1-4	0.9327	0.2959
1-5	x	0.2358
1-6	x	0.9326
1-7	0.9765	0.3066
2-3	0.6839	0.9934
2-4	0.8232	0.9995
2-5	x	0.9979
2-6	x	0.9919
2-7	0.9126	0.9996
3-4	1.0000	1.0000
3-5	x	1.0000
3-6	x	0.8072
3-7	0.9993	1.0000
4-5	x	1.0000
4-6	x	0.9157
4-7	1.0000	1.0000
5-6	x	0.8694
5-7	x	1.0000
6-7	x	0.9222

Slc26a6a2:

Table 19. *Mean Slc26a6a2 mRNA abundance (ma), mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon parr and smolt. Number of individuals (N) in each group and timepoint is included*

Sampling (day degrees)	Group (n)	Mean (ma)	SEM (ma)	Min (ma)	Max (ma)	P-value
1 (120)	Parr (7)	172.6	25.9	96.63	272	0.6269
	Smolt (9)	154.2	33.7	87.72	391	
2 (260)	Parr (8)	218.6	22.1	110.6	299	0.0002
	Smolt (6)	59.04	6.32	35.01	75.18	
3 (350)	Parr (8)	142.2	15.2	82.9	208	0.0001
	Smolt (9)	51.35	6.16	27.4	84	
4 (450)	Parr (10)	206	19	86.6	306	
	Smolt (10)	49.1	5.87	27.4	89.9	<.0001
5 (480)	Smolt (10)	25.76	4.18	12.4	54.8	x
6 (490)	Smolt (10)	13.59	2.78	3.15	27.3	x
7 (830)	Parr (10)	162	18.4	94.4	250	<.0001
	Smolt (10)	0.1	0.02	0.02	0.22	

Sampling	Parr	Smolt
1-2	0.9614	0.0304
1-3	0.9845	0.0008
1-4	0.9887	0.0002
1-5	x	<.0001
1-6	x	<.0001
1-7	1.0000	<.0001
2-3	0.5576	0.9974
2-4	1.0000	0.9866
2-5	x	0.0140
2-6	x	<.0001
2-7	0.8225	<.0001
3-4	0.6775	1.0000
3-5	x	0.0419
3-6	x	<.0001
3-7	0.9973	<.0001
4-5	x	0.0615
4-6	x	<.0001
4-7	0.9131	<.0001
5-6	x	0.0655
5-7	x	<.0001
6-7	x	<.0001