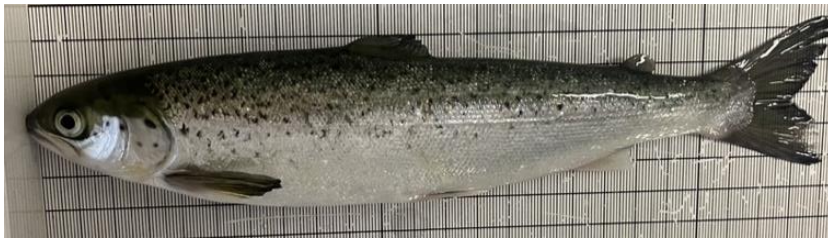
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

DEVELOPMENT OF OSMO- AND APPETITE REGULATION DURING TRANSFER OF SMOLT FROM FRESHWATER TO INCREASING SALINITIES



Jonna Hellsten

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Supervisor: Elisabeth Jönsson Bergman, Biological and environmental sciences
Examiner: Michael Axelsson, Biological and environmental sciences

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Abstract

Atlantic salmon (*Salmo salar*) is common in Scandinavian aquaculture and must be transferred from freshwater to seawater to grow properly. Despite being assessed as ready smolts, meaning they have gone through the physiological changes to prepare for SW transfer, the transfer result in a mortality rate of around 13-19%, and significant appetite- and growth reductions. The aim of this study was to get a timeline of appetite- and osmoregulatory development as salmon are transferred from freshwater to higher salinities, to detect potential mismatches between osmoregulatory tissues, and alterations in central appetite control that can explain the reduction in appetite and growth. Na^+/K^+-ATPase (NKA) activity using a kinetic enzyme assay, and gene expression of NKA α-isoforms *(α1a, α1b, α1c* and *α3a*) were measured in the proximal and distal intestine, gills, and kidney using qPCR. Appetite regulating factors (*npy, agrp, cart, cck-l, pomc-a* and *pomc-b*) were measured (qPCR) in the hypothalamus, from six time points: freshwater (0 ppt), brackish water (12 ppt) and 3, 6, 12 and 30 days after seawater transfer (30 ppt). The NKA activity and expected isoform expression were timely developed in the gill and intestine. The development of NKA activity and isoform expression in kidney have previously been scarcely studied. The isoform *nkaα1c* showed the most abundant expression in the kidney, peaking after three days in seawater, indicating a role of this isoform in renal hypo-osmotic regulation. The expression of *npy, cart* and *cck-l* increased temporarily upon transfer, followed by a decline after 30 days in seawater. The *agrp* expression was low in all timepoints until an increase after 30 days in seawater. The results indicate no mismatches in NKA activity or isoform development in the analyzed tissues. The changes in central appetite control suggest a temporary decrease in appetite upon seawater transfer increasing again after 30 days.

Sammanfattning

Atlantlax (*Salmo salar*) är en av de vanligaste odlade arterna i skandinavisk fiskodling. I odlingarna måste laxen flyttas från färskvatten till saltvatten för att få en optimal tillväxt. Trots att fisken bedöms vara smoltifierad, vilket innebär att den genomgått de fysiologiska förändringar som förbereder den för saltvattenöverföringen, innebär processen en dödlighet på ungefär 13-19%, samt minskad aptit och tillväxt. Syftet med studien var att få en tidslinje över Atlantlaxens utveckling av saltvattentolerans och aptitreglering under överföring från färskvatten till brack- och saltvatten, för att upptäcka potentiella mismatchningar mellan osmoreglerande vävnader, samt förändringar i den centrala aptitkontrollen, som kan förklara den reducerade aptiten och tillväxten. Sex provtagningar utfördes i: färskvatten (0 ppt), bräckt vatten (12 ppt), och sedan 3, 6, 12 och 30 dagar efter saltvattenöverföring (30 ppt). Genutrrycket av olika α-isoformer *(α1a, α1b, α1c* och *α3a*) mättes med qPCR och enzymaktivitet av Na^+/K^+-ATPas (NKA) mättes med en enzymaktivitetsanalys i den proximala och distala tarmen, gälen och njuren, och aptitreglerande faktorer (*npy, agrp, cart, cck-l, pomc-a* och *pomc-b*) mättes i hypotalamus. Resultaten visade inga mismatchningar i NKA-aktiviteten och uttrycket av de förväntade isoformerna i tarmen eller gälen. Isoform *α1c* var högt uttryckt vid saltvattenöverföring i njuren, vilket indikerar att denna isoform är inblandad i njurens roll i utvecklingen av saltvattentolerans. Uttrycket av *npy, cart* och *cck-l* ökade tillfälligt vid överföringen till saltvatten för att sedan sjunka efter 30 dagar, medan *agrp* var lågt uttryckt i alla tidpunkter för att sedan öka efter 30 dagar i saltvatten. Slutsatsen var att en mismatch i utveckling av aktivitet eller isoform-uttryck i de studerade vävnaderna inte ligger bakom dödligheten. Förändringarna i uttryck av aptitfaktorerna under- och efter saltvattenöverföringen indikerade en tillfällig minskning i aptit den närmsta tiden efter överföringen, följt av en ökning igen efter 30 dagar.

Introduction

Osmoregulatory challenges for freshwater, seawater, and anadromous fish

Most teleost fish are osmoregulators, meaning they keep a constant body osmolality of around 300-350 mOsmol/L regardless of the surrounding osmolality (Currie & Evans, 2021). In freshwater (FW), where osmolality can be as low as almost 0 mOsmol/L, the fish must counteract adverse gain of water and loss of ions due to the low osmolality of the surrounding environment (Kültz, 2015). This is accomplished by active uptake of ions, for example through the gills and from the feed in the gut, and disposal of water by production of large volumes of dilute urine. On the contrary, in seawater (SW), where osmolality in the oceans is around 1000 mOsmol/kg, the fish risks excessive gain of ions and loss of water (Evans, 2008). This calls for opposed means of regulation as compared to those in FW. To counteract the loss of water and gain of ions, the fish drink large volumes of water in combination with an increased intestinal ion-coupled water uptake, while producing low urine volumes, and actively secrete excess ions via their gills and kidneys (Marshall and Grosell, 2005; Kültz, 2015).

There are species that can live in both FW and SW despite the vast differences in physiological requirements. These are called euryhaline fish, and account for around 5% of teleost fish (McCormick, 2013). While some euryhaline fish temporarily acclimate to fluctuating salinities, others voluntarily migrate between waters of different salinity for more extended periods (Currie & Evans, 2021). Anadromous fish, for instance Atlantic salmon (*Salmo salar*) and the migrating form of rainbow trout, steelhead trout (*Oncorhynchus mykiss*), are born in FW, move to the sea as juveniles where they grow for 1-4 years before they sexually mature and migrate back to FW to spawn (McCormick, 2013). These migratory patterns provide the fish with the best of both worlds; the relatively safe FW for spawning, reproduction and early growth stages, and the high availability of nutritious food in the sea for growth and the energy demanding sexual maturation (Currie & Evans, 2021). Most anadromous fish are not born with the capacity to survive in SW. Instead, they must develop SW tolerance through a series of changes called smoltification, or parr smolt transformation, before migrating to the sea (McCormick, 2013). This transformation includes physiological, as well as morphological and behavioral changes that prepare the fish for life in SW.

Atlantic salmon – an anadromous fish in aquaculture

As the human population grow, the need of a sustainable production of nutritious food is rapidly increasing, where farming of seafood is of high interest. As mentioned above, Atlantic salmon (*Salmo salar*) is an anadromous fish, and one of the most common species farmed in Scandinavian aquaculture. To obtain sufficient growth and production efficiency of this salmon in aquaculture, SW transfer must be artificially executed when the fish have smoltified. As compared to the natural process, which takes place over a longer time and allows for a voluntary transition through a successive osmolarity change, the artificial transition is immediate and without regard to the individual smolt development. Consequently, aquacultural transfer of smolt to SW is relatively unsuccessful, with a mortality rate averaging 13-19% the months following the transfer, and risk for significant long-term growth stunting and appetite reduction after the procedure (Ytrestøyl *et al.*, 2020). As the physiology behind these adverse effects are not fully understood, further

investigation of the smoltification process and general physiological mechanisms in response to the transfer to SW is needed (Sommerset *et al*., 2019).

Smoltification physiology

As mentioned, smoltification involves physiological, as well as morphological and behavioral, changes. The Atlantic salmon has a life cycle that comprises six stages: egg, alevin, fry, parr, smolt, and adult. They spend the first four stages in FW. In the final FW-stage, the parr stage, they are characterized by the fish having dark vertical stripes and spots and maintaining territories in the stream. When the parr have reached a certain size, > 10 cm, early fall (August-September), they are prone to undergo a physiological pre-adaptation to life in seawater during the winter and coming spring, the parr-smolt transformation. During the smoltification the fish become leaner and silvery (see fig 1) and start to swim in schools with the current instead of against it. However, the focus in this thesis will be solely on the physiological mechanisms involved in smoltification and SW transfer. The physiological changes are highly related to osmoregulatory capacity, for instance increased ion secretion through the gills, increased drinking rate and water absorption capacity of the gut and increased water preservation and excretion of divalent ions in the kidney (Evans, 2010; Stefansson *et al*., 1998; Sundell & Sundh, 2012).

All these mechanisms are dependent on the ion transporter Na^+/K^+-ATPase (NKA), of which the activity generally increase in SW as compared to in FW. The developmental changes are regulated by hormones, reflected in pronounced changes in several endocrine systems before and during the parr-smolt transformation. The GH/IGF-I axis, consisting of growth hormone (GH) and insulin-like growth factor (IGF-I), have a well-known instrumental role in promoting lean growth and metabolism, among many other things. GH is produced by the pituitary and stimulates the release of IGF-I mainly from the liver. Their levels also increase during smoltification, and the two hormones seem to be crucial to obtain SW tolerance (McCormick *et al*., 2013). They promote hyperosmotic regulation by increasing the size and number of chloride secreting cells in the gills and inducing NKA activity (Björnsson *et al*., 2011). Moreover, metabolism and cardiovascular traits are altered for life in sea (McCormick *et al*., 2013; Morgenroth *et al*., 2022). Cortisol have also been shown to play an important role in the smoltification process, together with GH and IGF-I (McCormick, 2001). Treatment with exogenous cortisol has proved to induce NKA activity, similar to injection of GH. Interestingly, simultaneous injection of both GH and cortisol has a larger effect on NKA activity than the two in separate, suggesting they are working in synergy (McCormick, 2001). Moreover, cortisol injection studies have demonstrated that the hormone has a main role in stimulating water uptake in the intestines, most likely through promotion of NKA activity in the organ (Veillette *et al*., 1995).

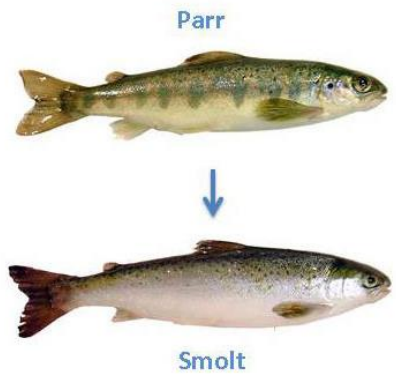

Figure 1: Atltantic salmon undergo a series of morphological, behavioral, and physiological changes, called smoltification, where they transform from freshwater-dwelling parr, into seawater tolerant smolts. The picture displays the typical parr- and smolt morphology, where the parr exhibit parr spots, and the smolt have a silvery coating (Photo: Fredrik Jutfelt).

The development of SW tolerance is reversible and will be temporary unless the fish reach SW within a close timepoint, a phenomenon called desmoltification (Stefansson *et al*., 1998). In indoor smolt facilities for aquaculture, temperature regulation and simulated light conditions are often used to obtain smolts at different times of the year (Sundell *et al*., 2003). Yearling fish that are kept under more, or less, natural photoperiod will undergo a more natural spring smoltification. However, underyearling fish can be treated with altered light conditions already during their first summer starting with short day photoperiod, simulating winter, followed by increased daylength to simulate spring conditions, which will produce a fall smolt.

The role of NKA in smoltification and SW tolerance

As the fish move from the hypoosmotic environment in FW to the hyperosmotic SW, ion-absorbing capacity must be exchanged to the opposite, from preventing loss of ions to the capacity to get rid of excess ions. The main osmoregulatory organs in fish are the gills, kidney, and gut (Marshall, 2022; Sundell & Sundh, 2012; Takvam *et al*., 2021). NKA is one of the ion transporters of high concentration in all three osmoregulatory organs and is the main osmoregulatory ion transporter in salmon smolts, as it is a key factor in both ion secretion and water uptake (McGowan *et al*., 2021). NKA, powered by ATP phosphorylation, pumps two potassium ions into the cell and three sodium ions out, generating a net efflux of positive ions, leading to a negative charge of the cell membrane on the inside with respect to the outside. NKA has an important direct function by pumping sodium out of the cell, and further contributes to salt secretion and ion coupled water uptake by driving another important ion transporter, $Na^+/K^+/2Cl^-$ (NKCC), the function of which is dependent on the electrochemical gradient generated by NKA (McCormick, 2013).

The NKA enzyme is composed of three essential subunits: alpha (α), beta (β), gamma (γ), where α is mainly involved in ion regulation, and β is involved in anchoring of the complex in membranes (Nilsen *et al*., 2007). There are different isoforms of the α- and β-subunits, and different α-isoforms have been suggested to have different roles in the gills of FW versus SW acclimated salmon due to their different ion regulatory capacity (Nilsen *et al*., 2007). There are multiple α-isoforms in Atlantic salmon, e.g. α-1a, α-1b, α-1c and α-3a, that also seems to have differential

tissue distribution (Nilsen *et al*., 2007). The γ subunit is also considered to affect ion transporting properties, where different isoforms have different affinities for Na^+ and K^+ (Yang *et al*., 2016).

Intestinal osmoregulation during smoltification and in SW

The intestine is the fish's main regulatory epithelia in fluid uptake and plays an important role in the development of hypo-osmoregulatory capacity (Sundell & Sundh, 2012). The mechanism of action in SW is the behavioral change of increased drinking rate together with increased fluid absorption in the intestine, for which NKA is considered the major driving force (Sundell & Sundh, 2012). The mechanisms behind the increased water absorption are increased, pre-adaptive, already during the smoltification, through increased NKA activity in the basolateral membrane (Sundell, 2003; Sundh *et al*., 2014; Madsen *et al*., 2011). NKA functions through secretion of Na^+ from the enterocyte, generating a concentration gradient of Na^+ necessary for the function of apical NKCC, that together with Cl-channels are involved in the buildup of an osmotic gradient in the lateral intercellular space (LIS) between the enterocyte [Fig. 2]. This gradient overrides the high osmolarity of the ingested and diluted seawater inside the intestinal lumen and creates water entry through the tight junctions and/or aquaporins in the apical membrane, inducing water absorption from the intestinal lumen (Takei, 2021). The increased water uptake capacity during smoltification, and SW exposure, is facilitated by the esophagus and cardiovascular system. In rainbow trout, the esophagus has been shown to reduce the salinity of the imbibed water by around 40%, which facilitates the subsequent water uptake in the intestine (Brijs *et al*., 2015). Furthermore, increased stroke volume and gastrointestinal blood flow facilitates the management of water and ions from the ingested water in the species (Brijs *et al*., 2015).

In the intestine, the α-1c isoform seem to be predominantly expressed and has been shown to increase during smoltification and upon SW exposure in Atlantic salmon (Tipsmark *et al*., 2010) and other euryhaline species (Urbina *et al*., 2012). Moreover, the smoltification-related hormone cortisol has been shown to increase the expression of isoform α-1c in the distal intestine in rainbow trout (Tipsmark *et al*., 2010). Isoform α-1a has displayed little to no expression in the intestine of Atlantic salmon, and does not seem to play an important role in intestinal osmoregulation, whereas α-1b seem to increase in the distal intestine in SW acclimation in the species (Sundh *et al*., 2014).

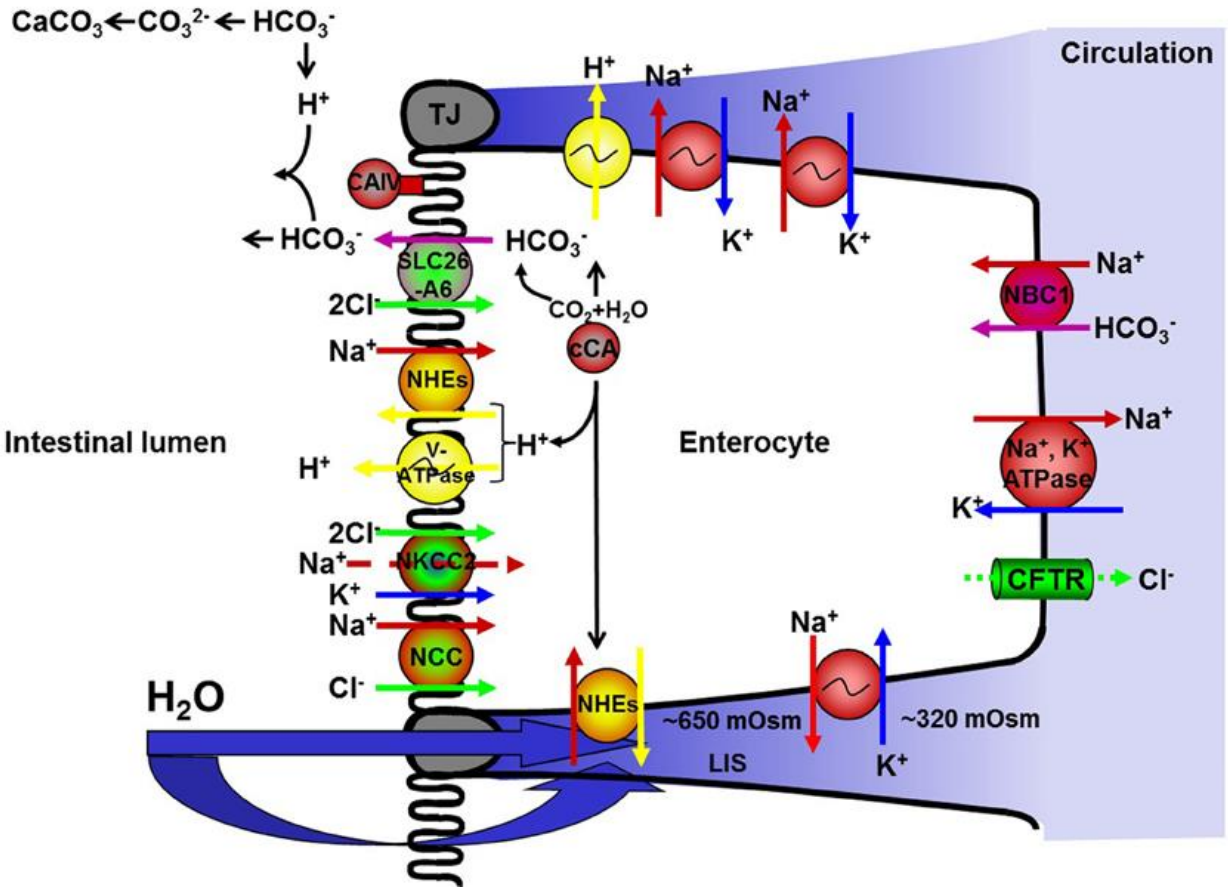

Figure 2: *Na^+/K^+-ATPase in the basolateral membrane transports sodium (Na^+) out of the cell, creating a Na^+ gradient over the apical membrane which drives the activity of the $Na^+/K^+/2Cl^-$ cotransporter. As the osmotic gradient over the cell is generated, water is absorbed through tight junctions and/or aquaporins between the enterocytes (Sundell & Sundh, 2012).*

Gill osmoregulation during smoltification and in SW

The gill epithelia contain a high density of ion transporters and is the main epithelia involved in the secretion of monovalent ions. During smoltification, NKA and the ion cotransporter, NKCC, levels are both significantly increased in the chloride cells of the gills, where they collaboratively function in ion secretion (Pelis *et al*., 2001). The NKCC is located in the basolateral membrane and transport potassium and two chloride ions into the cell using the sodium gradient built up by NKA. The chloride gradient generated by the NKCC allows for the ions to then leave the chloride cells through chloride specific channels in the apical membrane. This results in a negative outside of the cell that attracts sodium which leaves through the tight junctions (McCormick, 2013).

The α-1a and α-1b are the most abundantly expressed isoforms of NKA in the gills. The α-1a is expressed in FW, where it is involved in ion uptake, whereas α-1b is expressed in SW, with a role in ion excretion (McCormick *et al*., 2009; Nilsen *et al*., 2007). The presence of the nkaα1a isoform in SW is thought to be a solid indication that fish are not ready to be transferred, where high expression has been related to high mortality rates in salmon following the procedure (McGowan *et al*., 2021). Isoforms α-1c and α-3 do not seem to be involved in FW- or SW tolerance

orchestration in the gills, as they have been unaffected by smoltification development and SW transfer in previous studies (Nilsen *et al*., 2007; Richards *et al*., 2003).

Kidney osmoregulation during smoltification and in SW

As mentioned, the kidney functions in FW tolerance by producing large amounts of dilute urine, and thereby counteracting the passive gain of water that occurs in hypo osmotic environments. In SW, the kidney function is to reabsorb Na^+ for reabsorption of water as well as to secrete divalent ions (Takvam *et al*., 2021). The activity of renal NKA was unaffected by salinity change in a study conducted on Atlantic salmon (McCormick *et al*., 1989). However, it has been suggested that the activity would go down in response to acute exposure to SW, as a mean to reduce water loss through the kidney (McCartney, 1976). NKA α-isoforms have to my knowledge not been studied in the species.

Appetite regulation

The development of SW tolerance is a demanding process which requires a lot of energy. It is therefore important that fish sustain a sufficient feed intake. However, salmonid fishes display reduced appetite accompanied by increased levels of cortisol following SW transfer in aquaculture (Pankhurst *et al*., 2008; Usher *et al*., 1991). Appetite regulation during SW transfer is still unexplored, and is a crucial aspect of the process, as fish need the energy and nutrients to grow, and to develop the osmoregulatory capacity needed for a successful transition. Appetite regulation in teleost fish is orchestrated by the hypothalamus, which functions through integration of peripheral and central signals. Peripheral appetite regulation includes hormones, nutritional status, and gut satiety, as well as signal to the central control areas through endocrine signaling, vagal afferent nerves and through central nutrient sensing systems (Rønnestad *et al*., 2017). Among the most important peripheral hormones in appetite control are leptin and ghrelin (Rönnestad et al. 2017). The central control is exerted by neuropeptides that act orexigenic (appetite-stimulating) and anorexigenic (appetite-inhibiting) in specific neural networks in areas of the hypothalamus. First order neurons in these areas receive systemic signals, e.g. hormonal signals, and send forward the signals to higher order neurons for further processing. Orexigenic factors in first order neurons are neuropeptide Y (NPY) and agouti-related protein (AgRP), and anorexigenic factors are pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) as recently described in Atlantic salmon, and other vertebrates examined (Norland et al. 2023). Multiple appetite-regulating neuropeptides are present in complicated neural circuits of higher neurons. Examples are the anorexigenic corticotropin-releasing hormone (CRH) and CCK and the orexigenic orexin (Rønnestad *et al*., 2017). In a study by Valen *et al*. (2011), the expression of NPY displayed conflicting patterns in Atlantic salmon, where an increase was observed upon feeding. More recent data showed that fasting changes the expression of AgRP, NPY, CART and POMC implying a role in appetite regulation. However, they seem to have different roles in short- and long-term regulation of energy balance (Kalananthan, 2020; Kalananthan, 2020). Also, the *cck* expression has been shown to decrease in the hypothalamus in response to starvation, in line with it being an anorexigenic appetite factor (Murashita *et al*., 2009). The expression of the neuropeptide CART increases in response to feed intake, also consistent with an anorexigenic response (Murashita *et al*., 2011). However, our knowledge of how appetite is regulated in Atlantic salmon is still limited mainly to feeding/fasting experiments, with a lack of treatment studies.

Are all osmoregulatory organs ready for the transfer?

Upon transfer to SW, sufficient osmoregulatory capacity and sustained feeding is crucial for survival and continued growth of the smolt. A correct assessment of the readiness to be transferred is perhaps the most critical means of obtaining a successful SW transfer in aquaculture. The NKA enzymatic activity in gills has long been used as biomarker of smolt development, since its increase is accompanied by an increase in SW tolerance (Langdon & Thorpe, 1985). This testing is conducted by removal of gill filaments, directly measured in an ATPase enzyme activity assay, where a threshold value determines whether smoltification has occurred or not (McGowan *et al*., 2021). However, the role and timing of NKAs in the intestine is less known, and for the kidney information is lacking. It has been suggested that not all tissues have a common, full osmoregulatory capacity when the fish is transferred, reducing the animal's ability to cope with SW (Vargas-Lagos *et al*., 2018). Moreover, the classical enzyme activity assay does not discriminate between different NKA α-subunit isoforms, which might lead to deceiving results, as it could reflect the activity of NKA forms not favorable for hypo-osmoregulatory capacity.

Aim

The scientific aim of this project was to obtain a better knowledge of how osmoregulatory functions and appetite, with a focus on the tissue specific activities and isoforms of Na^+/K^+-ATPase and central appetite control, are developing in relation to exposure to increasing environmental salinities. The study aimed to gain more in depth basic physiological knowledge for implementation into salmon aquaculture procedures to increase smolt survival after SW transfer for increased ethical and economic sustainability

Scientific questions:

1. Is there a mismatch in the timing of development of intestinal, gill and kidney Na^+/K^+-ATPase activity and/or isoform expression upon transfer to brackish water and seawater?
2. Is the previously observed reduction in appetite and growth after seawater transfer linked to changes in central appetite regulation?

Materials and methods

Fish material and experimental design

Atlantic salmon (*Salmo salar*) +1 spring smolts (117 g) were obtained from Skagen Salmon, a fish farm in Skagen, Denmark, where they were hatched and kept in FW (salinity 0 ppt) in commercial tanks up until the start of the experiment. On June 21, the smolts were transported to Smögenlax, where they were immediately transferred to commercial tanks with a recirculating aquaculture system (RAS) containing BW (salinity 12 ppt). On July 26, the fish were transported to the experimental facilities at Department of Biological and Environmental Sciences (Bioenv), University of Gothenburg, where they were immediately transferred to SW (salinity 30 ppt). The fish were kept in a water temperature of 10˚C and a 12L:12D photoperiod throughout the entire experiment. In the farms, the fish were fed once per hour with an automatic feeder, and at the experimental facility they were hand fed, ad libitum, once per day with the same commercial feed, as they were fed in the farms.

Sampling protocol

In total, 72 fish were sampled, n=12 per timepoint. The samplings took place in Skagen, immediately prior to the first transportation (FW), at Smögenlax five days before the second transportation (BW), and then at Bioenv after 3, 6, 12 and 30 days following the SW transfer [Table 1].

Table 1: The table contains the date and water salinity of sample collections, and the duration in each salinity prior to sampling.

SAMPLING	FW	BW	SW 3 DAYS	SW 6 DAYS	SW 12 DAYS	SW 30 DAYS
DATE	21 June	21 July	29 July	1 August	7 August	29 August
SALINITY	0 ppt	12 ppt	30 ppt	30 ppt	30 ppt	30 ppt
TIME	∞	30 days	3 days	6 days	12 days	30 days

At each time point, 12 fish were randomly netted, one at a time, out of the tanks and immediately euthanized in 300 mg l^{-1} tricaine mesylate (MS-222, Sigma-Aldrich). In FW the MS-222 was buffered with 600 mg l^{-1} sodium bicarbonate. Mass (g) and fork length (L_F) (cm) was measured before sample collection was initiated. All samples were put on dry ice immediately after dissection and then stored in - 80℃ until analyses. Approximately five gill filaments were cut from the second gill arch on the left-hand side of the fish, and immediately put in 200 µl SEI-buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole; pH 7.3) for later Na-K-ATPase activity. Five additional filaments were cut and fresh frozen for gene expression analyses. The skull was cut open and the hypothalamus was dissected out. The fish was opened laterally to extract the intestine, which was cut to divide the proximal and distal parts where the proximal was defined as the region just behind the last pyloric caeca to the ileorectal valve and the distal as the region behind the ileorectal valve. The proximal and distal intestine were cut open longitudinally and rinsed in PBS (phosphate-buffered saline), and then the mucosa was scraped off with microscope glass slides and divided to two tubes: one empty for gene expression analyses, and one containing 200 µl intestinal SEI-buffer (200 mM glycine, 300 mM sucrose, 45 mM EDTA, 50 mM EGTA, 50 mM imidazole; pH 7.6) for NKA activity analyses. One pill of Complete™ protease inhibitor cocktail (04693116001, Roche) was added to every 50 ml of intestinal SEI-buffer just prior to sampling. Two small pieces of the kidney were pinched off from the distal side of the central part of the kidney. Approximately 1 mg was put in 200 µl SEI-buffer for NKA activity analyses and 1 mg was frozen as is for gene expression analyses.

NKA activity

The NKA activity was measured within 72 hours after sampling of the intestine, and after approximately a month in the gills and kidney. The assay builds on the principle that the NKA activity is fueled by hydrolysis of adenosine triphosphate (ATP), which is indirectly enzymatically coupled to the oxidation of β-nicotinamide adenin dinuclotide (NADH). The ADP (adenosine diphosphate) generated by the active NKA drives the conversion of phosphoenolpyruvate (PEP) to pyruvate by the enzyme, pyruvate kinase (PK). Lactate dehydrogenase (LDH) then utilizes oxidation of NADH to NAD and converts pyruvate into lactate. NADH, but not NAD, absorbs at a wavelength of 340 nm, which means its oxidation can be kinetically monitored in a spectrophotometer. Since the oxidation of one NADH molecule corresponds to one generated ADP molecule, the NADH levels directly mirrors the NKA activity. Thus, activity is assessed as

decrease in absorbance at 340 nm over time. The protocol for NKA assay measurements has been described in detail by McCormick (1993). Assay media A and B (AM-A, AM-B) were always prepared the same day as measurements were conducted (4 U/ml LDH, 5 U/ml PK, 0.7 mM ATP, 0.22 mM NADH, 2.8 mM PEP and 50 mM immidazole). The difference between AM-A and AM-B was that AM-B contained the specific NKA inhibitor ouabain (0.5 mM). AM-A will assess the activity of all ATPases in the samples while AM-B specifically blocks the NKA activity and the assay will thus assess the non-NKA, i.e., the residual ATPases. The difference in activity between AM-A and AM-B will therefore be the NKA activity. Every new batch of AM solution was efficiency controlled by running an ADP standard curve with four concentrations between 0-20 nmol ADP, to generate a slope value that describes the relationship between optical density and the amount of ADP, which was later used in the calculations. Samples were thawed on ice and 25 µl homogenizing buffer (sodium deoxycholate (0.1%)) was added to each tube before homogenization. A glass-glass homogenizer (20 strokes) was used for intestinal samples and a motor pestle (10 s) was used for gill and kidney samples. The samples were then centrifuged for three minutes in 3000 rcf and 4ºC, and supernatant was then transferred to new tubes to get rid of cell debris. The samples were pipetted in quadruplicates (10µl per well) to have duplicate measurements for both AM-A and AM-B. A 96 well micro plate was used (Greiner Bio-One, Germany). 150 µl AM-A and AM-B solution was added to the first and second duplicate pair respectively. Immediately prior to placing the plate in the plate reader (SpectraMAX 190, Molecular <devices, United Kingdom), 50 µl of a reaction salt solution was added to all wells (189 mM NaCl, 10.5 mM MgCl, 42 mM KCl and 50 mM imidazole). The absorbance was read at 340 nm at 25ºC, with 11 s intervals for 11 min. The optical density measurements were used to calculate NKA activity expressed in µmol ADP mg^{-1} protein h^{-1}. First, the part of the slope with the steepest curve is used for analysis. Then, the mean slope values were determined with (AM-B), and without (AM-A), oubain in the media, and converted from s^{-1} to min^{-1}. The AM-B slope values were subtracted from the AM-A slope values. The slope was divided by the ADP standard curve value:

$$\frac{\mathrm{Na}^{+} \,/\, K^{+} - \mathrm{ATPase}\ (\mathrm{mOD}\ 10\mu\mathrm{l}^{-1}\ \mathrm{min}^{-1})}{ADP\ standard\ curve\ (mOD\ nmole\ ADP^{-1})} = nmoles\ ADP\ \ 10\ \mu\mathrm{l}^{-1} min^{-1}$$

Protein levels were measured with the Pierce™ BCA Protein Assay Kit (23225, ThermoFisher) to get the NKA activity relative to the total amount of protein. The reagent was mixed immediately prior to analysis (50 parts A: 1 part B). Four BSA protein standards of concentrations between 0-20 µg/10µl was pipetted (10 µl/ well) in triplicates to a 96 well plate. Sample duplicates were pipetted to the same plate (10 µl/well) and 200 µl working reagent was added to all wells. The plate was placed on a plate shaker for 30 s and then incubated in 37ºC for 30 minutes. The plate was then placed in the plate reader and read with an endpoint assay at 562 nm. The measured protein level was then used to calculate the activity in µmol ADP mg^{-1} protein h^{-1}:

$$\frac{nmoles\ ADP\ 10\ \mu l^{-1}\ min^{-1}}{\mu g\ 10\ \mu l\ protein^{-1}} \times 60 = \mu moles\ ADP\ mg\ protein^{-1}\ hour^{-1}$$

RNA isolation, quantification, and integrity

RNeasy® Plus Mini Kit (Qiagen NV, Hilden, Germany) was used for RNA isolations, according to the manufacturers protocol. Samples were kept on dry ice before transfer to tubes with 600 µl lysis buffer (RLT and β-mercaptoethanol (β-ME) buffer, 100:1 ratio) and homogenizing beads (5 mm, sterile stainless steel). The samples were homogenized using TissueLyser II (Qiagen NV, Hilden, Germany) (3 min, 25 Hz). Genomic DNA was removed using the gDNA eliminator filter column, and the RNA was then washed with Buffer RW1, followed by Buffer RPE, before the RNA in the RNeasy spin column filter was eluted in nuclease free water. After isolation, RNA concentration was measured at 260 nm using the Nanodrop™ One/One (Thermo Fisher Scientific) spectrophotometer. High-concentrated samples (>600 ng μl^{-1}) were diluted with nuclease free water. RNA integrity of samples of concentrations lower than 50 ng μl^{-1}, or with a A260/280 ratio below 1.8, together with randomly chosen samples, was analyzed using Agilent Technologies 2200 TapeStation. Samples with low RIN-values were considered degraded and excluded from further analyses.

Complementary DNA synthesis

The iScript cDNA Synthesis Kit (Bio-Rad Laboratories Inc. USA) was used for synthesis of complementary DNA (cDNA). The RNA was diluted with H_20 to obtain a concentration of 1000 ng μl^{-1} RNA, and 5 µl iScript reaction solution (iScript and Reverse transcriptase, 4:1 ratio) was added (total reaction volume, 20 µl). Control reactions, with no reverse transcriptase (-RT), were ran with random samples from each RNA isolation group. The -RT samples were used as a control for DNA contamination. The reaction was run in MyCycler thermal cycler (Bio-Rad Laboratories Inc. USA) in accordance with the cDNA synthesis program: 5 min at 25ºC, 20 min at 46ºC, and 1 min at 95ºC. The cDNA was stored in -20ºC.

Real-time quantitative PCR

The primer sequences used were obtained from published articles, and primers were obtained from Eurofins Scientific [Suppl. table 1, Appendix 2]. The efficiency of all primer pairs was tested in all intended tissues prior to analysis, where an efficiency within a range of 95-110% was accepted. The primers that yielded an acceptable efficiency were used. The genes analyzed in the intestine, gill and kidney were Na^+/K^+-ATPase isoforms *α-1a*, *α-1b*, *α-1c and α-3a*, and in the hypothalamus: *npy*, *cart*, *cck-l*, *aslr*, *pomc-a2*, *pomc-b1* and *agrp-1*. Reverse transcriptase real-time quantitative polymerase chain reaction (RT-qPCR) was used to quantify the mRNA expression of the target genes. The qPCR reactions were carried out in duplicates, in Hard-shell 96 well plates (Bio-Rad Laboratories Inc. USA), using SsoAdvanced Universal SYBR® Green Supermix (Bio-Rad Laboratories Inc, USA). The reaction included 4 µl cDNA (12,5 ng μl^{-1}) and 6 µl reaction mix (SsoAdvanced Universal SYBR® Green Supermix, 0.5 µM forward primer, 0.5 µM reverse primer). Single-product confirmation was performed using the melt curves, and control reactions were included for every group of samples. Genomic DNA contamination was examined by replacing the cDNA sample with -RT, and primer-dimer control was carried out by replacing cDNA with water. The qPCR was run according to the following thermal cycle program: 95ºC for 3 min followed by 39 cycles of 10 s in 95ºC and 30 s of 60ºC, using CFX96 Connect Real-time PCR Detection system (Bio-Rad Laboratories Inc, USA). The cq-value mean of each duplicate

pair was calculated by the software (Bio-Rad CFX Manager 3.1), and the relative expression was calculated against the cq-values of the two reference genes, *elf-1α* and *β-actin*, to obtain a standardized relative expression.

Statistics

All statistical analysis were performed using SPSS (IBM SPSS Statistics, Version: 29.0.0.0 (241)). Given the assumptions of normality and equality of variances were met, the one-way analysis of variance (ANOVA) test was used to test for statistically significant differences between means of the different timepoints. If significant variance was detected, Tukey's post hoc was used to find which timepoints differed. Normal distribution was tested using Shapirro-Wilks test, and equality of variances was tested using Levene's test. All data had to be log-transformed to meet the assumption of normality. When log-transformation was unsuccessful, the non-parametric Kruskal-Wallis ANOVA was applied followed by Dunn's-Bonferroni post hoc. Pearson's correlation test was employed for analysis of bivariate correlations. Values are presented as mean ± standard error of the mean (SEM), and statistical significance was accepted at $P<0.05$.

Ethical permit

All experimental procedures were approved by the regional ethical committee for experimental animals under license number 5.8.18-08873/2022.

Results

The p-values from all performed Levene's tests, Shapiro-Wilks tests, and the tests of variances of means are presented in supplementary table 2 (Appendix 2).

Growth

The body mass did not significantly differ between the different time points (ANOVA: $F_{5,66}=0.761$, $P>0.05$) [Table 2]. The fork length (L_F) changed over time (ANOVA: $F_{5,66}=3.447$, $P<0.05$), and the post hoc test revealed a significant increase in length after 3 and 30 days in SW as compared to fish in FW [Table 2].

Table 2: Mean values (±SEM) of mass (g) and fork length (cm) in Atlantic salmon at the different timepoints, in different salinities. FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). n=12. *Significantly different from time 0 in FW, $P<0.05$.

	FW	BW	SW 3 DAYS	SW 6 DAYS	SW 12 DAYS	SW 30 DAYS
MASS (G)	116.6±5.4	119.2±10.9	123.9±6.5	108.3±8.0	120.4±7.9	122.0±5.1
LENGTH (CM)	20.6±0.4	22.0±0.7	22.5±0.4*	21.4±0.5	22.3±0.5	22.9±0.3*

NKA activity

Intestinal NKA activity

The NKA activity measurements in the proximal [Fig. 3A] and distal [Fig. 3B] intestine showed no significant differences in NKA activity between time points (ANOVA: $F_{4,47}=2.482$, $P>0.05$) and (ANOVA: $F_{4,47}=1.353$, $P>0.05$). The FW samples are not included in the analysis because of

high temperatures in the lab during measurements, which disturbed the temperature control in the plate reader.

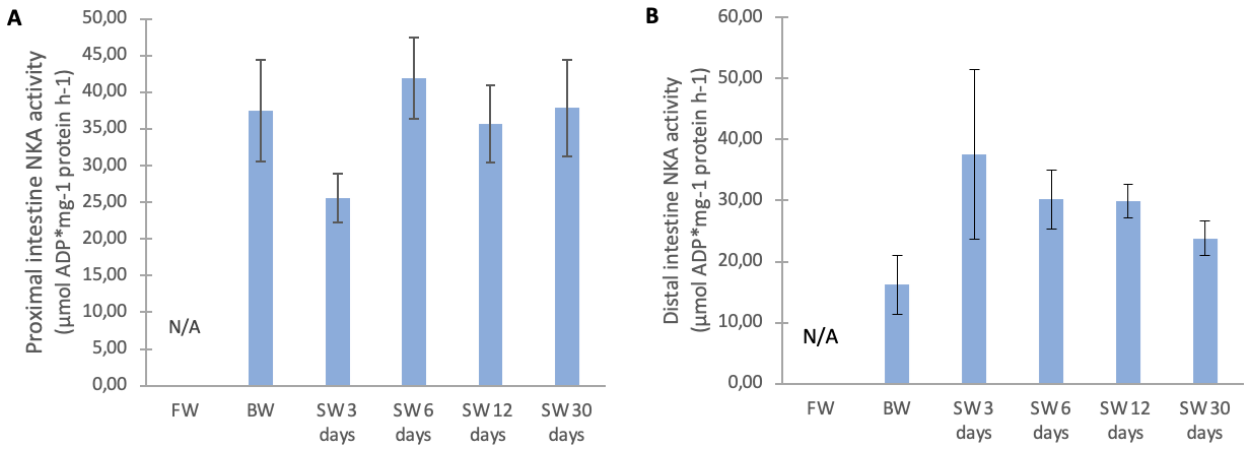

Figure 3: Mean values (±SEM) of NKA activity (µmol ADP mg^{-1} protein h^{-1}) in the (A) proximal intestine, and (B) distal intestine in Atlantic salmon at the six different timepoints. There were no significant differences between timepoints in any part of the intestine. N/A= not analyzed, FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (n_A=7-12, n_B=8-12).

Gill and kidney NKA activity

The NKA activity in the gills was significantly different between time points (Kruskal-Wallis test: $P<0.001$), with an increase in BW, compared with FW values of ($P<0.001$) [Fig. 4A]. After 3 days in SW, the activity increased compared to BW ($P<0.001$) [Fig. 4A]. The activity did not significantly differ between BW and 6- and 12 days after SW transfer but declined within 30 days ($P<0.05$). Compared to FW, the NKA activity was higher in BW- and all timepoints in SW ($P<0.05$). Significant differences were found in kidney NKA activity between timepoints (ANOVA: $F_{5,61}=15.635$, $P<0.001$) [Fig. 4B]. The NKA activity was lower in BW as compared to FW ($P<0.001$) [Fig. 4B]. Three days after SW transfer, the activity tended to be higher than in FW, although not significantly ($P=0.08$). The kidney displayed lower NKA activity again after 30 days in SW, as compared to FW and 3- and 6 days in SW ($P<0.05$).

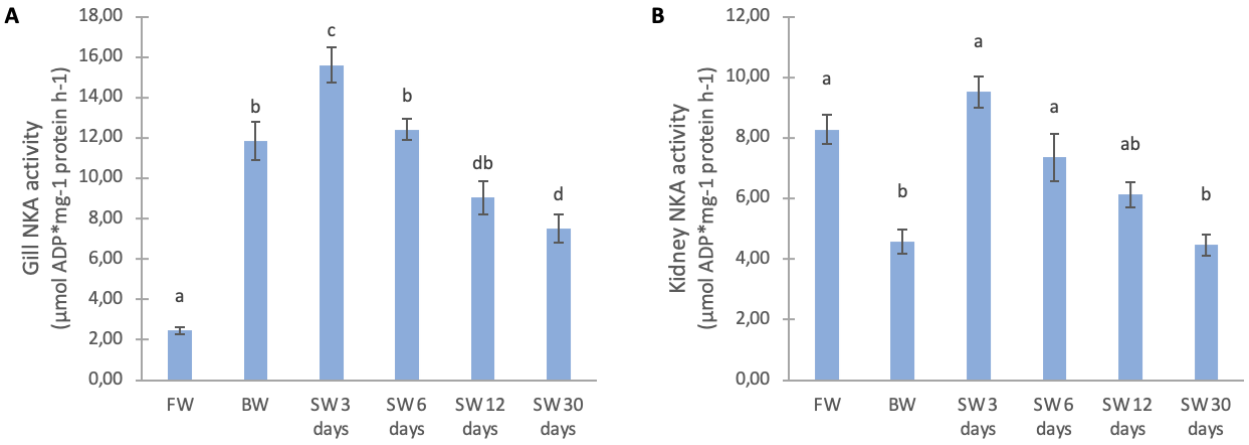

Figure 4: Mean values (±SEM) of NKA activity (µmol ADP mg^{-1} protein h^{-1}) in the (A) gills, and (B) kidney in Atlantic salmon at the six different timepoints. FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (Different lowercase letters denote significant differences (P<0.05), n=12).

NKA subunits - gene expression

Gene expression in the intestine

Analysis of RNA integrity revealed RNA degradation of the BW proximal intestine samples, and of FW and BW distal intestine samples, and these samples are therefore not included in the analyses. Further, the *nkaα1a* and *nkaα1b* subunits were below the level of detection in the proximal and distal intestine and were therefore not included. The most expressed subunit in both the proximal and distal intestine was subunit *nkaα1c*. Significant differences in *nkaα1c* expression levels between the time points were found in the proximal intestine (ANOVA: $F_{4,44}$=7.928, P<0.001) [Fig. 5A]. In the proximal intestine *nkaα1c* expression increased after 3 days in SW, as compared to FW (P<0.001). The increase remained after 6 days in SW (P<0.05), however, no significant differences were observed between FW and 12- and 30 days in SW. There were no significant differences in *nkaα3a* between time points in the proximal intestine (ANOVA: $F_{4,55}$=1.736, P>0.05). Although the difference was not significant, the expression measured 3 days after SW transfer was on average almost four times as high as in FW, but the individual variance at this particular timepoint was also very high [Fig. 5B].

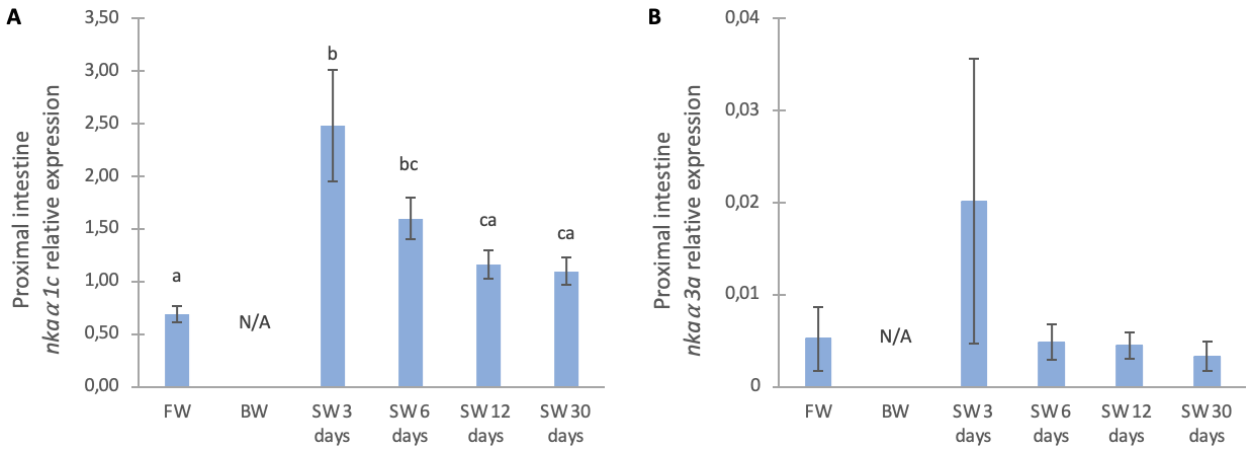

Figure 5: Mean values (±SEM) of NKA isoform (A) *nkaα1c*, and (B) *nkaα3*a relative expression in the proximal intestine in Atlantic salmon at the six different timepoints. Brackish water samples were not analyzed due to RNA degradation. N/A= not analyzed, FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (Different lowercase letters denote significant differences (P<0.05), n=7-12).

Significant differences in *nkaα1c* expression were found in the distal intestine (ANOVA: $F_{3,40}$=5.851, P<0.05) [Fig. 6A]. In the distal intestine, *nkaα1c* expression was higher after 3 days in SW, and significantly decreased after 12- and 30 days in SW (P<0.05). There were no significant differences observed in the *nkaα3a* expression in the distal intestine (ANOVA: $F_{3,40}$=1.305, P>0.05) [Fig. 6B].

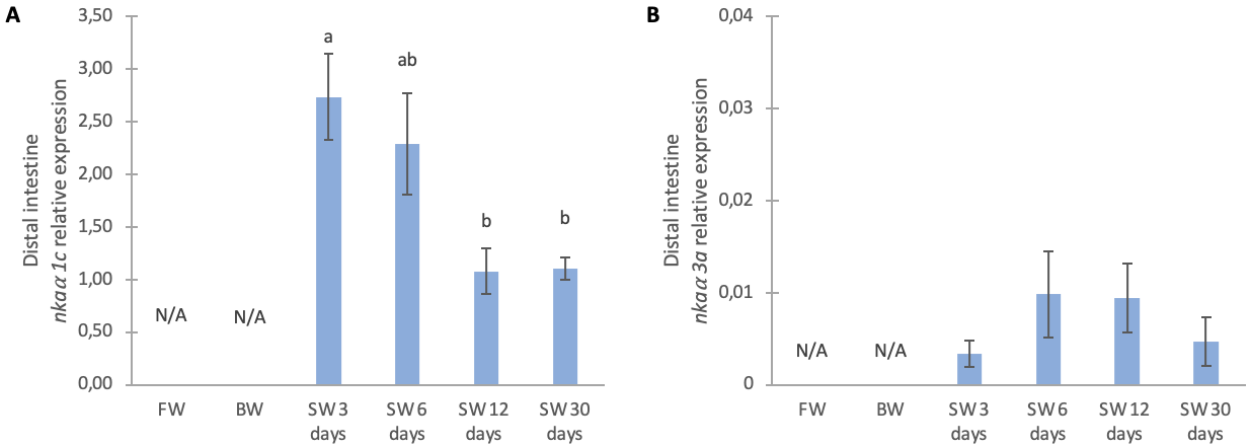

Figure 6: Mean values (±SEM) of NKA isoform (A) *nkaα1c*, and (B) *nkaα3*a relative expression in the distal intestine in Atlantic salmon at the six different timepoints. There were no significant differences between time points in *nkaα3*a expression in the proximal intestine. Freshwater and brackish water samples were not analyzed due to RNA degradation. N/A= not analyzed, FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (Different lowercase letters denote significant differences (P<0.05), n=10-12).

Gene expression in the gills

Differences in the expression of *nkaα1a* were found between time points in the gills (Kruskal-Wallis test: P<0.001) [Fig. 7A], and the post hoc revealed a higher expression in FW as compared to the other groups (P<0.001). There were no significant differences in the expression of *nkaα1a*

between any other groups (P>0.05). The expression of *nkaα1b* [Fig. 7B] was significantly different between time points in the gills (ANOVA: $F_{5,30}$=28.925, P<0.001). The post hoc revealed a lower expression in FW compared to the other time points (P<0.001), with a peak value in fish 3 days after SW transfer. There were no significant differences between BW and 3- and 6 days in SW. However, the *nkaα1b* expression decreased from the peak value after 12 and 30 days in SW (P<0.05). The *nkaα1c* isoform was expressed below detection values and are therefore not included in the analyses, and data is not shown. Significant differences in *nkaα3a* expression [Fig. 7C] were found between time points in the gills (ANOVA: $F_{5,66}$=26.075, P<0.001), with an increase after 3 days in SW, as compared to FW (P<0.001). After 30 days in SW the *nkaα3a* expression level had declined as compared to 3 days in SW (P<0.05) and did not differ from the FW expression level.

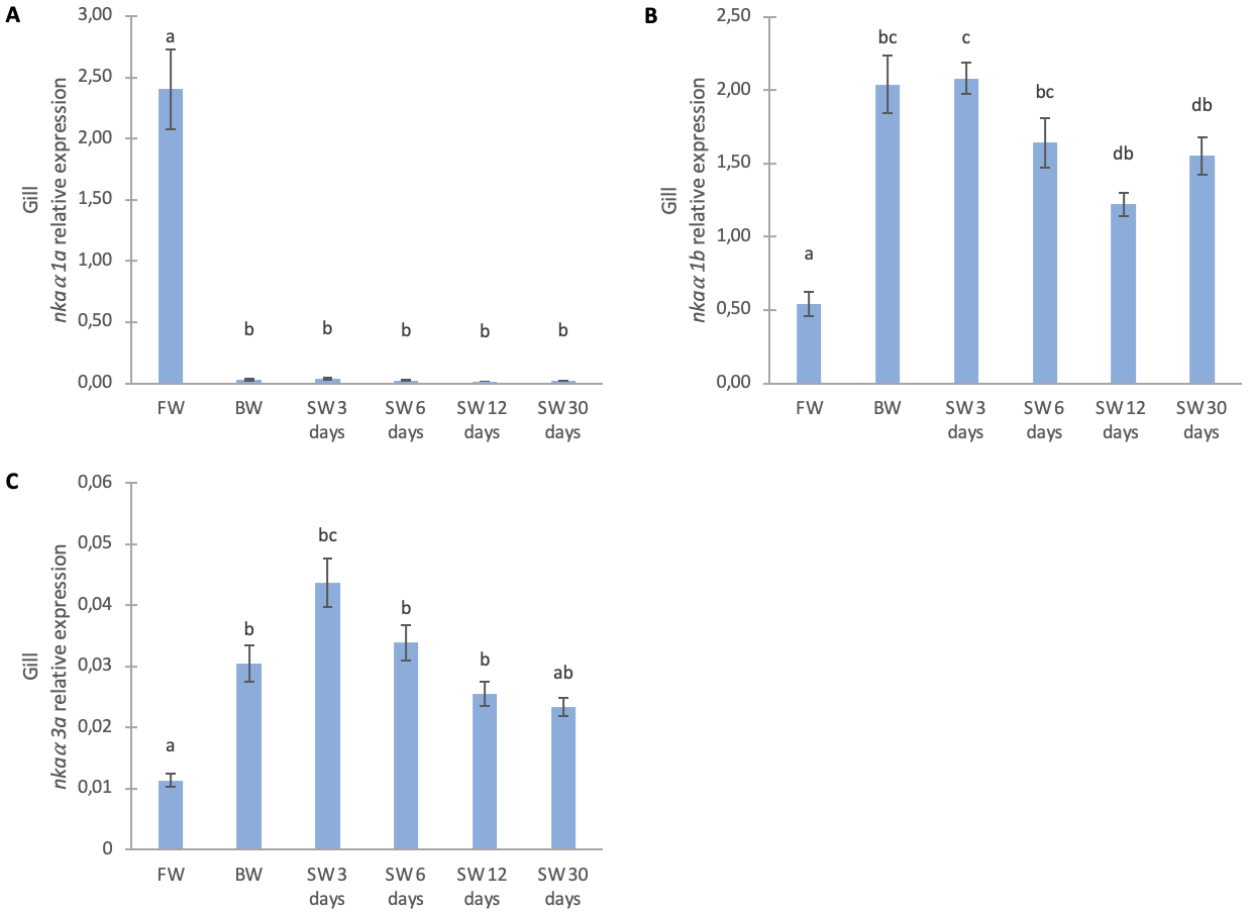

Figure 7: Mean values (±SEM) of NKA isoform (A) *nkaα1a*, (B) *nkaα1b*, and (C) *nkaα3*a relative expression in the gills in Atlantic salmon at the six different timepoints. FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (Different lowercase letters denote significant differences (P<0.05), n=12).

Gene expression in the kidney

The relative expression of *nkaα1a* varied significantly over time in the kidney (ANOVA: $F_{5,61}$=8.007, P<0.001). The expression was higher in BW as compared to all other timepoints (P<0.001) [Fig. 8A]. Significant differences between timepoints were also found for the *nkaα1b* expression (ANOVA: $F_{5,66}$=23.218, P<0.001) [Fig. 8B], where an increase in expression was

observed in BW and SW 3- and 6 days (P<0.05), to later come down to FW values again after 12- and 30 days of SW exposure. No significant differences between the peak values were detected (P>0.05). The expression of both *nkaα1c* (ANOVA: $F_{5,66}$=44.797, P<0.001) [Fig. 8C] and *nkaα3a* (ANOVA: $F_{5,66}$=4.445, P<0.001) [Fig. 8D] differed significantly over time in the kidney. The most abundantly expressed was the *nkaα1c* isoform, which reached expression levels much higher than the other isoforms, and its expression peaked 3 days following the SW transfer, in comparison to the other timepoints (P<0,05). The expression of *nkaα3a* peaked in BW (P<0.05) as compared to all other timepoints.

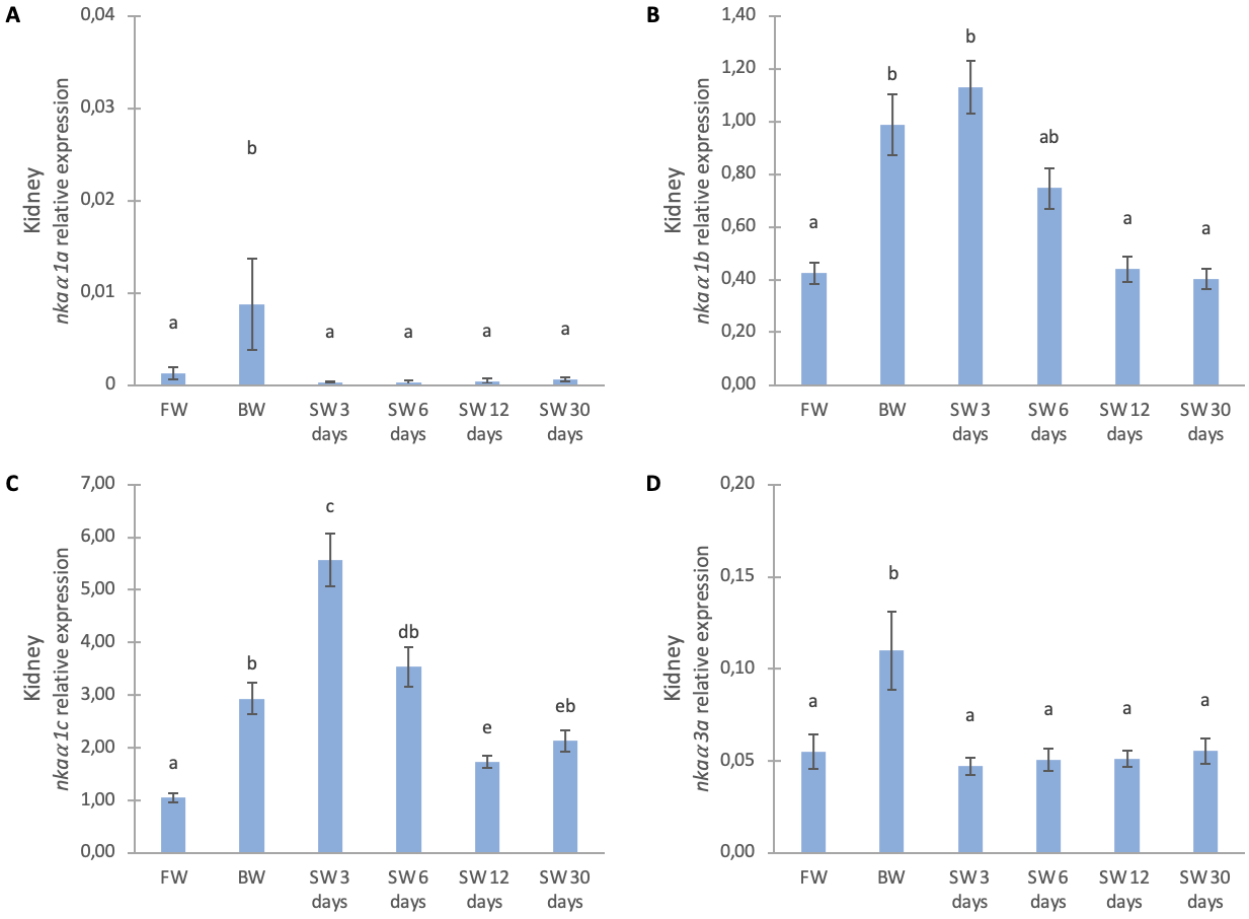

Figure 8: Mean values (±SEM) of NKA isoform (A) *nkaα1a*, (B) *nkaα1b*, (C) *nkaα1c* and (D) *nkaα3*a relative expression in the kidney in Atlantic salmon at the six different timepoints. FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (Different lowercase letters denote significant differences (P<0.05), n=12).

Correlations between NKA activity and isoform gene expression

Correlation tests were conducted for NKA activity and isoform expression in all tissues separately, and correlations were considered at R>0.4. In the gills, there was a positive correlation between both NKA activity and *nkaα1b* (r(72)=0.455, P<0.001) [Fig. 9A] and NKA activity and *nkaα3a* (r(72)=0.456, P<0.001) [Fig. 9B].

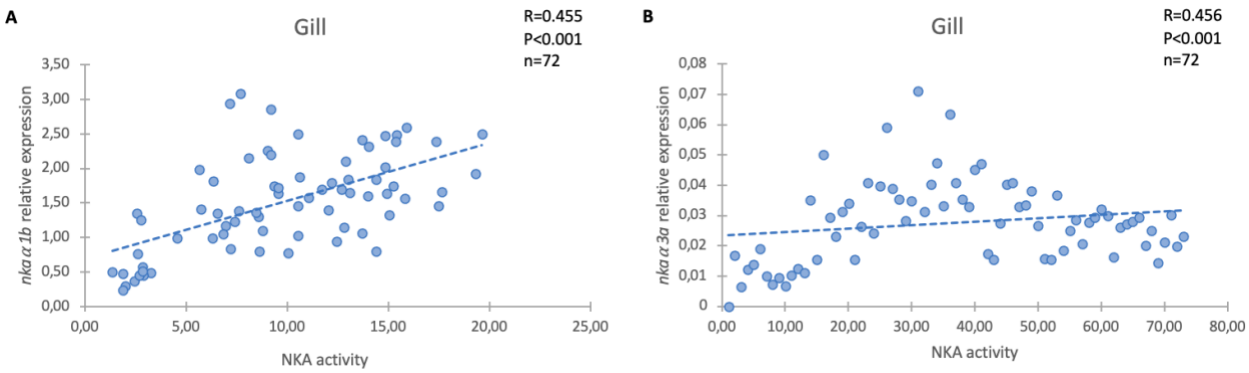

Figure 9: (A) Positive correlation (R=0.455) between NKA activity and *nkaα1b* expression in the gill. (B) Positive correlation (R=0.456) between NKA activity and *nkaα3a* expression in the gill.

The expression of *nkaα1b* and *nkaα3a* displayed a strong positive correlation (r(72)=0.726, *P*<0.001) [Fig. 10].

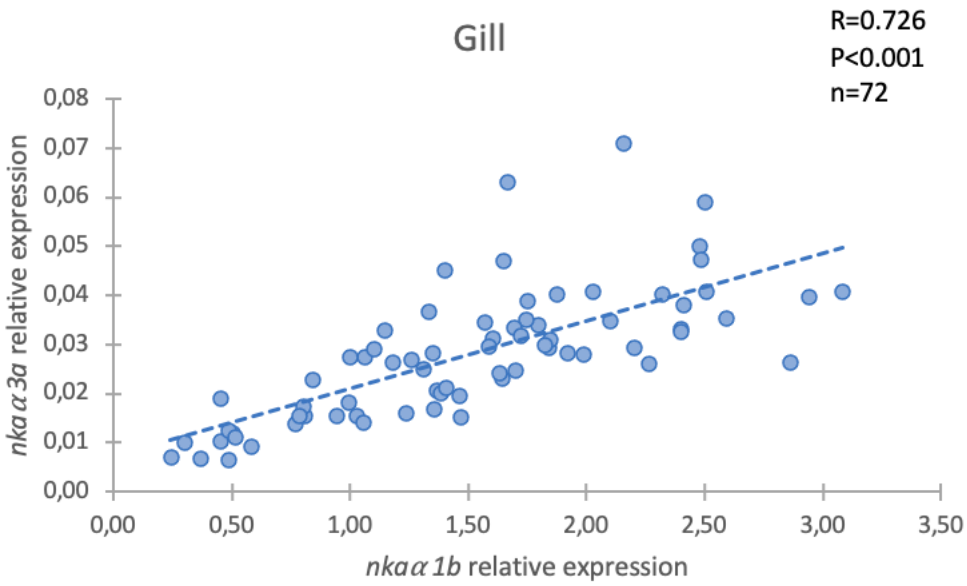

Figure 10: There was a strong positive correlation (R=0.726) between expression of *nkaα1b* and *nkaα3*a in the gill.

Expression of central appetite regulatory genes

The expression of *npy*, *cart*, *cck-l* and *agrp-1* differed significantly between timepoints (Kruskal-Wallis test: *P*<0.001). The post hoc tests revealed an increase in *npy* and *cart* expression from FW levels after 3 days in SW (*P*<0.05) [Fig. 11A-B]. There were no differences in *cck-l* expression between the first five timepoints, but after 30 days in SW the levels had declined compared to all other time points (*P*<0.05) [Fig. 11C]. The expression of *agrp-1* did not differ between FW, BW or after 3 days in SW, but increased after 6- and 12 days in SW, as compared to the first two samplings, and then increased as compared to all timepoints after 30 days [Fig. 11D].

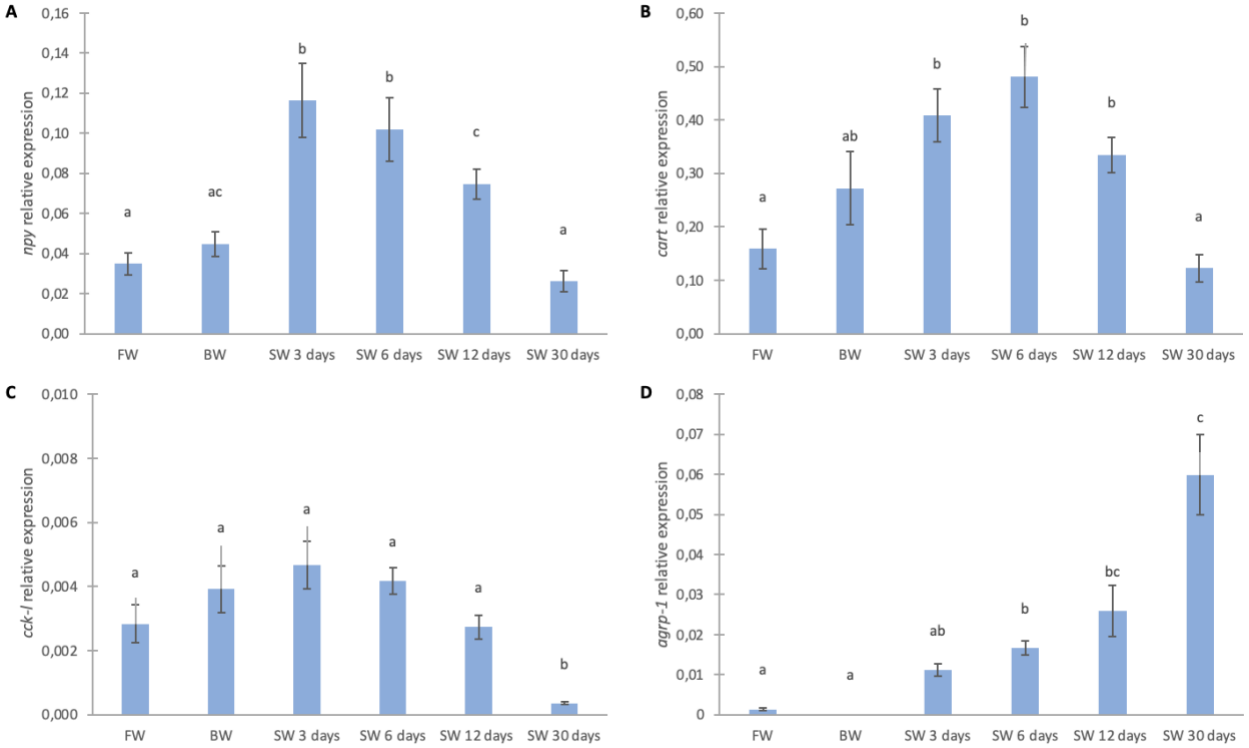

Figure 11: Mean values (±SEM) of hypothalamic expression of (A) *npy*, (B) *cart*, (C) *cck-l* and (D) *agrp-1* in Atlantic salmon at the six different timepoints. FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (Different lowercase letters denote significant differences (P<0.05), n=12).

The expression of *aslr* was excluded from the analyses due to high cq-values in the -RT control samples. The analyses of variances of means showed no significant differences over time in the expression of *pomc-a2* or *pomc-b1* due to high individual variance (Kruskal-Wallis: P>0.05) [Fig. 12A-B].

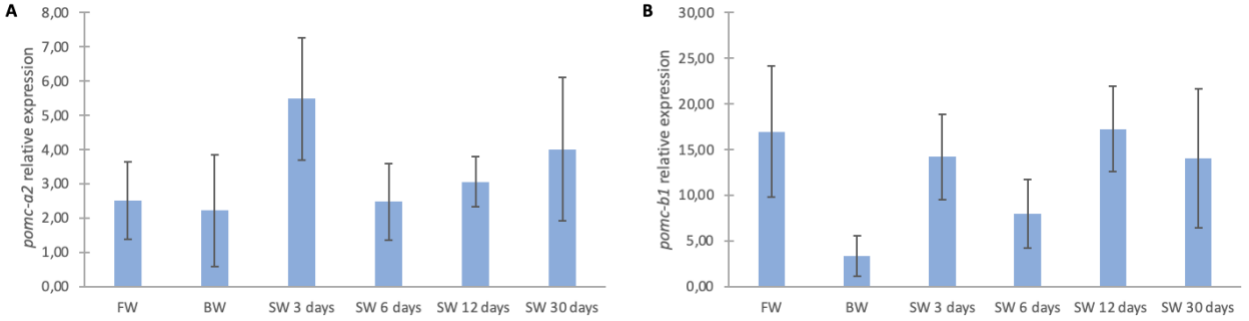

Figure 12: Mean values (±SEM) of hypothalamic expression of (A) *pomc-a2* and (B) *pomc-b1* in Atlantic salmon at the six different timepoints. There were no significant differences between timepoints. FW=freshwater, BW= brackish water (12 ppt) and SW= seawater (30 ppt). (P<0.05, n=12).

Discussion

Development of osmoregulatory functions during SW acclimation

NKA activity

In the proximal and distal intestine, the NKA-activity was similar between BW and SW at all samplings. Although there was no significant increase in activity in the proximal and distal intestine with increased salinity, the NAK-activity was high in BW and SW in the intestine when compared to in SW in other studies (Sundell *et al*., 2003; Takvam, 2020). Since we do not have the FW data to demonstrate an expected change from FW to increasing salinities, we cannot say where and when they reached the high activity. In a previous study, the proximal intestine has shown a similar activity pattern as in this study, where the activity tended to a decrease 2 days after SW (Takvam, 2020), as we also observed a non-significant tendency of a decrease after 3 days in SW, that quickly recovered within 6 days. The distal intestine was also analyzed in the mentioned study, where NKA activity peaked 1 day after transfer, to later corresponded to that measured in FW after 2 and 38 days in SW (Takvam, 2020), which is also similar to the pattern seen in our study, where the activity tended to temporarily increase after 3 days exposure. Although the differences are not statistically significant, both studies pointed to a similar pattern, where the NKA activity in the proximal intestine tend to decrease, whereas distal intestine increase, as an acute response to SW transfer. This is different from another study of the distal intestine where no changes in NAK-activity were observed 1 week after transfer to SW in Atlantic salmon smolts, but that it took longer time, up to 4 weeks, to reach a significant increase in SW (Sundell *et al*., 2003). In contrast, there were no indications of a long-term increase observed in the present study, which is in line with the study by Takvam (2020). Both previous studies of the intestinal NKA activity (Sundell *et al*., 2003; Takvam, 2020) were conducted on fish transferred immediately from FW to SW, and different results in different studies can for instance be linked to this. The intestinal NKA activity is known to display a preparatory increase during smoltification (Sundell *et al*., 2003). But the present study also shows that changes upon SW transfer calls for further investigation, with larger samples sizes, as the individual variance seem to be high. The trend of decreased activity in the proximal intestine after 3 days in SW compared with BW, although not statistically supported, is an interesting area to examine for future studies. If this type of decline in the activity takes place, it is important to understand how this affects the osmoregulatory capacity, and the overall homeostasis.

In kidney and gills, NKA-activity measurements peaked 3 days after sea water transfer and was elevated for the remaining experimental period although it gradually decreased up until 30 days after transfer. This indicates a similar timeline for induction of osmoregulatory response in these two tissues when the fish enters SW. Hence, the present study does not provide support that there are any mismatches in the induction of NKA activity between kidney and gills when transferred to SW. In a study where NAK-activity was measured 12 h, 24 h, 3 days and 7 days after SW transfer, the gill NKA activity was almost twice as high after 7 days as after 3 days in SW (Madsen *et al*., 2009). In our study, however, it peaked after 3 days and decreased already after 6 days again. There are a limited number of studies on transfer to BW, however one study was conducted on juvenile Coho salmon (*Oncorhynchus kisutch*), where gill NKA activity was measured upon moving fish from FW to BW and SW (Morgan and Iwama, 1998). After 7 days there were no significant differences in gill NAK-activity between any of the salinities (Morgan and Iwama, 1998). As we did not measure the short-term effects of the transfer from FW to BW, we cannot

directly compare with these data. However, the fact that there were no differences 7 days after transfer to a higher salinity is in line with our data, as the gill activity was almost identical in BW as after 6 days in SW in our study. Moreover, after 42 days, the activity was lower in BW as compared to both FW and SW values (Morgan and Iwama, 1998). One might hypothesize that this also indicates that fish in BW, which is nearly isosmotic, may require less osmoregulatory activity. But the present study does not support that interpretation with regards to the gills since our results showed that the gill NAK-activity after one month in SW were lower than after one month in BW. This could instead be an indication that the full acclimatization to increasing salinity takes time, at least 30 days for both gills and kidney.

The NKA activity in the kidney followed a different pattern when compared to the gills with regards to the response to being in BW. The results indicate that the activity is already high in FW, reduced when the fish are acclimated in BW, and then elevated again upon transfer to SW whereafter is gradually declines and stabilizes with time, as in the gills. Similar patterns for salmonids transferred between FW and SW have previously been observed where the high activity in FW was suggested to be a part of the smoltification process, (McCartney, 1976; Takvam, 2020). In that study, kidney NKA activity in smolts decreased distinctly 1 and 2 days after SW transfer, and then increased to FW values after 38 days acclimation (Takvam, 2020). However, they also simultaneously transferred salmon parr which had a higher kidney NAK-activity in FW compared with SW. The data from our study displays similar patterns as that of the parr salmon in the mentioned study, as the kidney NKA-activity is lower after one month acclimation in BW as well as one month in SW, compared to in FW. It is possible a high NKA activity in the kidney is not linked to SW tolerance, as in the other tissues, or that the studied fish were not fully smoltified. A high NKA activity in the kidney could be important during both FW and SW osmoregulation, as the amount of urine varies in different salinities contributing to regulation of water balance (Takvam *et al*., 2021), while fish in BW have less need for this. Therefore, one aspect to look at could be the relationship between NKA activity and the production of urine, as this is an unexplored area of research in salmonids. Moreover, the temporary decrease following the SW transfer in the study by Takvam (2020) was not seen in the present study, where the activity increased as an acute response to the transfer instead. The increase after 3 days in SW in this study is an interesting observation, as the previous observed decrease has been suggested to promote water conservation (McCartney, 1976). However, a major difference in experimental design is that the fish were transferred immediately from FW to SW in the previous experiment (Takvam, 2020), whereas fish in the current study were acclimated in BW before entering SW. Our results also differ from another study which was conducted on Atlantic salmon kept at three salinities for two months, FW (0 ppt), BW (10 ppt) or SW (33 ppt), the fish did not display any salinity-dependent differences in NKA activity (McCormick *et al*., 1989). A possible explanation for the decrease in activity in BW in our study could also be linked to the almost isosmotic environment which could be less demanding for the renal osmoregulation, however, this calls for further investigation, as the functional significance of the activity levels in different osmolarities of the kidney has not been defined.

The expression of NKA isoforms

The intestinal α-1a expression was below detection-level, which corresponds to previous observations that this isoform is not expressed in the proximal or distal intestine (Sundh *et al*., 2014; Tipsmark *et al*., 2010). The *α-1b* expression in the intestine was low, but measurable in the

study conducted by Sundh *et al*. (2014), however, intestinal α-1b does not seem to play a significant role in hypo-osmoregulation, as it did not change in response to SW transfer in the present study. The relative *nkaα1c* isoform expression was almost five times higher upon SW exposure than that in FW in the proximal intestine, confirming previous observations indicating a role of the α-1c isoform in hypo-osmoregulatory ability via the intestine (Sundh *et al*., 2014; Tipsmark *et al*., 2010). The expression of *nkaα1c* isoform in the proximal intestine is concentrated in the epithelial layer containing the enterocytes (Sundh *et al*., 2014). In the distal intestine, there was no data for the fish in FW and BW, which excludes the possibility to measure whether expression of the *α-1c* and *α-3a* isoforms change upon SW transfer. However, the *α-1c* expression is higher shortly after SW exposure compared to after 30 days of acclimation, similar to the expression pattern in the proximal intestine. The expression pattern similarity could indicate that the intestinal parts have a synched induction of *α-1c* expression upon SW transfer, however followed by a decrease again as the fish acclimated. The *α-3a* isoform expression did not significantly vary between salinities in the proximal or distal, intestine but there were tendencies of an increase after 3- and 6 days respectively, however with a big individual variance and still a relatively low expression which may indicate a less important role for that subunit.

The NKA isoform *α-1a* gene was abundantly expressed in the gills in FW, with a significant decrease in response to exposure to higher salinities, in accordance with previous studies (McCormick *et al*., 2009; Nilsen *et al*., 2007). This was expected given the function of this subunit as functioning in FW tolerance, where it is involved in the uptake of ions through the chloride cells. Furthermore, the expression was significantly lower and almost undetectable in BW and SW, which reflects a successful downregulation of the FW tolerance mechanism upon transfer to higher salinities. This coping mechanism appears to be a critical for the fish, as higher expression of the isoform in SW has been related to a higher mortality rate upon SW transfer (McGowan *et al*., 2021). Previous results of the expression pattern of *α-1a* have also been validated by Western blot protein analyses in the gills (McCormick *et al*., 2009). In the present study, the development of isoform *α-1b* expression in the gill was similar to previous studies and no delay in the induction was identified (McCormick *et al*., 2009; Nilsen *et al*., 2007). The increase upon exposure to higher salinity is an important mechanism for SW tolerance, which has also been further supported by Western blot protein analyses (McCormick *et al*., 2009). The α-1c expression was below detection values in this study, however, in a previous study *α-1c* expression was detected in the gill, and increased after SW acclimation (Nilsen *et al*., 2007). The gill *α-3a* isoform expression increased upon SW transfer, to later go down to FW values after 30 days acclimation, however the expression was consistently low in all timepoints (40-50 times lower than *α-1a* and *α-1b*) which makes it difficult to interpret whether the changes are functionally relevant.

The peaks of *α-1a* and *α3a* expression in BW in the kidney were significant. The expression of these genes was very low and the individual variance large. This, taken together with the fact that the current knowledge of their function in the kidney is unknown makes it difficult to draw any conclusions from these data. The expression pattern of *α-1a* and *α3a*, and observed decrease in kidney NKA activity in BW, calls for further investigation of the osmoregulatory function of the kidney in BW to understand the physiology behind these changes. The general salinity-related increase in expression of the *α-1c* isoform in the kidney is a novel finding, which indicates a significant role in renal mechanisms contributing to a hypo-osmoregulatory capacity. This seems

especially important in the acute phase of SW transfer, when there is a marked increase in its expression whereafter it levels off but remains higher than in FW. Its actual protein expression, specific localization in the nephrons, and the mechanisms of action, are still to be determined. One hypothesis that can be suggested is that the α-1c isoform is responsible for generating the osmotic gradients needed for an effective water absorption, similar to its suggested function in the intestine (Sundell & Sundh, 2012). Protein analysis of NKA α-1c have been performed in the intestine (Sundh *et al*., 2014) but not in the kidney. In the intestine, the isoform of the protein was abundant in the basal, and lateral, parts of the basolateral membrane of the enterocytes (Sundh *et al*., 2014), where they function in generating the Na^+ gradient utilized by the NKCC cotransporter to build up a high ion concentration over the enterocyte, which drives influx of water through tight junctions and thereby increase water uptake (Takei, 2021). In a study where fish were injected with the smoltification inducing hormones, cortisol, and growth hormone (GH), the intestinal expression of α-1c increased compared to the control group (Tipsmark *et al*., 2010). Taken together with the increase in expression of the isoform upon SW exposure, this further indicates a role of α-1c in the kidney in development of hypo-osmoregulatory capacity.

As for the isoform *α-3a*, the expression in the gill follows a similar pattern as the expression of *α-1b*, where it increased upon exposure to higher salinities, which was also emphasized by the high positive correlation between the two. In the intestine, there were no significant differences among the different samplings. However, a non-significant tendency of increased expression of *α-3a* shortly after SW transfer was observed, which could indicate an induction in the expression. In a previous study, the expression of *α-3* in the proximal intestine decreased upon SW transfer, and no changes were observed in the distal intestine (Sundh *et al*., 2014). In euryhaline eel, isoform *α-3* increases in the intestine upon SW transfer (Takei, 2021), but this has not been seen in Atlantic salmon (Sundh *et al*., 2014). The expression of *α-3a* in kidney responds in a different manner compared with the gills, being twice as high in BW than in FW or SW which is intriguing since the NAK-enzyme activity is lowest in BW in the kidney. It should be noted that the isoform expression in all the investigated tissues is low and has been most abundantly expressed in the brain in previous analyses (Tipsmark *et al*., 2010), it is possible it is an isoform involved in membrane physiology in nerve cells, rather than having an osmoregulatory function.

Appetite regulation

This is the first study to my knowledge that examines appetite regulatory gene expression in fish in different salinities. We predicted that hypothalamic gene expression of appetite regulating neuropeptides would change during the transfer to SW, as a possible mechanistic explanation to the observed decrease in appetite upon SW transfer that, for instance, lasts at least up to 30 days after transfer in Atlantic salmon smolts (Usher *et al.* 1991). The expectations were that orexigenic *npy* and *agrp-1* expression would decrease, and anorexigenic *pomc-a2*, *pomc-b1, cart* and *cck-l* would increase, in response to SW transfer. The results in this study are somewhat ambiguous as both the orexigenic *npy* and anorexigenic *cart* and *cck-l* expression peaked in response to the transfer, whereas *agr-1p* displayed a gradual increase from 3 days in SW and onward. Our results showed that *npy* expression increased initially upon SW transfer, which is usually interpreted as an indication of an increase in appetite, and then declined gradually as the fish acclimatize to full strength salinity. Previous treatment studies with NPY in rainbow trout and zebrafish increased food intake, as in mammals (Aldegunde and Mancebo, 2006; Yokobori *et al*., 2012). However

fasting studies imply that NPY might only have a minor stimulating effect on appetite in Atlantic salmon. This is based on observations of an increase of *npy* expression upon feeding, which could even be an indication that it has anorexigenic effects in the species (Valen *et al*., 2011). If so, our data would indicate an inhibiting effect on appetite upon SW transfer, exerted by NPY. The expression of *agrp-1*, however, did not significantly change after 3 days, but started to increase after 6 days in SW, and eventually peaked after 30 days. As AgRP has recently been suggested to be the most dominant orexigen and most reliable indicator of appetite in Atlantic salmon (Kalananthan 2020), due to its increase during both long- and short-term fasting, this gradual increase following SW exposure indicate that SW acclimation promotes appetite. This pattern would be consistent with the concurrent decrease in anorexigenic gene expression (*cart* and *cck-l*) at the end of the experiment and the potential return of appetite. Unfortunately, we did not have the possibility to measure food intake of the fish, but the growth data indicate that they did not consume enough to sustain body weight growth, indicating suppressed appetite [Table 2]. In line with the supposable *agrp-1* stimulated increase in appetite, c*ck-l* expression was also lowest after 30 days in seawater. Taken together, this study supports the suggested roles of AgRP, CART and CCK-L in central appetite regulation in Atlantic salmon smolt and the data can be an indication of a temporary decrease of appetite following transfer, and that they may regain appetite after 30 days, in accordance with previous observations (Usher *et al*., 1991).

The lack of significance from the Kruskal-Wallis test for variances of means of the *pomc-a2* expression was most likely due to the large in-group variance. After 3 days in SW, individual relative expression of *pomc-b1* varied between 0 and over 30. As POMC has been suggested to be a strong anorexigenic factor in Atlantic salmon, the data indicate a large individual variance in effects on central appetite control during SW transfer (Kalananthan *et al*., 2020). The large variances may also be partly explained by the involvement of POMC in the stress axis in Atlantic salmon, where it is cleaved to generate ACTH which stimulates cortisol synthesis and release (Madaro *et al*., 2015). Within salmon populations, bimodal stress coping characteristics have been observed, with a proactive and reactive behavior, and these characteristics have been related to post-stress cortisol levels (Damsgård *et al*., 2019). This bimodal distribution may be one contributing explanation as to why there is such a distinct individual variance within the different timepoints, as different individuals are more, or less, prone to stress. Moreover, as cortisol also plays an important role in the smolt development (McCormick, 2001; Veillette *et al*., 1995), this is also likely to affect the measured levels. As POMC is involved in many different systems and processes, i.e., it can be cleaved to several different peptides, it is a difficult factor to analyze regarding appetite. For future studies, analyses of the melanocortin-4 receptor expression would be a relevant addition, as it is through this receptor that POMC exert its appetite signaling (Khalanantan *et al*., 2020).

There are many factors involved in the central appetite control, where this study tested only a selection of factors that have been suggested as key players in previous studies (Murashita *et al*., 2009; Murashita *et al*., 2011; Valen *et al*., 2011; Khalanantan *et al*., 2020; Norland *et al*., 2022). A broader analysis of central appetite control should be conducted to fully understand how central appetite control is affected by SW transfer in the long term, and how changes in central appetite control affect feeding. Moreover, analysis of peripheral control factors, such as ghrelin and cortisol, together with effects on individual feeding and growth performance, need to be further investigated to better understand the effects of these factors, as well as possible explanations for

the large individual variance in *pomc* expression. The hypothalamic gene expression was generally low in the measured appetite factors. This could be due to neuron specific expression; the appetite regulatory areas are isolated in relatively small nuclei in the hypothalamus, which means that even though they are highly expressed in these nuclei, the detection is low in the hypothalamus as a whole (Valen *et al*., 2011; Norland *et al*., 2022).

Were the fish partly desmoltified?

Atlantic salmon smoltify during spring in nature, and as the studied smolts were not transferred to higher salinities until 21 June, there was a risk of desmoltification. Although not quantified, morphological signs of desmoltification were noticed during the first sampling, as the fish were not fully silvered, and had pale parr spots. During the second sampling, when the smolts had been kept in BW for a month, no such traits were noticed. Moreover, the gill NKA activity in this study is lower in FW as compared to other studies, where smolts have been analyzed prior to transfer (Handeland *et al*., 2013; Madsen *et al*., 2009). This can be due to inter-laboratory differences but is also a possible indication that the fish had partly desmoltified, as the gill NKA activity is generally high in smolts.

Future perspectives

It is not standard procedure to transfer the smolts to BW before they are transferred to SW, and the fish in this study were possibly better prepared for the transfer to SW due to the stepwise change of osmolarity. In most farms, fish are transferred immediately from FW to SW, and there can be differences in the times of induction of SW mechanisms if the fish are exposed to a more challenging transfer (Tom Ole Nilsen, personal communication). However, as a decrease in gill NKA activity has been observed in BW, it is possible this intermediate stage slows down osmoregulatory mechanisms (Morgan and Iwama, 1998), which could be a disadvantage when the fish are later transferred to SW. Moreover, the fish in this study were spring smolt, which are suggested to be more robust than out-off-season smolt, and potential mismatches in the studied mechanisms could be present in off-season-smolt. Furthermore, if the fish were partly desmoltified, this indicates an even more robust induction of the development of SW mechanisms, as there were still no delays detected. Taken together, a similar experiment but with an immediate transfer from FW to SW, where both spring smolt and off-season-smolt are compared, could lead to interesting findings. For instance, if out-off season smolt production imposes extra stress on the fish or that the practices used do not lead to robust smolt from a physiological viewpoint. However, the intermediate transfer to BW needs to be further investigated too, as this is part of some farms protocol, and the effects are scarcely understood. There are also fundamental biological questions regarding osmoregulatory mechanisms at different salinities that are worthy to address, not the least the function of the kidney.

There were no group-wise mismatches in the induction of SW tolerance among the three tissues, however, there were big individual variances within the groups. Although no fish died from the transfer, it is possible that individuals with lower induction of a given mechanism are more sensitive, and would suffer from consequential issues, such as growth stunting. This is something that should be taken into consideration when readiness is assessed, as only some individuals suffer from adverse effects of the transfer, and it is likely the ones who does display physiological differences in response to SW exposure, compared to the ones who grow well after the procedure. Further investigation as to how individual induction strength, and timing, of the studied

mechanisms, together with stress coping, relate to ion homeostatic status would give a more detailed picture of how the fish are affected by the transfer.

Furthermore, gene transcription data is one way to obtain indications of the function of NAK-isoforms, as upregulated expression is expected to indicate a higher demand of the function of the given protein. However, these results need to be further verified and developed by looking at down-stream factors, such as protein presence and localization, and characteristics of the proteins function. This especially applies to the kidney, where the number of studies is most limited, although it is one of the main osmoregulatory organs. To better understand its function, the volume and concentration of urine produced by the kidney should be analyzed before, and following, SW transfer. Moreover, to further confirm the readiness upon transfer, plasma ion concentration should be measured to confirm whether the different inductions reflect a successful osmoregulation, and if the peaks in induction of the studied mechanisms upon transfer are related to a temporary failure to sustain ion homeostasis upon SW exposure.

An aspect that is not part of the study is the role of the cardiovascular system in seawater acclimation. It would be interesting to find out whether Atlantic salmon exhibit a similar increase in gastrointestinal blood flow that has been observed upon SW transfer in rainbow trout (Brijs *et al*., 2015), since this is suggested to be of importance for water absorption. However, as stress often lead to a decrease in blood flow in the gastrointestinal tract (Brijs *et al*., 2019). It is important to understand how the perfusion of the intestine is affected by the SW transfer in aquaculture, as it is likely to play a role in how effective the intestinal water absorption orchestrated by the NKA is.

Conclusions

There were no mismatches among the tissues in the development of any of the analyzed osmoregulatory mechanisms, as the analyzed hypo-osmoregulatory functions were induced shortly after SW transfer. Understanding the reasons for the mortality and growth stunting upon SW transfer in aquaculture smolt is important to obtain an efficient smolt production as well as for the health and welfare of the fish, and other physiological mechanisms should be considered to solve the current issues related to transfer. The fish in this study were exposed to a stepwise salinity increase, which should be further investigated in future studies, as the knowledge of how this affects the outcome of SW transfer is scarce. The central appetite control was affected by SW transfer, where an increase in expression of anorexigenic factors, *cart* and *cck-l*, and a low expression of orexigenic factor *agrp-1*, were observed upon SW exposure. As the SW tolerance mechanisms are energy demanding, reduced appetite is a potential threat to the capacity of remaining homeostasis. After SW acclimation, these relationships altered to the opposite, indicating a stimulation of appetite exerted by the central control system at this point. The central appetite control is a complex system with many central factors as well as peripheral signals. Wide analyses of these factors, and how they work together, together with studies of feed intake need to be conducted to better understand how appetite and growth is affected by SW transfer in aquaculture.

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Appendix 1: Popular Science summary

Atlantic salmon must be transferred to seawater in aquaculture – but how do we do it?

As the human population grows, the need of a sustainable production of nutritious food is rapidly increasing. One nutritious, and well liked, food source is seafood, which is also one of the most debated foods in terms of sustainability. With the increasing demand for seafood comes the issue of overfishing, which means that human fishery harvests more fish than can be reproduced. Does this mean we have to stop consuming seafood all together? No, not necessarily. If we produce seafood, such as salmon, by sustainably farming fish in aquaculture we can provide a substantial amount, without disturbing the natural populations.

Atlantic salmon is one of the species that is grown in aquaculture in Sweden, and in the wild, the Atlantic salmon is born in freshwater but later migrate to the sea where they grow and sexually mature. This is a great challenge for the fish, as they are first exposed to a much lower salinity than of their internal concentration in freshwater, which expose the fish to the risk of loss of ions and excessive gain of water. When they move to seawater, they instead need to get rid of excessive salts and counteract water loss.

As the migration to seawater is important for development and growth, it is necessary to include it in aquaculture, by transferring the fish from freshwater to seawater. However, this process has not been successful, and the mortality after the transfer is high. Before they are transferred, they are assessed to have developed the capacity to survive in seawater through a process called smoltification. This involves mechanisms such as salt excretion and water uptake, where the gills, kidney, intestine, and skin are the main tissues in charge. Although much is known of how fish cope with salinity stress, it is now crucial to find out exactly what happens during seawater transfer, in order to facilitate a successful procedure.

An important factor in this is a protein called sodium/potassium-ATPase. The fascinating thing with these proteins is that they can alter their function depending on a part, that can be exchanged. This is a very interesting phenomena, and we wanted to see if the protein's activity, and different forms, were correctly developed upon seawater transfer. We measured the protein activity and gene expression of the different subunits, as well as known appetite control factors, over time during a seawater transfer.

We found no mismatches in the activity or part expression, which indicates that this is not the reason for the issues observed after seawater transfer. However, we did see changes in the central appetite control, which is likely to lead to a reduced appetite. The reduction in appetite indicate that the fish were stressed from the transfer, but the cause could not be detected in this study. This can either be due to a lack of knowledge of how these mechanisms actually work, or because the issues lie within other regulatory mechanisms. Either way, this calls for further investigation, as we need to obtain a seafood production that is sustainable both economically, and in terms of animal welfare

Appendix 2: Supplementary tables

Supplementary table 1: *Overview of all primers used in this study (obtained from Eurofins, Sweden). All primers were efficiency tested and run at 60 ℃.*

GENE	PRIMER SEQUENCES	AMPLICON LENGTH (BP)
ELF	F 5'-GTGCTGTGCTTATCGTTGCT-3'	20
	R 5'-GGCTCTGTGGAGTCCATCTT-3'	20
B-ACTIN	F 5'-CCAAAGCCAACAGGGAGAA-3'	19
	R 5'-AGGGACAACACTGCCTGGAT-3'	20
NKAα1A	F 5'-CCAGGATCACTCAATGTCACTCT-3'	23
	R 5'-GCTATCAAAGGCAAATGAGTTTAATATCATTGTAA-3'	35
NKAα1B	F 5'-GCTACATCTCAACCAACAACATTACAC-3'	27
	R 5'-TGCAGCTGAGTGCACCAT -3'	18
NKAα1C	F 5'-AGGGAGACGTACTACTAGAAAGCAT-3'	25
	R 5'-CAGAACTTAAAATTCCGAGCAGCAA-3'	25
NKAα3A	F 5'-GGAGACCAGCAGAGGAACAG-3'	20
	R 5'-CCCTACCAGCCCTCTGAGT-3'	19
NPY	F 5'-ACTGGCCAAGTATTACTCCGCTCTCA-3'	26
	R 5'-CTGTGGGAGCGTGTCTGTGCTCTCCTTCAG-3'	30
CART	F 5'-AGCAACTGCTTGGAGCACTACATGAC-3'	26
	R 5'-CAGTCGCACATTTTGCCGATTCTCGCGCCC-3'	30
CCK-L	F 5'-CAGCCACAAGATAAAGGACAGAGA-3'	24
	R 5'-GGTCCGTATGTTTCTATGAGGAGTACG-3'	27
ASLR	F 5'-GCAACTTGCTGGTTATCATTTCAGTG-3'	26
	R 5'-GTGACAGATGTAGCAGTAGCGGTTG-3'	25
POMC-A2	F 5'-ATACTTTTGAAACAGCGTGACGA-3'	23
	R 5'-CAACGAGGATTCTCCCAGCA-3'	20
POMC-B1	F 5'-CTTCCGCTGGGGCAAACC -3'	18
	R 5'-CTCCCGCCATCTTGTCCT -3'	18
AGRP-1	F 5'-ATGGTCATCTCAGTATTCCCAT-3'	22
	R 5'-AGAGAGCCTTTACCGATATCTG-3'	22

Supplementary table 2*: Results from Levene's test of homogeneity of variances, Shapiro-Wilks test of normal distribution and One-way ANOVA and Kruskal-Wallis test of variances of means for all analyzed data. Homogeneity of variances and normal distribution was assumed at P>0.05 and significant variances of means were accepted at P<0.05.*

	Levene's	**Shapiro-Wilk**	**ANOVA**	**Kruskal-Wallis**
	P-value	*P*-value	*P*-value	*P*-value
MASS (G)	0.341	0.370	0.581	-
FORK LENGTH (CM)	0.386	0.654	0.008*	-
PROXIMAL NKA ACTIVITY	0.957	0.521	0.265	-
DISTAL NKA ACTIVITY	0.364	0.198	0.056	-
GILL NKA ACTIVITY	0.256	0.024*	-	<0.001**
KIDNEY NKA ACTIVITY	0.351	0.764	<0.001**	-
PROXIMAL *NKAα1C*	0.257	0.240	<0.001**	-
PROXIMAL *NKAα3A*	0.435	0.602	0.155	-
DISTAL *NKAα1C*	0.215	0.179	0.002*	-
DISTAL *NKAα3A*	0.827	0.991	0.286	-
GILL *NKAα1A*	0.053	0.034*	-	<0.001**
GILL *NKAα1B*	0.452	0.053	<0.001**	-
GILL *NKAα3A*	0.856	0.324	<0.001**	-
KIDNEY *NKAα1A*	0.634	0.085	<0.001**	-
KIDNEY *NKAα1B*	0.990	0.087	<0.001**	-
KIDNEY *NKAα1C*	0.726	0.194	<0.001**	-
KIDNEY *NKAα3A*	0.070	0.900	0.001**	-
NPY	0.215	0.002*	-	<0.001**
CART	0.126	0.022*	-	<0.001**
CCK-L	0.134	<0.001**	-	<0.001**

LEPTIN RECEPTOR	0.254	0.792	0.187	-
POMC-A2	0.375	<0.001**	-	0.120
POMC-B1	0.627	<0.001**	-	0.154
AGRP-1	0.137	<0.001**	-	<0.001**