Development of Na+, K+ ATPase enzyme activity, and the effects of different transfer times and short-term starvation on gill, kidney, and intestine during smoltification and SW acclimation of Atlantic salmon (Salmo salar L.)

Master thesis in aquaculture biology

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Abstract

Atlantic salmon (*Salmo salar L.)* displays an anadrome lifecycle. To prepare for marine environments, they undergo parr-smolt transformation (smoltification), a developmental stage that entails several physiological changes. Development of seawater (SW) tolerance is crucial for SW survival, and is linked to changes in the gills, intestine, and kidney. Ensuring proper development of SW tolerance is key to producing high quality smolts. Very few studies have addressed changes in osmoregulatory functions of the gills, intestine, and kidney in smolt concurrently, and none have considered the implication of short-term starvation before SW transfer

This study aims to investigate the effect of different transfer times and short-term starvation Nka enzyme activity during smolt development, and 6 weeks after SW exposure. Juvenile salmon were subjected to a 6-week short day photoperiod (12 hours light: 12 hours dark). a 24 hours light (summer signal) was used during the remaining experimental period. Salmon were split into three groups: Early (168dd), Normal (348dd) and Late (516dd) transfer groups. Prior to SW transfer, smolts were either fed normally (fed treatment), or starved for four days (starvation treatment). A classic smoltification related decrease in condition factor was shown in all groups at SW transfer. Weight and specific growth rate indicated an increased scope of growth for salmon in seawater. Gill Na+, K+ -ATPase (Nka) peaked at 516dd, significantly higher than the early and normal groups. However, Nka activity decreased in this group in SW, but increased significantly at 348dd and was sufficient at 168dd. Kidney Nka activity peaked at 516dd FW, but significantly lower in SW, and a peak at 348dd in SW. The intestine however peaked at 516dd in both FW and SW, as well as large, significant drop in Nka activity at 168dd SW. This indicates that the kidney and intestine require longer to acquire optimal osmoregulatory capacity under intensive out of season smolt production protocols. An optimal transfer time may be between 348 and 516dd, as desmoltification may have occurred in the gills and kidney at 516dd. Different Nka activity patterns have been demonstrated in the gills, kidney, and intestine, but further comprehensive studies are required in all organs.

Short-term starvation showed significant increases in Nka activity compared to the fed group the gills, kidney, especially the distal intestine group. The distal intestine retained some enhanced Nka activity after 6 weeks of SW exposure, suggesting it may have some physiological benefit. The mechanism behind these increases is not known and requires extensive research.

Content

Glossary

Cyprinus carpio – Common carp

Oncorhynchus mykiss – Rainbow trout

Salmo salar – Atlantic salmon

Smoltification – parr-smolt transformation

Takifugu Obsurus – Obscure pufferfish

Abbreviation list

AM – Assay mixture

dd – Day degrees

FTS- Flow-through system

FW – Freshwater

GFR – Glomerulus filtration rate

GH- Growth hormone

IGF-1 -insulin-like growth factor 1

ICs- ionocytes

LDH – Lactic Dehydrogenase

mOsm/L – milliosmole per litre

NADH - β-Nicotinamide Adenine Dinuclotide

NKA – Na+, K+ -ATPase

NKA-a1a –NKAα-1 isoform

NKA-a1b –NKAb-1 isoform

NKCC – Na+, K+, 2CL- cotransporter

PEP – Phosphenylpyruvate

PK – Pyravate Kinase

RAS – Recirculating aquaculture systems

KCL- saltmix

SW – Seawater

Th3- triiodothyronine

Th4 - thyroxine

UFR – Urine filtration rate

Introduction

Since its early beginning in the late 1960`s the Norwegian aquaculture sector has grown exponentially and are currently valued at 81 billion Norwegian kroner (Norwegian Seafood Council, 2023, No access). Norwegian seafood is an important export commodity valued at about 151 billion NOK where salmonid-based aquaculture (Atlantic salmon; Salmo salar and Rainbow trout; Oncorhynchus mykiss), is the main contributor (Norwegian Seafood Council). The rapid growth in the sector has led to significant development of coastal communities, directly employing more than 50 000 people across the Norwegian coast (Robertsen et al., 2023). In 1985 farms produced on average 100k-300k smolt each year, at 30-50 g (Bergheim et al., 2009). However, in 2000 the number increased to 500k-2million smolt per farm, with an average weight of 70-120 grams, highlighting the huge technological leaps (Bergheim et al., 2009). A key element to this growth has been the development of technology and production regimes in land-based facilities (Bergheim et al., 2009).

While the industry was facilitated by rapid expansion of infrastructure and capital investment, research of salmon biology has also contributed significantly to the development. The ability to better manage massive challenges such as salmon lice, high mortality, control of environmental factors, and disease has allowed mass scale production of today, using land-based facilities for production of smolt, and sea cages for adult fish (Sommerset et al., 2023). Furthermore, the study of osmoregulation has been of significant importance to produce salmon smolt of optimal quality (McCormick et al., 2018). Manipulation of photoperiodic stimulation of the parr-smolt transformation has led to more possible transfers of smolt to Seawater through degree-days (dd), stimulating seasonal cycles (Mørkøre & Rørvik, 2001).

1.1 lifecycle and smoltification

Atlantic salmon (Salmo salar) displays an anadromous lifecycle, where juveniles migrate from freshwater (FW) to seawater (SW) environments, while spawning adults engage in the opposite migratory process (Mccormick, 2013). The eggs, juvenile salmon called fry or parr, as well as smolt spend their time in FW. Adult individuals migrate to SW for improved feeding conditions in which food are more abundant (Hoar, 1988). During the transition from FW to SW environments salmon undergo a process called smoltification in order to survive the large increase in salinity associate with a marine environment.

Smoltification is a developmental process in which juvenile fish undergo changes which prepare them for seawater entry (Hoar, 1988). The timing of this process is determined mainly

by temperature and daylength, though salinity, water current, endogenous rhythms and size play and important part as well ((Hoar, 1988; Mccormick, 2013)). In nature the process is initiated during winter where fish experience a decrease in daylength while the smoltification process is initiated during spring where the daylength increase. This acts as the 'zeitgieber' that triggers developmental events that prepare salmon for migration to SW (Björnsson et al., 1989).

During the smoltification process the fish undergoes several behavioral, morphological, and physiological preparations (Mccormick, 2013; Nilsen et al., 2007; Sundh et al., 2014). These include changes in the salmon's gills, and in their kidney and gut, which enable them to regulate their body fluids and electrolytes facing significantly higher salinity levels in the SW (Mccormick, 2013; Nilsen et al., 2007; Sundh et al., 2014; Takvam et al., 2021a; Takvam et al., 2021b).The salmon also loses its parr-marks, and they develop a silvery color in the skin/scale (Björnsson et al., 2011). The fish also adapts a streamlined body shape (reduction in condition factor) and starts schooling behavior and downward migration. These changes are facilitated by hormonal changes, mainly by the hormones growth hormone (GH), triiodothyronine (Th3) and thyroxine (Th4), cortisol and insulin-like growth factor (IGF1) (Mccormick, 2013; Young et al., 1989). Through the neuroendocrine axis, these hormones stimulate the processes that prepare the smolt for the SW environment. These changes are facilitated through photoperiod and temperature change, which enables smoltification (fig.1), and with this knowledge it is possible to produce out of season smolt (Stefansson et al., 1991).

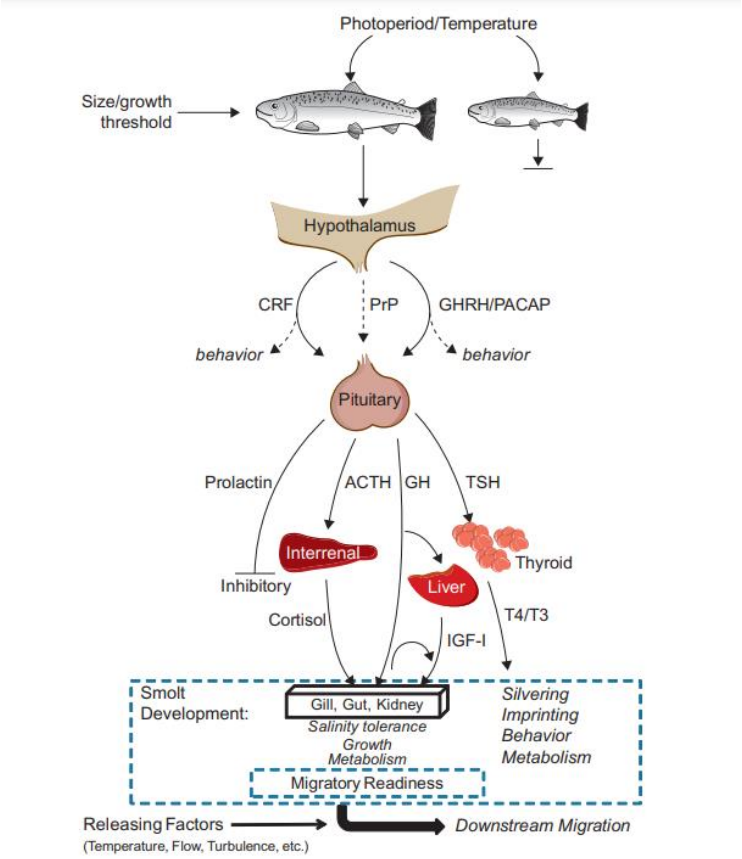

Figure 1 overview of hormonal control of smoltification. Photoperiod and temperature facilitate hormone cascades, which promotes physiological, morphological, and behavioral changes. The thyroid hormones impact scale silvering, imprinting, behavior, and metabolism. The interrenal and liver hormones facilitate changes in the gill, kidney, and intestine, as well as growth, metabolism and migratory behavior (Mccormick, 2013).

1.1.2 production of out of season smolt

By artificially manipulating the photoperiod (winter period followed by spring period) in land-based facilities the farmer provides the necessary signals to initiate the smoltification and its associated physiological changes to prepare the fish for SW. Thus, this knowledge could be utilized in order to produce out of season smolt, meaning farms could produce smolt independent of the natural smoltification cycle all year round (Stefansson et al., 1991). This could be done by imitating the natural winter and spring period, first by exposing parr to 6 weeks of artificial winter signal (12 hours light and 12 hours of darkness; 12L:12D), then by a 4-6 week artificial spring signal (24 hours light and 0 hours dark; 24L:0D) (Ytrestøyl et al., 2023). When smoltification is nearly complete, the salmon is in the smolt window (about 250–350-degree days; dd), in which preparedness for SW transfer is optimal, and the fish should be transferred to SW (McCormick et al., 1989). If fish are kept in FW, desmoltification can occur, where physiological changes associated with smoltification could revert (Mortensen & Damsgård, 1998). This could have unknown consequences for the fishes osmoregulatory

capacity which may predispose them to disease and the response to handling (i.e. sea lice treatments) during the SW phase. From a fish health perspective, it is vital to retain osmoregulatory capacity (seawater tolerance) before transfer to SW, as reduced osmoregulatory function may reduce growth and survival after SW transfer (Handeland et al., 1998).

1.2 Osmoregulation

Osmoregulation is key to maintain internal salt and water homeostasis in fish. Thus, maintaining stable levels of water and salts in the fish is crucial to perform a range of biological processes such as function of biomembranes, enzymes and O2 transport (Edwards & Marshall, 2012; Mccormick, 2013). This is particularly important for salmonids as they move between FW and SW environments as they need to osmoregulate in both low and high salinity environments. Thus, salmonids spend a considerable amount of energy first through smoltification in FW and secondly after entering SW regulating salt and water balance through the gills, intestine, and kidney (Fridman, 2020). Teleosts regulate toward an internal range of 300 – 350 mOsm/kg, (fig.2) which differs from their respective environmental osmolalities). FW has a range of 3-5 mOsm/kg, while SW has 1000 mOsm/kg, thus bringing different challenges to salmon in their FW and SW life stages (Edwards & Marshall, 2012; Tipsmark, Sørensen, & Madsen, 2010). In FW, fry, parr and smolt conduct hyper-osmoregulation, to counteract loss of ions to the environment by diffusion, as well as water gained by osmosis (fig.2). Consequently, large amounts of dilute urine is produced, and ions are gained by active transport through the gill epithelium while the intestine, and the kidney removes excess water and reabsorbing Na and Cl. Once in SW, fish gain ions through diffusion and loses water through osmosis. Thus, they drink water and filtrate out excess ions mainly through the gills (Na and Cl), as well as the intestine (Water) and kidney (Mg2+, SO42- and Ca2+)(Edwards & Marshall, 2012; Mccormick, 2013; K. S. Sundell & Sundh, 2012; Takvam et al., 2021b). In salmonids, Na+/K+-ATPase (NKA) is present in all the osmoregulatory organs (Mccormick, 2013). NKA is located in ionocytes (ICs) in the gills, in enterocytes in the intestine, and in nephron tubule cells in the kidneys(Evans et al., 2005; Hoar, 1988; K. S. Sundell & Sundh, 2012). NKA is associated and indirectly involved in the transport of many water and different solutes, and it has been strongly correlated with salinity tolerance and therefore a common metric for assessing smolt development and quality (McCormick, 2013).

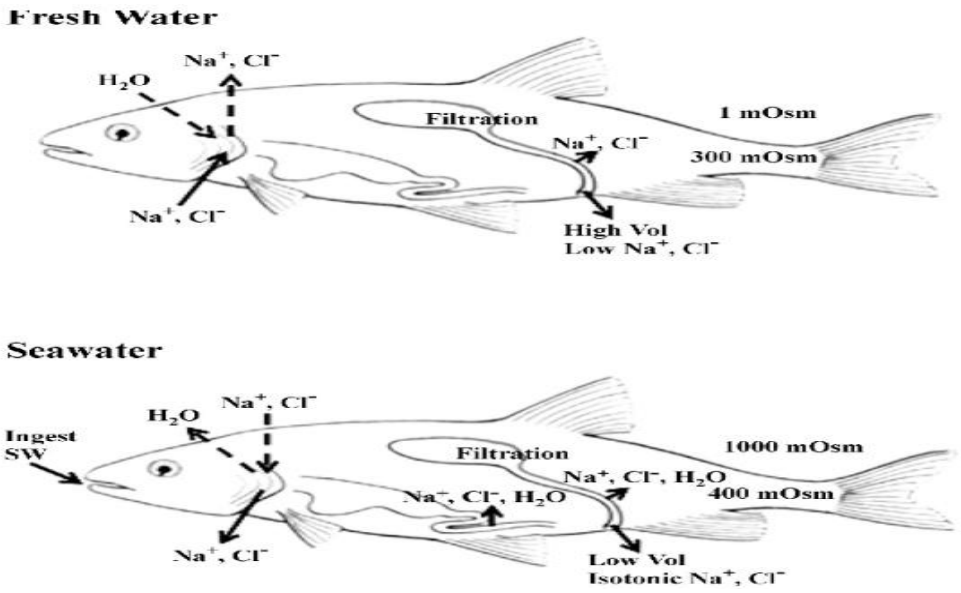

Figure 2; overview of osmoregulatory function in teleosts. FW teleosts actively transport NaCL through their gills, and water through diffusion. High salt concentration is also retained by High glomerular filtration rate (GFR) in the kidney, retaining salts while expelling excess water to retain homeostasis. In SW Na+. K+, cl- is actively expelled by the gills, while the intestine absorbs water and salts. low GFR and urine flow rate (UFR) in the kidney ensure minimal loss of water while expelling excess divalent ions to ensure homeostasis (Evans, 2008).

1.2.1 gills

Osmoregulation in the gills occurs across the secondary lamella, located at the gill arches (fig.3). The process is facilitated by the ionocytes (ICs), which is the site ion absorption in FW fish, and ion secretion from SW fish (Evans et al., 2005). The ion regulation is regulated by three main cellular components; Na+/K+-ATPase (NKA), $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), and apical cystic fibrosis transmembrane conductance regulator (CFTR) (Mccormick, 2013; Nilsen et al., 2007). Na+/K+-ATPase (NKA) is an enzyme localized within the basolateral membrane, in which two Na+ ions are changed for two K+ ions by active transportation. This creates a negative transmembrane potential within the IC, thus allowing Na+ an Cl- secretion (Fridman, 2020). After being transported into the extracellular fluid, Na+ leaves the gill through paracellular pathways into SW or is absorbed into the bloodstream in FW. Furthermore, the basolateral $Na^+/K^+/2Cl^-$ cotransporter (NKCC1), utilizes the sodium gradient to transport Cl- into the IC. Cl- will then leave the IC through and apically located Cl-channel, the apical cystic fibrosis transmembrane conductance regulator (CFTR) (Evans et al., 2005; McCormick et al., 2013; Nilsen et al., 2007). In FW, Cl- will diffuse back into the extracellular fluid instead.

NKA consists of two main isomers, called NKAa1a and NKAa1b. NKAa1a is most prevalent in FW, and NKAa1b in SW (fig.3). However, there is a considerable amount of NKAa1b as well in FW CCs. They are, however, lying inactive beneath pavement cells. As smoltification occurs, both NKAa1a and NKAa1b are found in great numbers. As the smolt migrate to SW, and NKA activity increases, NKAa1a levels in the secondary lamella will decrease, as NKAa1b numbers increase greatly (Mccormick, 2013; Nilsen et al., 2007), suggesting plasticity in NKAa1a, allowing them to change into the NKAa1b isoform (fig.3). This is consistent with enhanced salinity tolerance and is frequently used as an indicator of seawater tolerance in salmon smolt, linking NKAa1a to FW osmoregulation, and NKAa1b to SW osmoregulation.

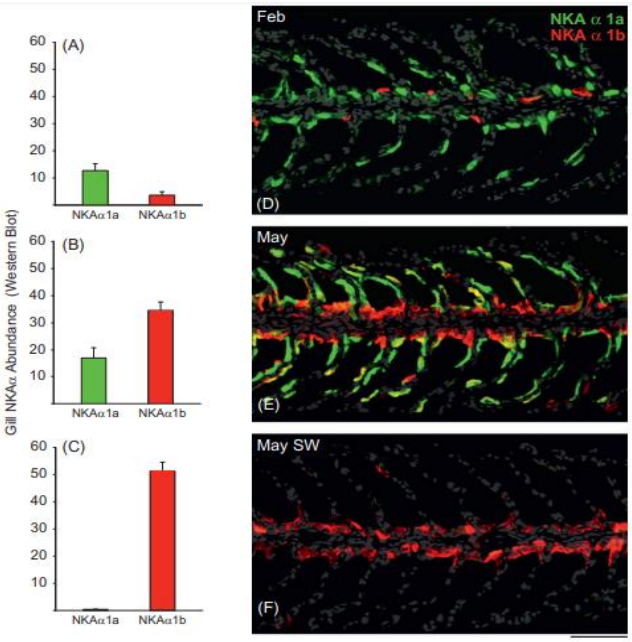

Figure 3; abundance and localization of NKAa1a and NKAa1b from FW stage (D), during peak smoltification (E), and after 2 weeks of SW exposure. The figure illustrates how NKA1a is most prevalent in FW, both in the secondary lamella and the gill arch. During smoltification, the NKAa1b isomer is most prevalent along the gill arch, however NKAa1a is still active. After sufficient time exposed to SW, only NKAa1b activity is prevalent (Mccormick, 2013).

1.2.2 kidney

The kidney plays an important role in FW teleosts by removing excess water while reabsorbing both monovalent and divalent ions (kidney reabsorbs about 95 % of all NaCl from the preurine) (Takvam et al., 2021b). In SW teleosts the kidney retain water by reducing filtrations rates (reduced GFR and UFR) while removing excess divalent ions such as sulfate, magnesium, and calcium(Edwards & Marshall, 2012; Takvam et al., 2021b). the kidney is located alongside the dorsal wall of the body cavity and is comprised of the head kidney, followed posteriorly by the trunk kidney (Martinez, 2017; Resende et al., 2010). While the head kidney functions mainly

as a hematopoietic organ, the trunk kidney contains nephrons and ducts responsible for the excretion and osmoregulatory functions (Martinez, 2017; Resende et al., 2010). The functional units of the kidney are nephrons (fig.5). Nephrons consist of several parts, the first being the glomerulus, here small capillaries transport blood to the vascular pole of the glomerulus where the blood filters plasma through the small pores of the bowman`s capsule ((Brown et al., 1983); Takvam et al 2021a). Once blood have been filtered through the bowman`s capsule it is often referred to as the "preurine". After this the preurine is further modified passing through the proximal tubule, distal tubule and collecting tubule before entering the collecting duct (fig.5). Ultimately the urine enters the paired mesonephric duct which then merge forming the urine bladder where urine is periodically discharged through the urinary papilla (Curtis & Wood, 1991; Demarest & Machen, 1984). While these structures are mostly shared by both FW and SW teleosts, they have different functions according to their environment ((Takvam et al., 2021a)).

In FW, the main goal of the kidney is to reabsorb ions, as well as produce dilute urine to remove water. This is firstly done by filtrating large amounts of blood in the glomerulus, hence high GFR. The early proximal tubule reabsorbs Na/Cl (fig.5) through the absorptive NKCC isomer, NKCC2 (meditated by NKA) as well as water, macromolecules, and glucose (Takvam et al., 2021b). In the proximal tubule, Na and cl are reabsorbed, as well as K+ and HCO3–, along with water. Divalent ions such as Mg2+, SO4 2- and Ca2+ are also reabsorbed here (Edwards & Marshall, 2012; Takvam et al., 2021b). The distal tubule has the same function, by absorbing mainly Na/Cl, meditated by extensive basolateral NKA, apical NKCC2, and NaCl cotransporter for NA+, as well as basolateral kidney-specific Cl− channels for Cl-(Hickman & Trump, 1969) (fig.4). The impermeable parts of the distal segment prevent water from following the ions being reabsorbed, thus diluting the urine heavily, to a concentration approx. 4–16 ml//kg/h. This results in high urine flow rate of (UFR) of 1–6 ml/kg/h, which contains small amounts of Na/Cl, large amounts of water are expelled (fig.5).

In SW, the main function is to excrete excess ions gained from drinking SW, especially the divalent ions, such as Mg2+ Ca2+, SO4 2- which easily can get toxic for the fish (Edwards & Marshall, 2012; Takvamet al., 2021a). Firstly, the glomerular has a low GFR (0.5-2.0 ml/h/kg) in order to conserve water (Takvam et al., 2021b; Talbot et al., 1992). In the early proximal tubule, Na/Cl is absorbed, while divalent ions are excreted, both followed by water. The distal segment has greatly increased permeability, allowing water to be absorbed together with NA/Cl, enabled by NKA, NKCC2, and the apical Cl- channel. Due to this reabsorption, SW teleosts produces urine which is iso-tonic to the blood plasma (300-410 mOsm/L).

NKA is evidently important in kidney function in teleosts, however, knowledge of NKA activity changes during smoltification in salmonid kidneys is very limited (McCartney, 1976; Takvam et al., 2021b), though recent studies suggest that there is a small increase in both NKAa1a and NKAa1b activity for smolt (Madsen et al., 2020). However, it has been shown to decrease shortly after transfer to SW, which suggests a requirement for reduction of monovalent ion transportin nephron tubules, due to rapid GFR and UFR reduction in SW (Takvam et al., 2021a). In order to acclimates to higher salinites the kidney must remove excess divalent ions such as Mg2+, Ca2+ and SO42- to maintain normal plasma levels (Takvam et al., 2021).This is suggested to correlate with high Nka enzyme activity shown after 1 month in SW(Takvam et al., 2021a). This is supported by studies in SW mengfu where NKA has been linked to transport of sulfate (SO42-) by creating a sufficient gradient which can drive apical secretion of SO42-, by Slc26a6a in mefugu (Kato et al., 2009).

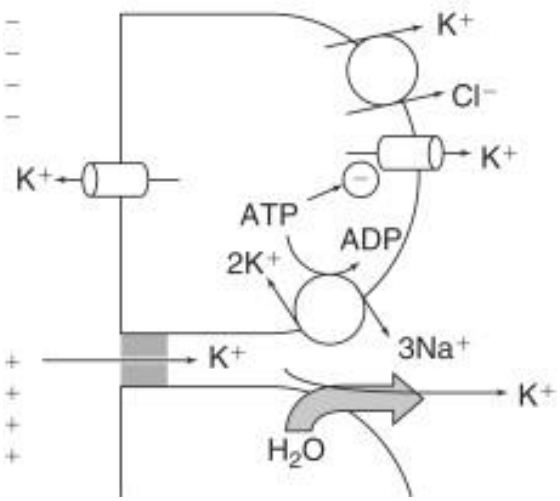

Figure 4: illustration of mayor ion transport pathways in the kidney, modeled on the distal tubule. Basolateral Na+/K+-ATPase creates a gradient that drives Cl- transport, as well as Absorption of NA+,K+ and Cl- via NKCC2 (Moe et al., 2009).

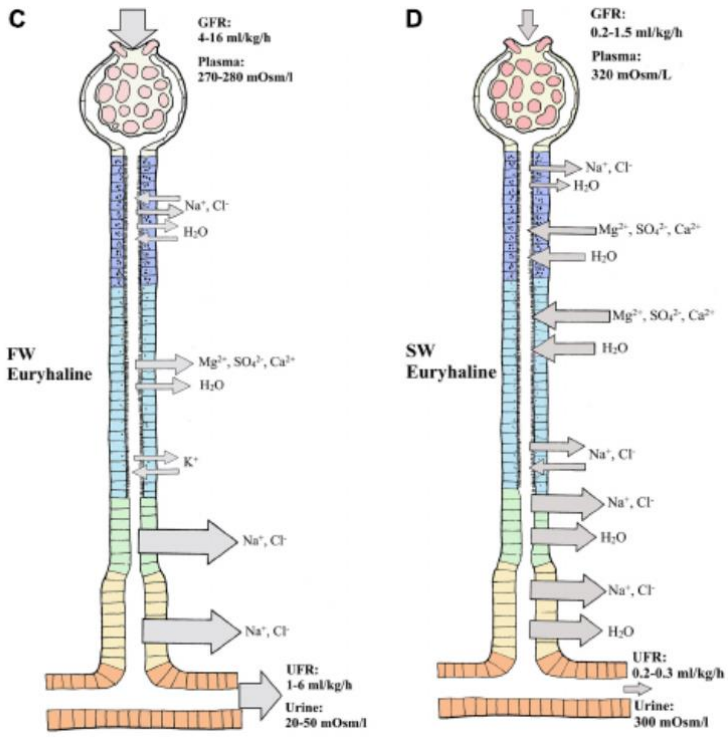

Figure 5; overview of FW I and SW (D) euryhaline fish nephrons. The figure illustrates the glomeruli (head), followed by proximal tubules (Dark blue), proximal tubule 2 (Light blue), Distal tubule (green), collecting tubule (yellow, and collecting duct(orange). Ion and water flow from each segment is illustrated, as well as GFR, UFR, plasma osmolality, as well as urine osmolality (Takvam et al., 2021b).

1.2.3 intestine

The intestine is commonly separated into two main segments; the proximal intestine, which extends from the pyloric caeca to the 'thicker' segment of the intestine, called distal intestine, recognized by increased diameter, annular rings, and darker mucosa (Bakke-Mckellep et al., 2000). They are the main osmoregulatory parts of the intestine, facilitated by its functional unit, the enterocyte (Sundell & Sundh, 2012). The teleost intestine is responsible for nutrient uptake, as well as ion and water balance. Teleosts in FW absorb NaCl, magnesium, calcium, etc. from feed to offset passive ion loss across the gills and kidney, but drink limited amounts of water (Perrott et al., 1992). Absorption of ions are mainly facilitated by NKCC2 and the Na/Cl cotransporter, carrying Na and Cl into the enterocyte. Some Cl- is also transported through the apical HCO3-/Cl- exchanger, as in the gills and kidney (Mccormick, 2013). These processes are facilitated by NKA, creating an electrical gradient, which is required to transport Na and Cl into the extracellular fluid (fig.6).

During smoltification, NKA enzyme activity increases, mostly associated with increases in the nkaa1c isomer in both the distal and proximal intestine, while NKAa1a and b are not regulated to the same extent during smoltification and subsequent SW transfer (Sundh et al., 2014; Tipsmark et al., 2010). Abundance of Nkaα1c appears to be slightly reduced at the peak of smoltification, suggesting a mechanism to prevent excess water uptake while the smolt still resides in FW (Sundh et al., 2014). Furthermore, water is transported from the lumen paracellularly via tight junctions (Sundell et al., 2003). In SW the drinking reflex, found in euryhaline fish, results in increased drinking of water. However, water cannot be actively absorbed alone, and must be coupled with ion transportation. Thus, SW euryhalines intestinal NKA activity increases, coupled with a decrease in transepithelial resistance after SW exposure, suggesting a shift from paracellular transport of water in FW, to a transcellular one in SW (Sundell & Sundh, 2012). A Recent study has also highlighted the importance of tight junctions and aquaporins in the salmonid intestine (Sundell & Sundh, 2021). While it is known that paracellular permeability is mainly regulated by affecting tight junctions (TJs) (Daugherty and Mrsny, 1999; Anderson et al., 2004), incorporation of aquaporins into the intestinal epithelium are suggested to maintain waterflow when water transport shifts from para- to transcellular pathway, and thus maintaining high NKA activity (Fig.6) (Sundell & Sundh, 2012).

Additionally, significant net HCO3(-) secretion is another aspect of the intestine's osmoregulatory function (Fig.6). This is thought to stimulate extra fluid absorption both directly through Cl(-)/HCO(3)(-) exchange, as well as indirectly, through precipitation of ingested Ca(2+) as CaCO(3), thus establishing the osmotic gradient for further fluid absorption (Whittamore et al., 2010).

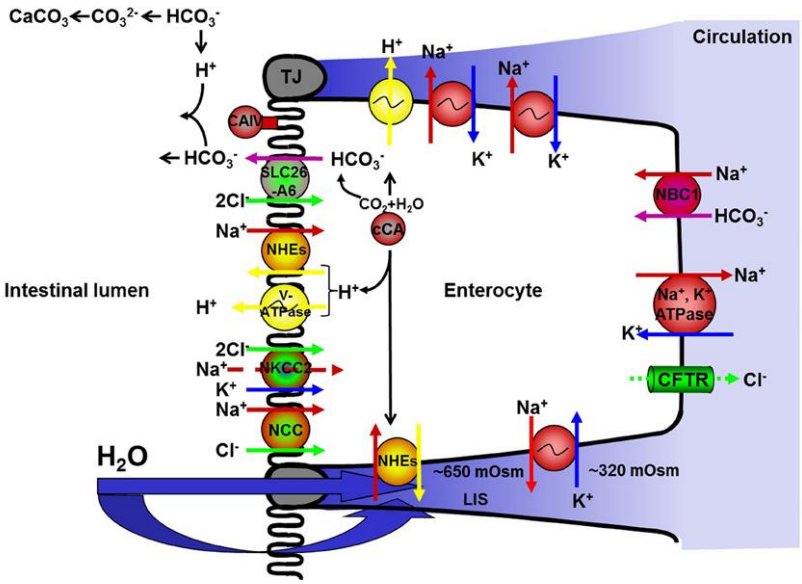

Figure 6: Ion transport pathways in the intestine, localized in the enterocytes. NaCl transport into the enterocyte is facilitated by the sodium gradient produced by NKA. Cl is also exchanged for HCO3-, which is then transported into the extracellular fluid. The transcellular, as well as the paracellular pathways of H2O are also illustrated, driven by the NKA sodium gradient (Sundell & Sundh, 2012).

1.4 Flow-through facilities, and problems in modern production protocols regarding smolt physiology

Traditionally the gills have been the main organ investigated when evaluating seawater tolerance as well as smolt quality (Nilsen et al., 2007; MCccormick, 2013), while limited attention have been given to evaluating the correct transfer timepoint investigating the osmoregulatory capacity in the all three organs Correct timing of smolts is often referred to as the "smolt window" (discussed earlier) indicates the time in which smolts have optimal SW tolerance (High Gill NKA enzyme activity) and are ready for SW transfer (McCormick, 2013). Usually, this time window is between 250 and 350 dd in gills, but neither kidney nor intestine are evaluated in terms of the optimal transfer window. Studies show that a winter signal (12:12LD) followed by six weeks of LD24:0 at optimal temperature (14 ° c) is necessary to minimize effects on growth and mortality (van rijn et al., 2021). However, this is only based on measurements in the gills, and a more holistic approach including both kidney and intestine is needed to better understand the correct timing of SW transfer. Studies in both intestine and kidney indicate that they are not completely synchronized with the gills (Sundell et al 2003; Takvam, 2020; Takvam et al 2021b; Takvam et al., 2023), in fact they may show peak levels several weeks later compared to gills (Takvam et al., 2023). Thus, if smolt are transferred too early or too late this will impact the overall osmoregulatory functions (e.g SW tolerance in gills,

kidney and intestine) obtained during smoltification may be reduced leading to lower SW survival.

Traditionally, smolt are reared in flow-through systems (FTS). FTS retrieve water from a river/lake, which is led through the plant to provide highly oxygenated water, as well as filtering out metabolites (Sandvold, 2016). Production protocols have allowed for more intensive production, through use of contiouse light regimes, as well as adding salt to feed/water, and providing higher temperature (Handeland et al 2004; Striberny et al 2021; Ytresøyl et al 2023). Some new protocols delay transfer of smolt to SW cages to 500g, or even 1000g as so-called 'post smolts'(Bjørndal & Tusvik, 2020), compared to the industry standard of 70-130g (Bergheim et al., 2009). Collectively, about 91 million fish die before harvest combining the mortality of FW and SW phase in aquaculture (Fish health Report, 2023). Exposing smolt to continuous light, higher temperature, salinity, either through feed or by adding small amounts the water has become common practice. However, many of these practices are unnatural for the fish in this part of their life-cycle and should be used with caution.

Starvation prior to SW transfer is part of common production protocol (Tom Ole Nilsen and Marius Takvam, per comm.). Routinely, starvation is conducted 2-4 days before transport, in order to empty the gut, to lower risks of poor water quality from waste products, as well as crowding, pumping, delousing, transportation and slaughter (Noble et al., 2018; Waagbø et al., 2017). Short term starvation also initiates lower metabolism, and may also increase stress tolerance, due to an improved aerobic scope (Hvas et al., 2020). Furthermore, studies have been conducted on weight and fatty acid composition (Einen et al., 1998), oxygen consumption (Cook et al., 2000), as well as endocrine responses (Hevrøy et al., 2011). However, no studies have examined the effects of short-term starvation on osmoregulatory capacity in the gills, kidney, and gut together.

With an ever-increasing demand for better quality smolts, higher production volumes and more frequent smolt transfer to SW, there is a great need to understand smolt physiology, and how this may interact with the rapid changes in production protocols. Correct timing of SW transfer is vital in commercial salmon aquaculture where increased mortality rates and poor growth performance commonly have been linked to reduced SW tolerance in the first weeks of SW (van rijn et al., 2021). From transfer to SW roughly 16% die (54 million fish) before the end of their production cycle, which can be linked to insufficient smolt quality, as well as disease (ytrestøl et al., 2019). In addition, there is a need to understand the impact of starvation on all

three osmoregulatory organs before transfer to SW. Currently there is limited knowledge on the impact smolt quality have for overall SW performance (evaluation of all three organs). In addition, more than 37 million smolts die before transfer to SW. Collectively, about 91 million fish die before harvest combining the mortality of FW and SW phase in aquaculture (Fish health Report 2023).

1.5 goal of study

This study seeks to further expand our knowledge of the three main osmoregulatory organs: the gill, intestine and kidney during smoltification and after 6 weeks of seawater. Firstly, we aim to investigate how short-term starvation (4 days) affect osmoregulatory capability. This is measured by calculating NKA activity in the osmoregulatory organs gill, intestine and kidney in smolt transferred as early (158 dd), peak (350 dd) and late (518 dd) in the smoltification process. Each starvation group is coupled with a fed group. Hence, we aim to investigate the effect of starvation as well timing of SW transfer on overall smolt quality, using the NKA enzyme activity protocol in gill, intestine and kidney (Takvam, 2020; Takvam et al., 2023). These measurements will be coupled with and detailed growth performance data (Weight, condition factor Specific growth rate, Feed conversion ratio) for smolts and after 6 weeks SW exposure.

Objective 1; determine NKA enzyme activity in the three main osmoregulatory organs; the gills, kidney and intestine during smolt development (Early, Normal and late transfer)

Increased Nka activity in the gills is well established in Atlantic salmon during smoltification, and thus is used as the main indicator of smolt development (Mccormick, 2013; Nilsen et al., 2007). The kidney also has some studies showing increased Nka activity (Madsen et al., 2020; Takvam et al., 2021a). Finally, Intestinal Nka activity has been shown to increase(Sundh et al., 2014; Tipsmark, Sørensen, & Madsen, 2010). Therefore, I predict a steady increase of Nka activity across the 3 transfer times, possibly with a large difference between Early and Normal, and a less significant one between Normal and Late. Perhaps some desmoltification will occur at the late transfer time.

Objective 2: determine the effects of short-term starvation (4 days) on osmoregulatory capabilities across the different transfer times (early, normal and late) in gill, intestine and kidney.

Short-term starvation is common industry practice to empty the gut and reduce metabolites in transfer tanks, but few studies have been conducted on the effect of short-term starvation in the

osmoregulatory organs. To my knowledge, only 1 study has examined this relation, and only examined the gills, finding no increase in branchial Nka activity (Breves et al., 2016). Thus, it difficult to predict the effect of starvation, however I hypothesize that the intestine will show significant up-regulation of Nka activity, as dearly needed salts are absorbed from feed in FW to offset passive ion loss across the gills and kidney facilitated by Nka (Mccormick, 2013; Sundell & Sundh, 2012). Furthermore, I expect the most compensatory Nka activity increase to be in the proximal intestine, as this region is the main segment for nutrient absorption (Bakke-Mckellep et al., 2000; Bjørgen et al., 2020).

Objective 3: Determine how different transfer times (early, normal and late) effect on short-term (6 weeks) SW performance.

Transfer to SW is a critical phase in aquaculture, and optimal timing of SW transfer of smolt is essential for growth and feed intake in marine environments (McCormick, 2013). SW has an osmolality of 1000 mOsm/kg (Edwards & Marshall, 2012), thus requiring high Nka values to prevent dehydration (Grosell, 2010; Perry et al., 2003). Thus, I predict activity will be higher in the normal and late groups, but lower in the early group, as smolts may not yet be ready for SW transfer.

Objective 4: determine the effect of short-term starvation (4 days) on short-term (6 weeks) SW performance.

Yet again, no studies have examined the relation between short-term starvation on short term SW exposure. I believe that if any significant differences are shown in smolts, increased Nka activity may be retained after 6 weeks of SW exposure (though slightly lower). General growth performance should be similar, even though short-term starvation may initially lower weight of fish. Compensatory growth is shown in fish after short-term starvation, and thus I hypothesize no significant changes in growth after 6 weeks SW exposure (Azodi et al., 2015; Maclean & Metcalfe, 2001; Oh et al., 2008; Tian & Qin, 2003)

Materials and methods

2.1 Fish stock, experimental design and sampling protocol

2.1.1 Fish stock

Atlantic samlon (Salmo salar L.) were obtained from the stock 'Benchmark Crossbreed', were delivered to skretting Aquaculture Research Centre at Lerang, Norway. The fish were delivered as eyed egg, and hatched and further reared as fry, and further grown into parr using commercial feed. When parr reached a weight of ~20g they were pit tagged and induced to a 6-week winter signal (12:12), thus marking the start of the experiment.

2.1.2 Experimental design

After pit tagging, fish were randomly sorted into 450 L tanks, with N= 75 fish per tank and given standard diet group. The fish were divided into two subgroups prior to sampling, fed and 4-days starved (fig.7). Each experimental group was in triplicate tanks, giving 36 tanks in total. The tanks had a concurrent flowthrough of 400– 600 L / hour, with an oxygen saturation of 95-120%, at 12°c throughout the entire experiment, and an average salinity of 0.04ppt in FW. At ~70-80g fish were vaccinated with Pharmaq ALPHA JECT micro 6 (0.05 µl dose) during the winter signal period (12D:12L).The experiment followed a classic square wave photoperiodic induction of smoltification (Stefansson et al 1991). Tanks were exposed to a 6-week winter signal (12D:12L) photoperiod regime, and then changed to 24H daylight (24:0) regime to induce smoltification. Fish were sampled at 4 intervals (fig. 7). The first sampling was of baseline smolt (4-6th Jan) after vaccination, followed by early transfer (17-19 Jan, 158 dd), normal transfer (peak smolt; 30.jan-01.feb, 350 dd) and finally late smolt (14-15 feb., 518 dd). Following each sampling, the group was exposed to SW (33.8 ppt). Finally, in the scope of this project, weight, length, condition factor, SGR, and FCR were measured by skretting after each sampling, as well as after 6 weeks of SW exposure, which were then transported to a common garden for further data collection after 6 months (will not be included in the thesis).

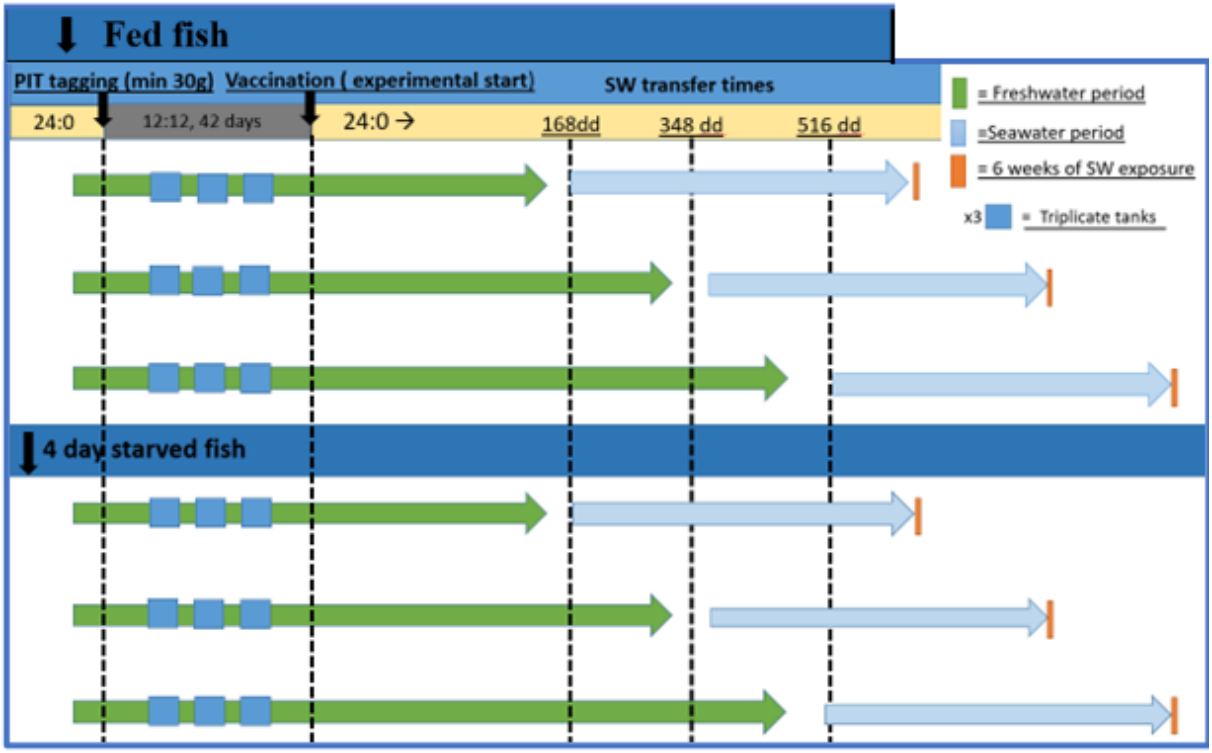

***Figure 7: Overview of experimental design**. Parr were exposed to 24h lights, then PIT tagged. The winter signal was then induced for 6 weeks. A 24:0 light regime remained the remainder of the experiment. Triplicates were set up for fed and 4-day starved fish. Fish were exposed to SW after three separate time intervals, early (168dd), normal (348dd) and late transfer (516dd). After 6 weeks of seawater (SW) exposure, final samples were taken, and fish were transported to a common garden.*

2.1.3 Sampling protocol

For each group four fish (from each triplicate) were extracted from each tank (i.e N=12 per treatment) of the two groups (starved & non-starved) over the course of two days, thus totaling 24 fish per sampling. Fish were retrieved from their tanks with a simple net and quickly euthanized using an overdose of tricaine sedation (6x normal dose). Plasma was retrieved from the caudal vein, and then centrifuged and frozen on dry ice and stored at -80 for future analysis. Moreover, samples for NKA enzyme activity measuring where collected for gill, anterior and posterior intestine, and kidney. The second gill arch on the left side, which was then stored in SEI buffer, frozen on dry ice and promptly stored in -80 freezer. The kidney tissue was sampled in the posterior part (area D to E), due to it containing the highest NKA activity of the kidney to standardize the sample area (Takvam, 2020). The samples were also stored in SEI buffer, frozen on dry ice and stored in -80 freezer. The intestine was dissected out before feed and feces was removed. It was then cut horizontally, and the mucosal side was facing up. Finally, a small sample was extracted from both the anterior- and posterior intestine, by gentle scrapings of the

mucosal side using a glass microscope slide to maximize abundance of the enterocytes. The intestinal tissue were stored in a modified intestinal SEI buffer (200 mM glycine, 45 mM EDTA, 50 mM EGTA, 300 mM sucrose, 50 mM imidazole; including 1 tablet Complete™ protease inhibitor cocktail (04693124001 Roche) for each 10 mL batch of intestinal SEI buffer) to minimize degradation during lysis and homogenization. Samples were stored at -80°C freezer until analysis. For more information regarding sampling procedure please see Takvam et al., 2023 (submitted to Reviews in Aquaculture).

2.2 Calculations

In addition, measurements of length and weight were performed for each sampling point. Weight and length were measured using a simple weight and ruler, shortly after euthanasia.

Several data parameters were provided from skretting, which were processes and analyzed using the following formulas:

Condition factor:

Ful'on's condition factor was calculated according to the standard formula (nach et al., 2006):

$$CF = (\text{W x } l^{-3}) \text{ x } 100$$

In which W=weight, and L = length (cm)

Specific growth rate

Specific growth rate (SGR) was calculated using this formula:

$$SGR = \frac{(lnW2 - lnW1)}{(t2 - t1)} * 100$$

In which t_1 and t_2 are the start and endpoint sampling, and W_2 and W_1 are the natural logarithm of weight (g) measured at times t_2 and t_1 respectively.

Feed conversion ratio

Feed conversion ratio (FCR) measures how well fish utilize feed for growth, and is calculated using the following formula:

$$FCR = \frac{F}{Wg}$$

With F (g) being dry matter of feed, during a certain period, and Wg indicating weight gain over said period

2.3 Sample analysis

2.3.1 NKA enzyme activity (gills, kidney, intestine)

After samples were collected and frozen in -80°c, NKA enzyme activity was measured using the microassay method of McCormick (1993), completed by using a Spark microplate reader (Tecan, Mannedorf, Switzerland), regulated to 25°c. The modified and standardized NKA enzyme activity protocol was used for measurements in gills, intestine and kidney (Takvam et al 2023).

Prior to NKA analysis, an assay mixture was prepared. It is composed of 5.0 U/ml Pyruvate Kinase (PK) and 4.0 U/ml Lactic Dehydrogenase (LDH), 0.22 mM β-Nicotinamide Adenine Dinuclotide (NADH), 0.7 mM Adenosine Trisphosphate (ATP), 21 mM phosphenylpyruvate (PEP), and 50 mM immidazol. After all chemicals were mixed, the Assay mix was split into two containers solution A and B where IB was mixed into Solution A (AM), while solution B (AM-O) was mixed with ouabain according to protocol. Further, an ADP standard curve was made. 2.7ml was mixed with 900 µL of salt-mix (KCL) and placed in a 25°c water bath for ~5 minutes. Then, 200 µL of the solution was then pipetted into quadruplicates in a NUNC plate, mixed with the 10 µL of each ADP standard (table 1).

Tablel 1: concentrations of ADP standards for quality validation of assay mix

conc.	**IB (µl)**	**4 mM ADP stock solutiion (µl)**
0	200	0
5	175	25
10	150	50
20	100	100

The microplate was then analyzed using the spark microplate reader, applying the ADP-standard protocol. The standard curve was always kept between 17-18 mOD/nmole ADP.

Shortly prior to ATPase analysis, samples (N=24) were retrieved from -80°c freezer for thawing on ice. 8.1ml AM and AM-O were mixed with 2.7ml salt solution (find buffer components and concentrations). Roughly (n=4-6) gill filaments, kidney tissue (1.2-1.6 mg) and

anterior/posterior intestine (1-1.2 mg) was used for the analysis according to the modified protocol by Takvam and authors (2023). Following dissection and weighing, addition of 25 ml SEID buffer (concentrations of buffer) to all samples. Sampled were then homogenized by mortar pestle for 10-20 seconds, and vials were centrifuged (2 minute, 4 °c, 6000 g for gills, 4 minutes, 4°c, 7500g for kidney and 4 minutes, 4°c, 8000g for intestine). Then, 10 µL of each sample was then pipetted in quadruplicates in 96-well NUNC plates, as well as in triplicates in costar plates for protein concentration. After finalizing sample pipetting, 200 µL of solution A and B were added in duplicates and measured on the plate reader (Spark microplate reader, Tecan, Mannedorf, Switzerland) 340 nm at 25°c, in 10s intervals for 60 cycles (10 minutes). The output of the plate reader machine was measured as mOD/10µl/min (mOD = milli optical density unit), and is used to calculate ADP production according to this formula:

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

Finally, protein concentration of the samples was calculated using the pierce BCA protein assay kit. BSA standards were also made according to protocol (table 2)

Table 2: BSA standard concentrations

µg/10 µL	**2mg/ml BSA standard**	**DiH_2O**
0	-	100 µL
5	25 µL	75 µL
10	50 µL	50 µL
20	100 µL	-

Then 50 parts of A was mixed with 1 part of B (20 mL A with 400 µL B for 1 microplate), and 200 µL was pipetted into each well. After pipetting, the plate was placed on a plate shaker for 30 seconds, covered in parafilm and placed in the Spark multimode microplate reader at 540nm. NKA Enzyme activity was calculated in a standardized excel sheet, using these formulas:

$$\frac{nmoles\ ADP/\ 10\mu\text{L/min}}{\mu\text{g/10}\mu\text{L protein}} = \mu\text{molesADP/mg protein/min}$$

$$\mu\text{molesADP/mg/min (60 min)} = \mu\text{molesADP/mg protein/hour}$$

2.4 statistical analysis

Data collected was structured in Excel (version 2304), which was then imported into RStudio (version 4.3.0) for statistical analysis and plotting. The following packages were used: dplyr, ggplot2, nlme, readxl, writexl, stringr, emmeans, tibble, data.table, gridextra, and tidyverse. Response variables for analysis were Nka activity, length, weight, specific growth rate (SGR), and feed conversion ratio (FCR). Linear models (Two-Way ANNOVA) analysis was fitted between each response variable and the predictor variables treatment and degree days for sampling. Two different models were fitted to the data set for each response variable. The first model used treatment and degree days as predictor variables. The second model used treatment and sampling as predictor variables. For each model, to determine distribution of each response variable, normality (Q-Q- plots), homogeneity of variance (scale location plots), or influential outliers (residuals vs leverage with cook's distance) was used.to assure that the best fitted model was used for further analysis. Finally, a Tukey's HSD post-hoc test was applied to test the models for significance between treatments. For significant differences between each FW timepoint (early transfer; 156 dd, normal transfer; 348 dd and late transfer; 516 dd) and final SW sampling (6 weeks SW; early, normal, and late transfer) and growth data an unpaired t-test was used. For t-tests, a Shapiro–Wilk test was used to determine normality, as well as a Kolmogorov–Smirnov test to determine empirical distribution. Of these criteria were not met a non-parametric Mann-Whitney test was applied. To determine statistical significance between treatments, the standard p-value of $p<0.05$ was applied, which was marked with an asterisk (*) in figures in the results ($p < 0,05$ (*), $p < 0,01$ (**) and $p < 0,001$(***)) for both regular and colored asterisks. Non-identical letters were used for significant difference between timepoints/samplings in each group. Data was presented with standard error of mean.

3. Results

3.1 Weight, length, condition factor and Specific growth rate (SGR)

3.1.1 Weight

The early transfer group displayed an increase from SW transfer (168dd) to SW end in both fed and starved groups, from 87.7 ±1.06 g to 143±1.71 g ($p<0.0001$) and starved group, from 79.0±1.13 g to 133.1±2.12 g ($p<0.0001$). Significant differences were found between the fed/starved group at SW transfer (168dd) ($p=0.00269$) and SW end ($p<0.0001$). The normal transfer group exhibited the same pattern, increasing from 112.3±1.28 g to 174.4±2.43 g in the fed group ($p<0.0005$) and from 100.5±1.67 g to 180.5±3.12 g starved groups ($p<0.0001$). The late transfer group showed differences between each sampling point for both groups. The fed group went from 81.1±0.96 g to 134.0±1.61 g ($p<0.0001$) and peaking at 215.0±2.96 g ($p<0.0005$). The starved group went from 78.2±1.00 g to 117.0±1.56 g ($p<0.0001$), and finally peaking at 203.1±2.83 ($p<0.0001$). Significant differences were found between the SW end fed/starved groups ($p<0.0038$).

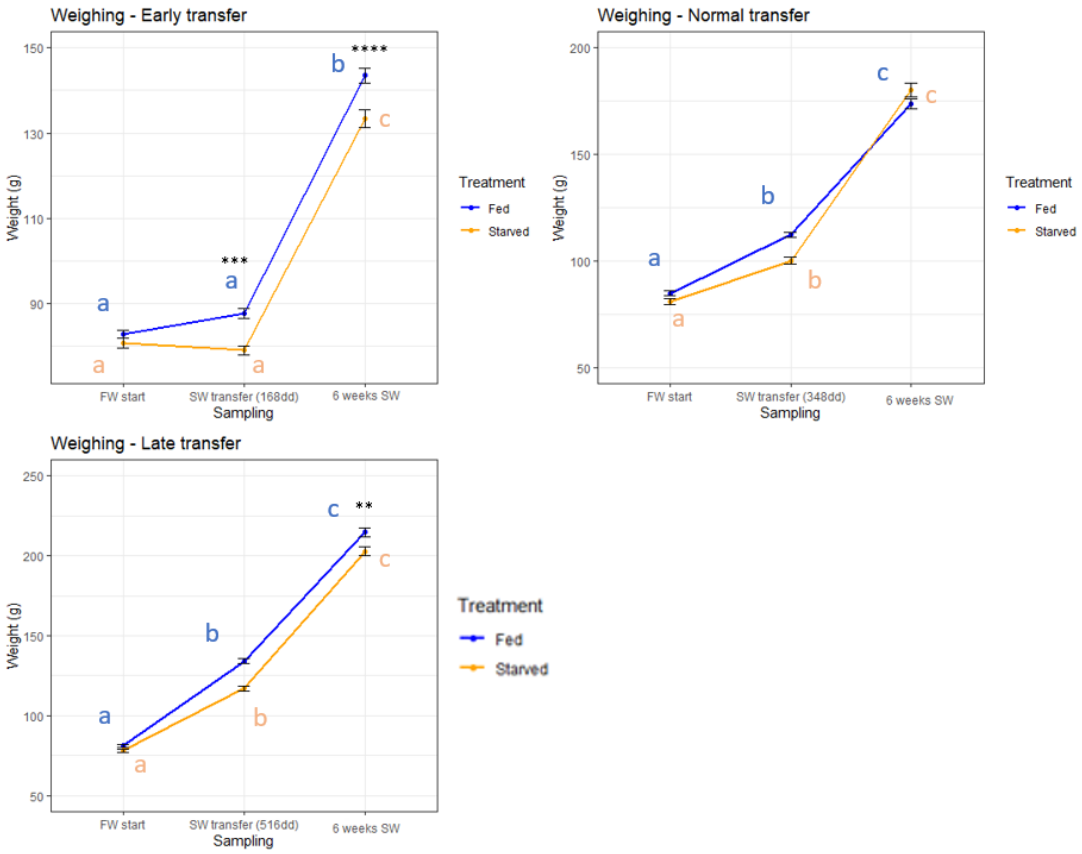

***Figure 8: Weight of Atlantic salmon smolt (grams, g) in FW (FW start – SW transfer), and after 6 weeks of SW exposure**. Different small letters indicate significant differences between timepoints in FW for the starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed. Each datapoint is represented as mean +- standard error of mean (SEM).*

3.1.2 Length

The early transfer group showed an increase from SW transfer (168dd) for both groups (fed/starved). The fed group went from 18.5±0.145 g cm to 22.8±0.116 g ($p<0.0001$), while the starved group went from 18.1±0.165 cm to 22.0±0.164 cm ($p<0.0001$). at SW end, the fed group is significantly higher than the starved group ($p>0.0001$). The normal group exhibited significant differences for each sampling point, for both groups. The fed group gradually increased from 18.5±0.315 to 19.8±0.142 ($p<0.0001$) and peaking at 24.2±0.182 ($p<0.0001$). a slight significant difference was shown between the fed/starved group at SW transfer (348dd). ($p<0.0334$). The same pattern is shown in the late transfer group. The fed group increased from 17.8±0.214 cm to 21.6±0.165 cm ($p<0.0001$) and peaking at 26.0±0.160 cm ($p<0.0001$). the starved group also increased from 18.9±0.387 cm to 21.4±0.168 cm ($p<0.0001$) and peaking at 25.5±0.132 cm ($p<0.0001$). statistical differences were found between fed/starved groups at FW start ($p<0.0167$) and SW end ($p<0.0108$)

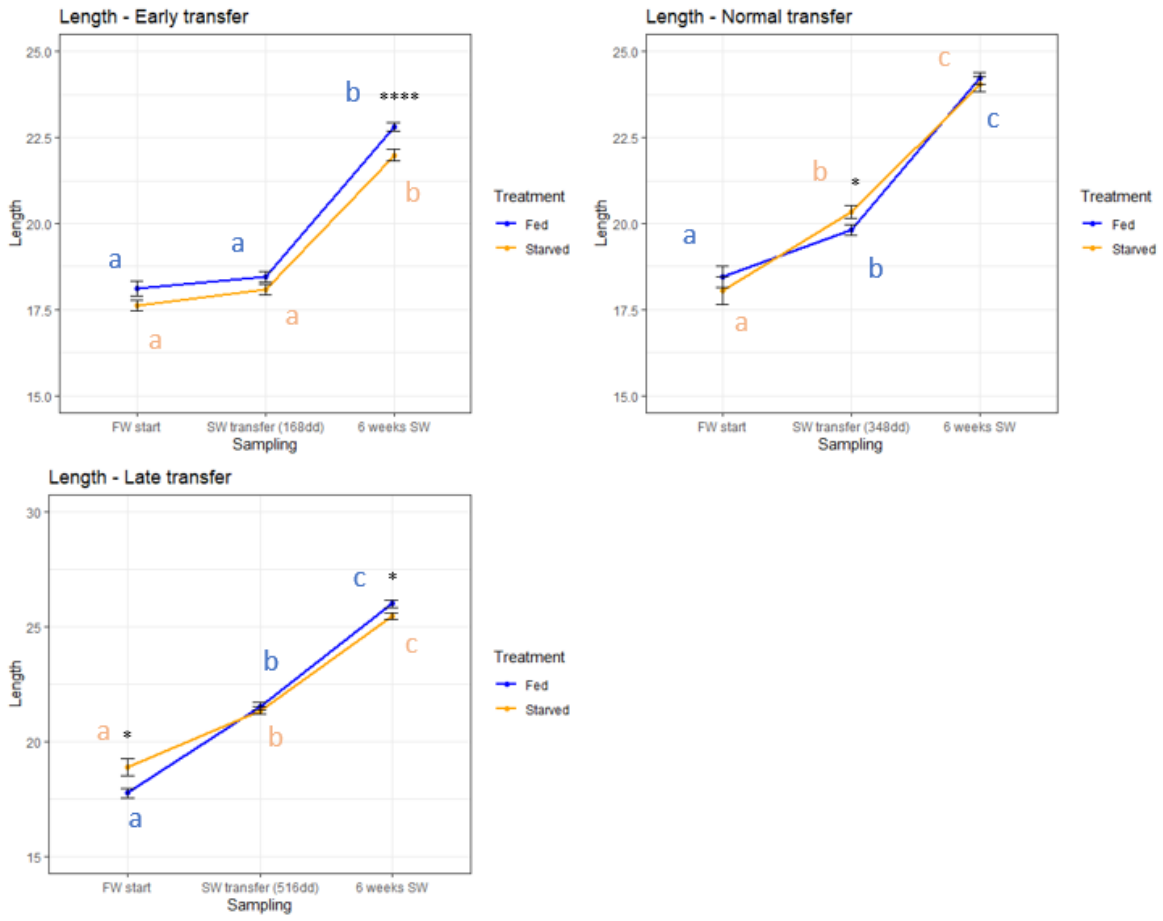

Figure 9: Length of Atlantic salmon smolt (centimeters, cm) in FW (FW start - SW transfer), and after 6 weeks of SW exposure*. Different small letters indicate significant differences between timepoints in FW for the starved, and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed). Each datapoint is represented as mean +- standard error of mean (SEM).*

3.1.3 Condition factor

The early group shows a clear decrease of condition factor for the early fed group, from 1.40±0.011 (158dd) to 1.27±0.0007 ($p<0.0001$). The starved group showed a steady decrease from FW start to SW end, from 1.44±0.024 to 1.34±0.017 ($p<0.0019$), bottoming at 1.25±0.009 ($p<0.0001$). a significant difference was found between fed/starved groups at SW transfer (168dd) ($p<0.0038$). The normal transfer group showed a decrease of condition factor from 1.40±0.025 (248dd) to 1.25±0.015 ($p<0.0001$). The starved group showed a decrease from FW start, 1.42±0.049 to 1.27±0.017 ($p<0.0001$). Furthermore, a significant difference was found between the starved/fed group at SW transfer (348dd) ($p<0.0001$). The late transfer group showed a decrease in the fed group, from FW start, 1.46±0-046, to 1.29±0.009 ($p<0.0001$). The starved group showed no significant differences.

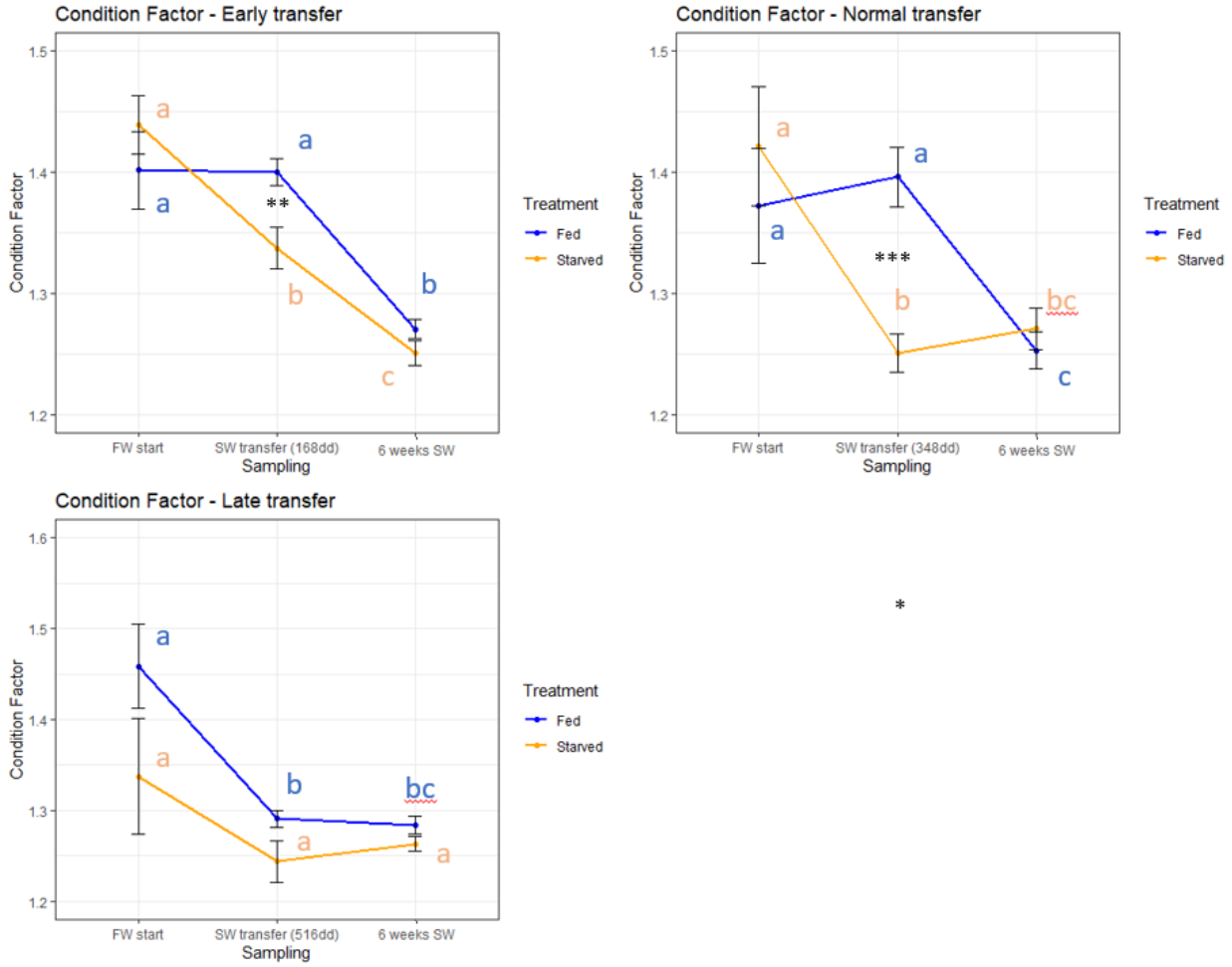

***Figure 10: Condition factor of Atlantic salmon smolt in FW (FW start - SW transfer), and after 6 weeks of SW exposure**. Different small letters indicate significant differences between timepoints in FW for the starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed. Each datapoint is represented as mean ± standard error of mean (SEM).*

3.1.4 Specific Growth Rate

The early transfer group shows a massive drop of SGR in the fed early group, between FW start, 1.20±0.015 (%/day) to 0.42±0.033 (%/day) ($p<0.0001$). at 6 weeks SW though, it drastically increased again to 1.23±0.023 (%/day) ($p<0.0001$). The starved group exhibits the same pattern. From 1.16±0.018 (%/day) at FW start, it drops into the negatives, at -0.16±0.019 (%/day) ($p<0.0001$) but recovers at SW end (1.30±0.035 (%/day)) ($p<0.0001$). A statistically significant difference was found between the fed/starved group at 168dd ($p<0.0001$). Similar patterns are shown in the normal transfer group as the early group. The fed group decreases from 1.26±0.013 (%/day) to 1.01±0.031 (%/day) ($p<0.0001$), yet no difference from SW transfer to 6 weeks SW. The starved group decreased from 1.15±0.018 (%/day) at FW start, to 1.01±0.031 (%/day) at SW transfer (348dd) ($p<0.0001$), then increased to 1.44±0.028 (%/day) ($p<0.0001$). Significant differences were found between fed/starved groups at each sampling time ($p<0.0001$ at each sampling time). At the late transfer group, SGR hovered closely between each point of the fed group, yet the differences were recorded as highly significant. It went from 1.15±0.0014 (%/day) to 1.20±0.0015 ($p<0.0001$), to 1.18±0.024 (%/day) ($p<0.0001$). The starved group decreases from 1.09±0.016 (%/day) to 0.96±0.019(%/day) ($p<0.0001$), then increasing to 1.36±0.0019 (%/day) ($p<0.0005$)

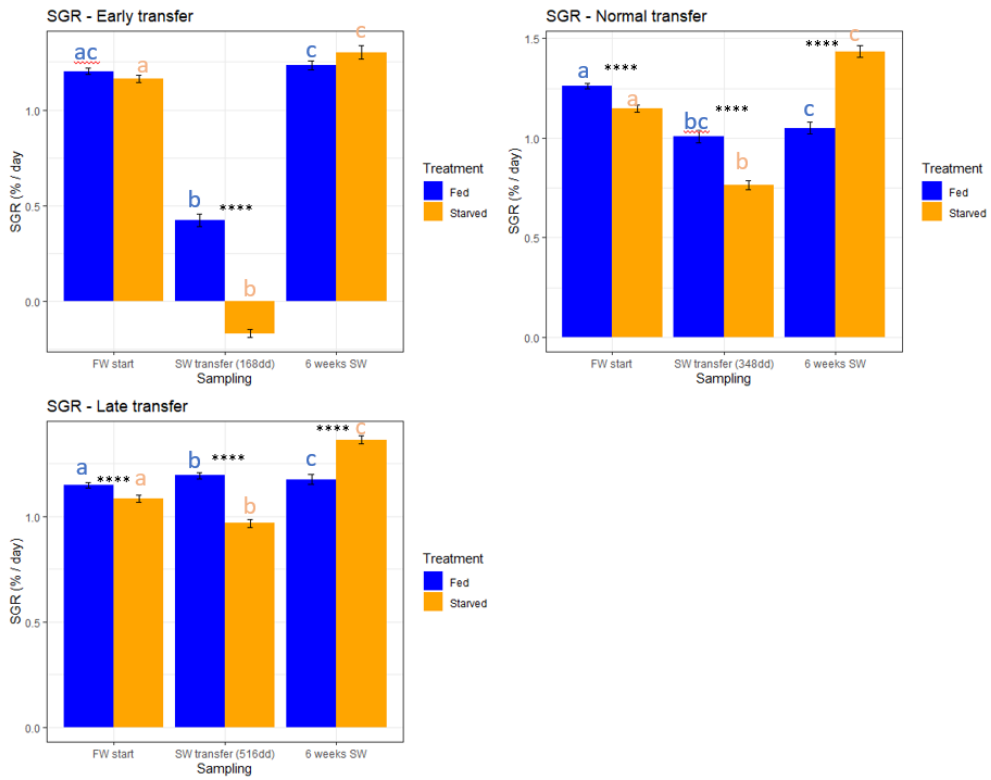

Figure 11: Specific growth rate (% / day) of Atlantic salmon smolt in FW (FW start - SW transfer), and after 6 weeks of SW exposure*. Different small letters indicate significant differences between timepoints in FW for the*

*starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed. Each datapoint is represented as mean ± standard error of mean (SEM).*

3.3.5 Feed conversion rate

Due to lacking data, statistical analysis of specific growth rate (SGR) was not possible. However, some general trends can be observed. SGR decreased from FW start to SW transfer but remained similar from SW transfer to 6 weeks of SW exposure.

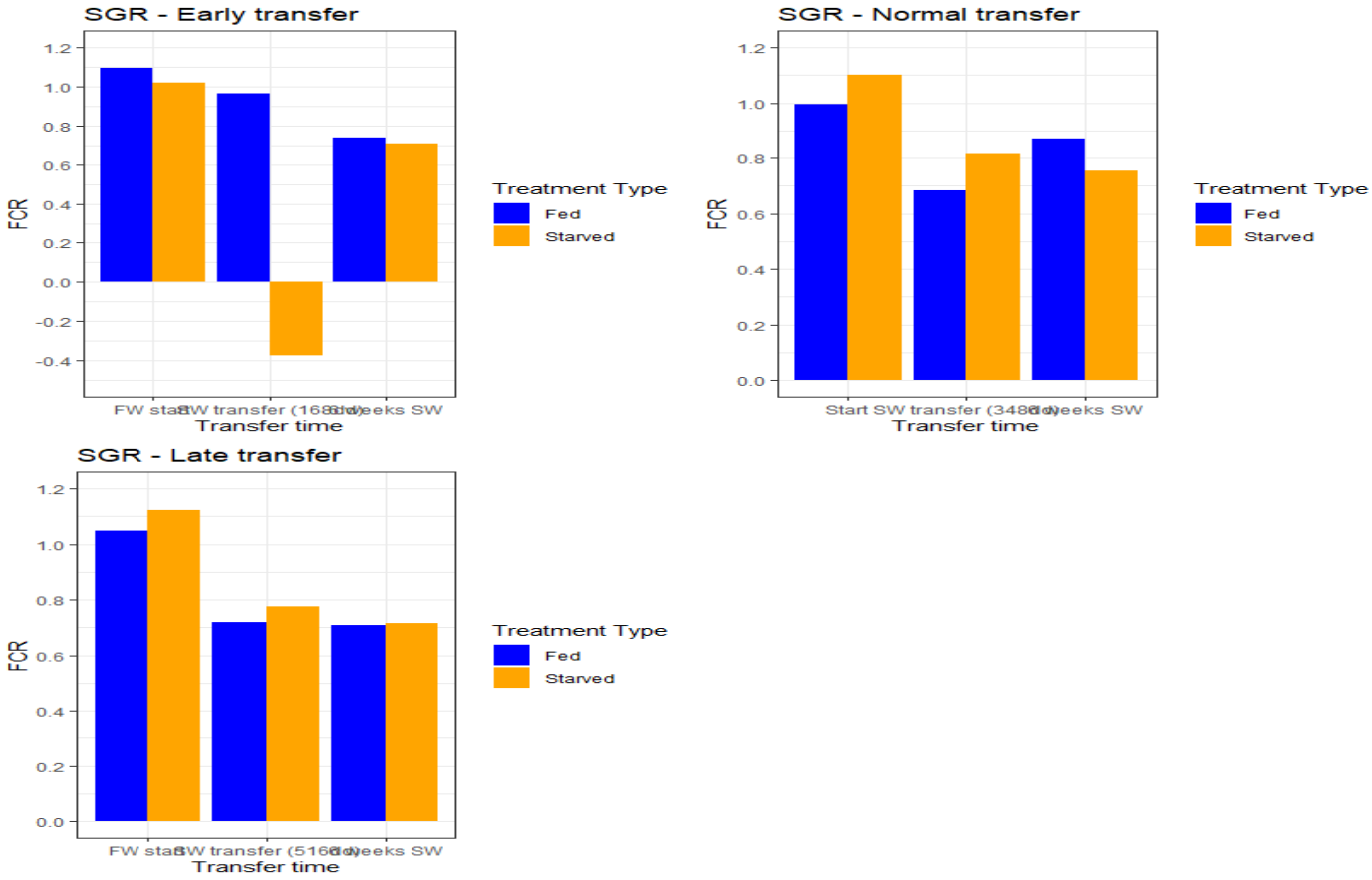

Figure 12: Specific growth rate (SGR) of early, normal and late transfer groups. *Samplings were done at FW start, differing SW transfer times (168dd, 348dd,516dd) and 6 weeks after SW transfer. Samples were collected for fed and starved fish. Statistical significance was not possible to determine.*

3.2 Nka enzyme activity in the gills, intestine, and kidney

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

Gill Nka enzyme activity levels steadily increased in both groups (fed/starved). In the fed group, activity went from 8.14±1.05 (168dd) to 10.65±1.05 (348dd) (p=0.2145) and reaching a peak of19.66±1.05 (516dd) (p=0.0001). Significant increases were not achieved until 516dd. In the starved group, activity went from 6.41±1.05 (168dd) to 14.58±1.05 (348dd) (p=<0.0001) and reaching a peak of 21.01±1.05 (516dd) (p=0.0001). All groups showed significant differences. The SW, the fed group activity was 13.96±0.45 at 168dd, a significant decrease from its FW phase (p=0.0001). At 348 dd, activity was 21.76±11.23, a significant increase form FW (p=<0.0001). at 516dd, activity was 10.41±0.61, significantly lower than in FW (p=0.0213). The starved group showed activity of 16.10±0.88at 168dd, significantly lower than in FW (p=<0.0001). At 348 dd, activity was 24.44±0.69, significantly higher than in FW (p=<0.0001). At 516dd, activity was 12.05±0.54, not significantly different from FW (p=0.3837). At 348 dd the fed and starved group in SW showed a significant difference (p=0.0271)

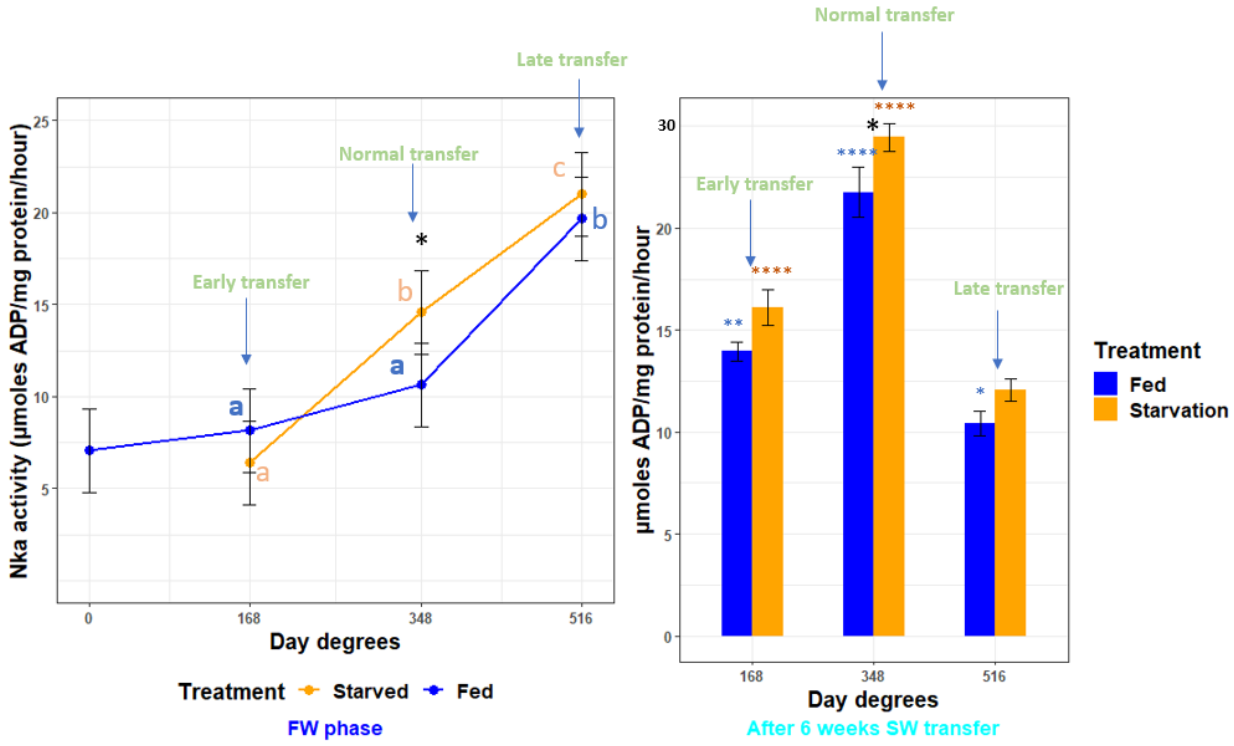

Figure 13: Gill Nka enzyme activity (µmoles ADP/mg protein/hour) of Atlantic salmon smolt in FW, and after 6 weeks of SW exposure. *Different small letters indicate significant differences between timepoints in FW for the*

*starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed), and colored Asterisks indicate significant differences between FW and SW groups at each sampling point. Each datapoint is represented as mean ± standard error of mean (SEM), and N=10-12. Baseline (0) is included in the figure for reference, but not considered statistically.*

3.1.2 Kidney Nka activity

Gill Nka enzyme activity levels did not increase until 516dd in both groups (fed/starved. In the fed group, activity went from 8.15±0.64 (168dd) to 6.41± 0.64(348dd) (p=0.9367) and reaching a peak of19.67± 0.64(516dd) (p=<0.0001). Significant increases were not achieved until 516dd. In the starved group, activity went from 6.41±0.64 (168dd) to 14.58±0.78 (348dd)(p=0.9625), and reaching a peak of21.02± 0.64(516dd) (p=<0.0001). Significant increases were not achieved until 516dd. The SW, the fed group activity was 13.96±0.46at 168dd, a non-significant change (p=0.1546). At 348 dd, activity was 21.77±1.23, a significant increase form FW (p=<0.0001). at 516dd, activity was 10.42±0.62, significantly lower than in FW (p=0.0079). The starved group showed activity of 16.10±0.84 at 168dd, a non-significant change (p=0.8874). At 348 dd, activity was 24.45±0.69, significantly higher than in FW (p=0.0003). At 516dd, activity was 12.05±0.55, significantly different from FW(p=<0.0001).

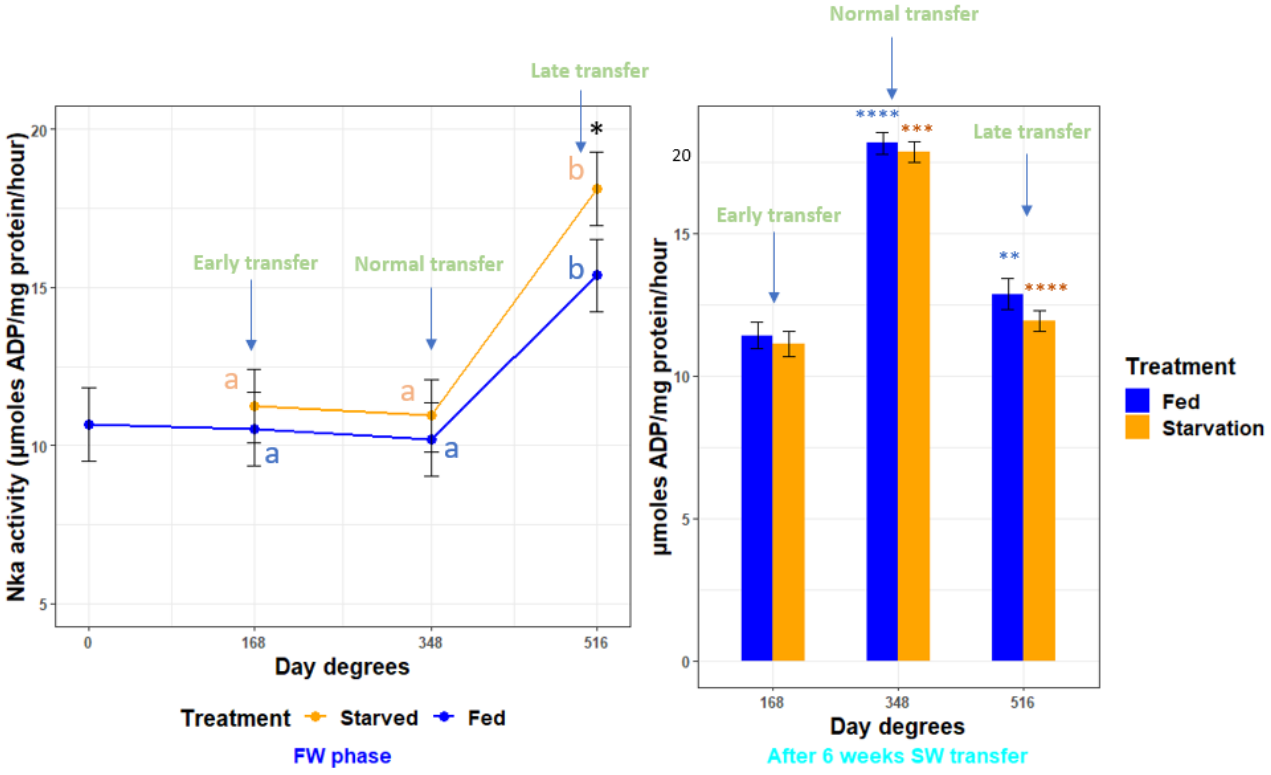

Figure 14: Kidney Nka enzyme activity (µmoles ADP/mg protein/hour) of Atlantic salmon smolt in FW, and after 6 weeks of SW exposure*. Different small letters indicate significant differeneces between timepoints in FW for the starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant*

differences between groups (starved/fed), and colored Asterisks indicate significant differences between FW and SW groups at each sampling point. Each datapoint is represented as mean ± standard error of mean (SEM), and N=10-12. Baseline (0) is included in the figure for reference, but not considered statistically.

3.1.3 proximal intestine Nka activity

Proximal Nka enzyme activity levels steadily increased in both groups (fed/starved. In the fed group, activity went from 10.99±2.11 (168dd) to 13.91± 2.02 (348dd) (p=0.5658) and reaching a peak of21.41± 2.02(516dd) (p=0.0264). Significant increases were not achieved until 516dd. In the starved group, activity went from 12.20±2.02 (168dd) to 16.52±2.48 (348dd) (p=0.3801) and reaching a peak of 25.10± 2.02 (516dd) (p=0.0230). Significant increases were not achieved until 516dd. The SW, the fed group activity was 6.58±0.86 at 168dd, a significant decrease from its FW phase (p=0.0024). At 348 dd, activity was 19.56±1.07, a significant increase form FW (p=0.0241). at 516dd, activity was 24.44±1.18, significantly higher than in FW(p=0.0109). The starved group showed activity of 6.65±0.72 at 168dd, significantly lower than in FW (p=0.0009) at 348 dd, activity was 17.58±1.04, a non-significant difference from FW (p=0.0979). At 516dd, activity was 26.78±1.69, not significantly different from FW(p=0.3274). While non-significant, clear differences are shown between the starved and fed group in FW.

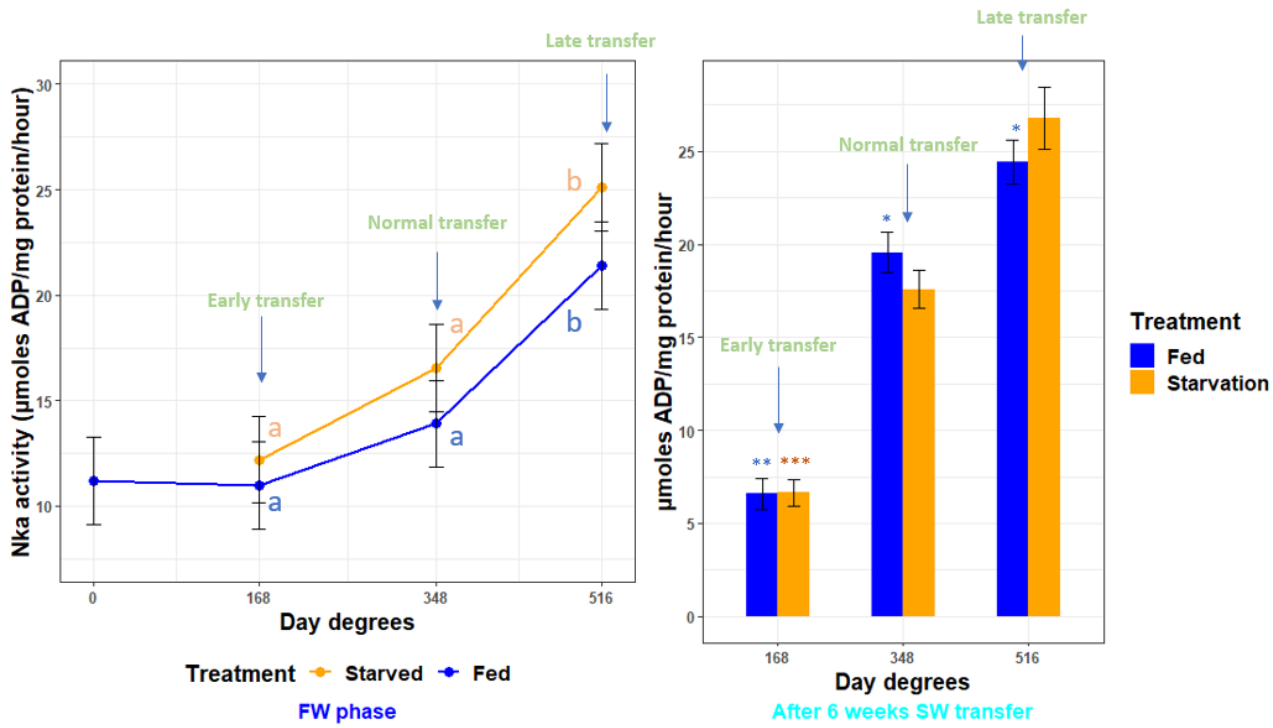

Figure 15: Proximal intestine Nka enzyme activity (µmoles ADP/mg protein/hour) of Atlantic salmon smolt in FW, and after 6 weeks of SW exposure. *Different small letters indicate significant differences between*

*timepoints in FW for the starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed), and colored Asterisks indicate significant differences between FW and SW groups at each sampling point. Each datapoint is represented as mean ± standard error of mean (SEM), and N=10-12. Baseline (0) is included in the figure for reference, but not considered statistically.*

3.1.4 distal intestine Nka activity

Distal intestine Nka enzyme activity levels steadily increased in both groups (fed/starved. In the fed group, activity went from3.87±1.07 (168dd) to 8.15± 1.02 (348dd) (p=0.0123) and then decreasing to 5.91± 1.02 (516dd) (p=0.3544). Significant increases were apparent between 168dd and 516dd, but not 348dd. In the starved group, activity went from 10.33±1.02 (168dd) to 12.72±1.25 (348dd) (p=0.30009), and then peaking at 516 dd at 14.78±1.02 (p=0.0073). Significant differences were shown between early 168dd and 348dd, as well as 348-516dd. In SW, the fed group activity was 1.98±0.33at 168dd, a significant decrease from its FW phase (p=0.0003). At 348 dd, activity was 2.25±0.36, also a significant decrease form FW (p=0.0029). at 516dd, activity was 7.14, not significantly lower than in FW (p=0.7987). The starved group showed activity of 2.5±0.5 at 168dd, significantly lower than in FW (p=<0.0001). at 348 dd, activity was 3.75±0.81, significantly lower than in FW (p=0.0002). At 516dd, activity was 8.23±0.46, again significantly lower than in FW (p=0.0016). The starved group shows significant differences from the fed group at all transfer times in FW.

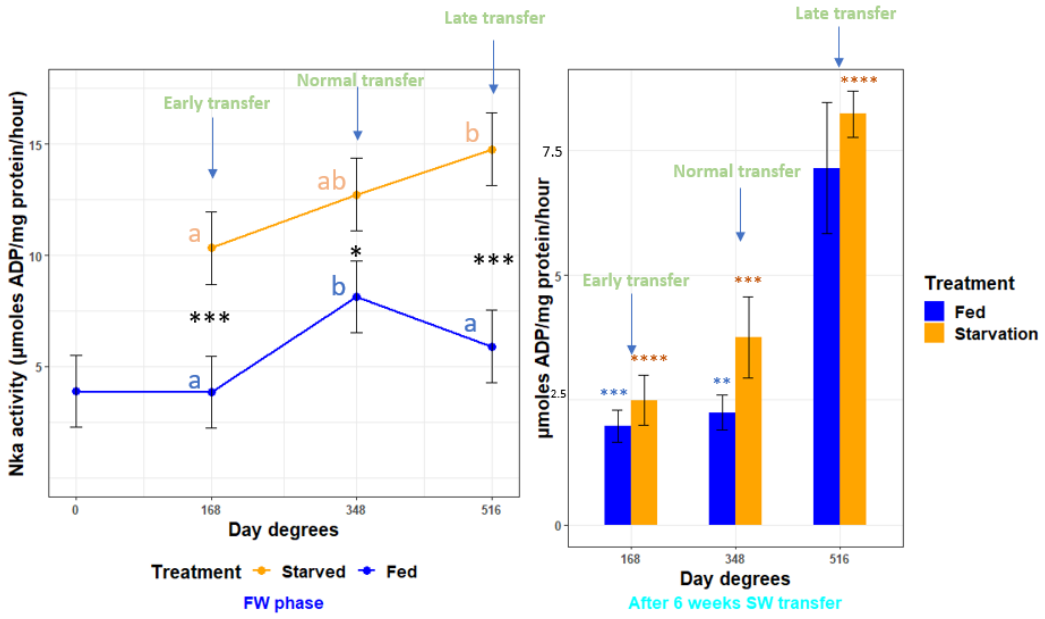

Figure 16: Distal intestine Nka enzyme activity (µmoles ADP/mg protein/hour) of Atlantic salmon smolt in FW, and after 6 weeks of SW exposure. *Different small letters indicate significant differences between*

*timepoints in FW for the starved and fed group. Asterisk *p<0.05 ** p<0.01 *** p<0,001 **** p<0.0001 indicate significant differences between groups (starved/fed), and colored Asterisks indicate significant differences between FW and SW groups at each sampling point. Each datapoint is represented as mean ± standard error of mean (SEM), and N=10-12. Baseline (0) is included in the figure for reference, but not considered statistically.*

4. Discussion

4.1 Considerations of material and methods

4.1.1 Experimental design

The experimental setup includes the use of replicate tanks with randomized placement. The setup is maintained from the initiation of the experiment, until after 6 weeks of SW exposure. Triplicates of each treatment were used, to reduce potential rick of tank effects. Significant tank effect has been in studies which measure growth (Weight, length, condition factor, SGR, FCR) thus proving its significance (Thorarensen et al., 2015). During the experimental phase, two tanks from starved group (early and normal transfer) were given salinity for a short time-period due to an error. Thus, these tanks had to be excluded from the experiment leaving duplicates for commercial group starved for early (168 dd) and normal transfer (348 dd). This could lead to statistical errors, and one cannot fully rule out tank effects. However, duplicate studies on smolt development usually show limited tank effects and produce significant results with high confidence (Bell et al., 1997, 2003; Duncan et al., 1999; Remo et al., 2014). As this study mainly focus on smolt development and not growth it has been decided that duplicates for these groups was sufficient.

The experiment utilized the traditional photoperiodic square wave regime and water temperature (12°C), which is known to stimulate smoltification, as well as induce proper rate of smolt development (Ebbesson et al., 2007; Stefansson et al., 2007), thus producing similar rearing environment to that of industry standards. Furthermore, the temperature of 12°c was chosen to avoid limitations of photoperiodic control from low temperature (Mccormick & Moriyama, 2000), as well as premature maturation from elevated temperatures (Fjelldal et al., 2011). To avoid low oxygen levels, flow rates of 0.6L/kg/min is recommended for stocking densities above 50kg m^3. The experiment had values varying from 6.67-10L/kg/min, thus providing ample oxygen saturation (95-120 %). Oxygen was constantly monitored and kept above 95%.

The sampling protocol for this experiment is consistent with standardized protocols used in similar studies (Takvam et al., 2021a; Takvam et al. 2023). For sedation/euthanasia, Tricaine Pharmaq was used. Furthermore, water from each respective tank was used to ensure optimal water quality. During sedation, bicarbonate (Na2CO3) was added in the same amount as Tricaine to buffer the solution. For euthanasia 6x normal dose of Tricaine pharmaq was used. As criteria for death, opercula movement and eye rolling reflex was used. These procedures

were to ensure proper handling and fish welfare. After length and weight measurement, tissue samples were harvested and promptly put on dry ice and stored at -80°c to prevent tissue degradation.

4.1.2 Nka enzyme activity in the gills, kidney, and intestine

Nka activity in the gills, kidney and intestine were measured using the micro assay method of Mccormick (McCormick, 1993) which has been further optimized in Takvam et al (2021; 2023). The activity represents conversion of adenosine triphosphate (ATP) into adenosine diphosphate (ADP) and Phosphate (P). Further, pyruvate kinase converts ADP and Phosphoenolpyruvate into Pyruvate and ATP. Finally, Lactate dehydrogenase converts Pyruvate and NADH into Lactate and NAD^+

This occurs through the following subreactions:

$$ATP + H20 \xrightarrow{Na^+K^+ATPase} ADP + Pi \text{ (I)}$$

$$ADP + Phosphoenalpyruvate \xrightarrow{Pyruvase\ kinase} Pyruvate + ATP \text{ (II)}$$

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

The reaction is highly sensitive to ouabain and is enzymatically coupled with oxidation of nicotinamide adenine dinucleotide (NADH)., Thus, it can be measured by a temperature controlled (25°c) microplate reader. Prior to Nka measurements, a standard curve (range 17-18 mOD/nmole ADP) to ensure proper quality of assay mixture. Due to time constraints and large sample size in the project, analysis was conducted by one other student with the assistance of the supervisors. A strict lab protocol was followed by all students to standardized lab routines, to minimize error.

The McCormick micro assay method is most widely used, requiring only 4-5 gill filaments, while producing similar fold changes to that of the Zaugg method (Zaugg, 1982). Thus, it is possible to assay more samples within a reasonable time limit. However, gill filaments increase in size in relative to fish size, and human error may lead to collection of excessive/insufficient tissue. Consequently, higher variance may occur in the dataset, which may lead to higher error rates. Thus, standardized weighing of gill tissue may provide more consistent data. In the current study fish did not exceed 140 grams so standardized weighing was not required. However, if fish exceeds 200 grams standard weighing of the gill filaments are recommended (See Takvam et al 2023 for more information).

The McCormick method is used mostly on gill tissue. Therefore, optimization of kidney Nka analysis has been developed for use on kidney (Takvam et al 2021a). As a result, the kidney was divided into five areas (A, B, C, D, E) (Fig.9), according to activity patterns. Data demonstrated higher Nka activity in the posterior kidney, which was determined to reflect increased density of nephron more accurately than the anterior kidney. Furthermore, tissue from area D & E were determined more suitable (Fig.9), and thus used in this experiment. Moreover, consistency is of high importance, as to not sample areas with relatively lower Nka activity. For Nka analysis, samples were measured at 10-15 milligrams (mg) for standardization purposes (Takvam et al 2021).

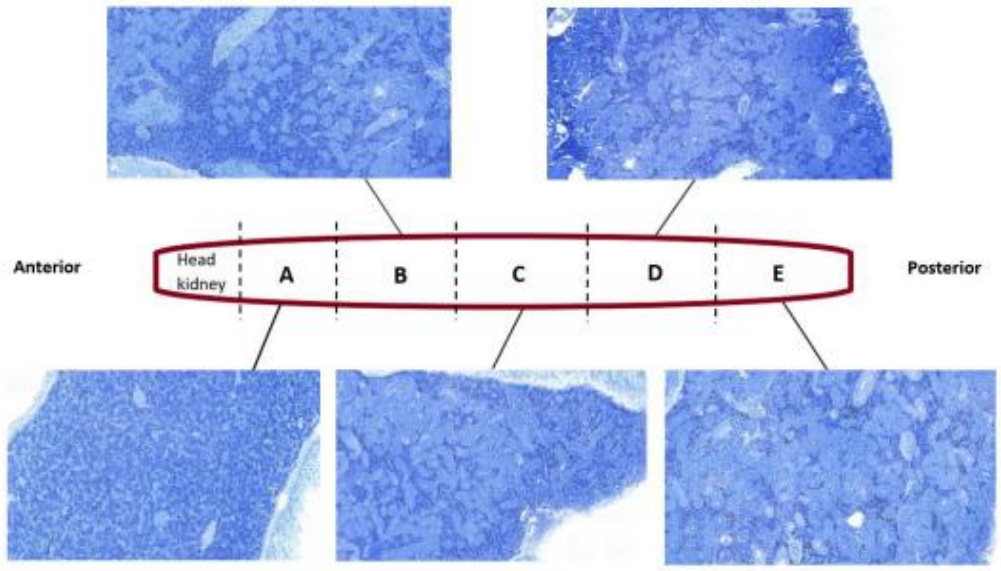

Figure 17; Overview showing the different sections of the kidney. Kidney tissue sections stained with toluidine blue allowed visualization of nephron density and hematopoietic tissue in the kidney (Takvam et al., 2021a).

The intestinal tissue required extra precaution, as the digestive properties of the intestine lead to rapid degradation of tissue (Takvam et al., 2023). Thus, samples were preserved in a modified SEI buffer (200 mM glycine, 45 mM EDTA, 50 mM EGTA, 300 mM sucrose, 50 mM imidazole, including 1 tablet CompleteTM protease inhibitor cocktail (04693124001 Roche)). Furthermore, samples were rapidly transported from site of sampling, to be analyzed the following 2-3 days. Former methods show large variation and/or inconsistent assay performance, and therefore this method was applied (Takvam et al., 2023).

4.1.3 Statistical analysis

Two-Way Annova analysis were applied when analyzing the dataset, as it was determined to be the best fit. A two way-Annova analysis of variance has four assumptions; 1) The

populations from which the samples were obtained must be normally or approximately normally distributed. 2) The samples must be independent. 3) The variances of the populations must be equal. 4) The groups must have the same sample size. As mentioned earlier, for each model, to determine distribution of each response variable, normality (Q-Q- plots), homogeneity of variance (scale location plots), or influential outliers (residuals vs leverage with cook's distance) was used, thus satisfying #1-3. Sample sizes were standardized for each treatment group (N=12), thus satisfying #4. When analyzing growth parameters, SW samples, and differences between FW/SW an unpaired t-test was utilized. The most important criteria of unpaired t-tests are gaussian distribution (1) and unpaired data (2). Gaussian distribution was mostly satisfied, but in some cases a non-parametric Mann-Whitney test was applied instead, to provide more accurate statistics. Additionally, significance value of <0.05 was selected as it is commonly used in biological studies (Fay & Gerow, 2013). However, variations can be large in biological studies, which could affect the statistical outcome. In cases which some of the triplicate tanks (N=4, 12 total) were terminated from the experiment prior to sampling, N=6, 12 total was instead used on the remaining duplicate tanks to achieve sufficient statistical power.

4.2 Discussion of results

4.2.1 Weight, length, condition factor, SGR and FCR

The findings of growth performance, showing an increase in weight and length, as well as reduced condition factor, is consistent with smolt attributes. Smolt are known to adapt a slimmer, silvery body (Mccormick, 2013; Stefansson et al., 1991). This growth further corresponds with growth patterns of smolt exposed to classic photoperiod protocols, for stimulation of smoltification (Stefansson et al., 1991). Experimental protocols were identical for all groups, except for SW transfer time (dd) and treatment (starved, non-starved at end of FW phase), and thus, differences stem from these parameters. All transfer groups showed consistent growth in FW (FW start - SW transfer) both starved and non-starved, except for the early transfer group. This may indicate incomplete smoltification, as they were transferred at 168dd, and peak transfer time for good smolt parameters are suggested to be at 280-350dd (Handeland et al., 2004; Stefansson et al., 1998). Growth dramatically increased after SW transfer, likely due to the increased scope of growth for salmon in seawater(Handeland et al., 2003; Mccormick et al., 1998). Specific growth rate (SGR) declines significantly at FW end (transition to SW for each group). Reduced SGR of smolts at SW transfer may in part be due to the energetic demands of smolt development, which include increased basal metabolic rate,

increased activity, and reduced lipid content (Mccormick, 2013). The extreme drop of SGR in the early transfer may be a greater energy % is dedicated to smoltification yet is difficult to justify being completely physiological. It is possible that sampling may have selected mostly large fish for weighing at 168dd, and small fish at 348dd.This would also explain the negative FCR at the same sampling point. Furthermore, condition factor was lower at each starved SW transfer group. This may be simply due to emptying of the gut sac which occurs between 49-96 after feed deprivation, (Valente et al., 2012) lowering weight, as similar studies have shown (Kalananthan et al., 2020).

4.2.2 Nka enzyme activity in the gills, intestine and kidney

The Na+/K+- ATPase (NKA) pump is a critical component in regulating intracellular ionic gradients used for cellular homeostasis and plays a role in the secondary transport of various compounds (Skou & Esmann, 1992). Though Nka activity increases during smoltification, Full capacity is not achieved until SW, as activation in FW would result in considerable problems in maintaining homeostasis. This study measured Nka activity in the gills, intestine, and kidney, at three different SW transfer times (prior to SW exposure), and 6 weeks after SW acclimation in both starved and non-starved fish. Historically, Nka activity in the gills have been a focal point for monitoring smolt development, given its hypo-osmoregulatory ability (McCormick, 2013; Stefansson et al., 1998, 2007; Striberny et al., 2021). Several studies have also been conducted on the role of Nka in the intestine, especially in relation to water and monovalent ion absorption (Sundell & Sundh, 2012; Sundh et al., 2014). Furthermore, Nka activity in the kidney has been sparsely studied ((Madsen et al., 2020; McCartney, 1976; McCormick et al., 1989; Takvam et al., 2021a). To better understand optimal transfer time of Atlantic salmon, all mentioned tissues should be equally addressed, and assessed during smolt development.

4.2.3 Nka enzyme activity in gills, intestine and kidney in during smoltification

No studies have been conducted on the gills, intestine and kidney simultaneously. However, work from a master thesis documented overall increase in gill, kidney, and proximal and distal intestinal Nka enzyme activity, which is consistent with the developmental progression of hypo-osmoregulatory capacity in Atlantic salmon during smoltification (Takvam, 2020 (Mccormick et al., 1998).and similar to that found in the current study. Recently the results from several of studies done the last years on Nka enzyme activity assay in all three organs have been submitted to Reviews in Aquaculture (Takvam et al., 2023).

Nka activity levels steadily increased during smoltification, which parallels findings in other studies (Mccormick, 2013; McCormick et al., 1989; Nilsen et al., 2007). Peak Nka activity was registered at 518d dd, however it was adequate at 348 dd. The kidney, however, did not show any significant Nka activity increase between 168-348dd. While a significant increase was first observed at 518 dd compared to 348 dd the kidney reached sufficient activity levels, for both starved and non-starved group. An identical pattern was observed in the proximal and distal intestine, except for the fed late transfer group, which showed a significant decrease in Nka activity. Clearly, all three organs show significant increases during smoltification in which all three organs reach peak levels at 518 dd. However, in both proximal and distal intestine the staved group (4 days) consistently show higher Nka activity although only significant in the distal intestine at all transfer times.

4.2.3.1 NKA enzyme activity in the gills

The smolt window is defined as the timeframe during which fish are fully acclimated for seawater (SW) transfer, making this period for survival and optimal growth in SW (Stefansson et al., 2008). This is important as to secure optimal pumping capacity in dealing higher Na and Cl ions in SW in which the gill have a key role in secretion to maintain stable plasma levels of Na and Cl in the blood (Nilsen et al 2007; Hiroi et al 2012; McCormick et al 2013). Handeland et al., (2004) determined that a period of approximately 350 degree-days at 12 degrees was needed for smolts to reach peak gill NKA activity levels from when the smolt-related increase was first noticed(Handeland et al., 2004). In the current study the Nka enzyme activity levels in gills continued to increase until 516 dd at the same temperature which indicate that in fact the gills reach peak levels at 516 dd rather than 350 dd at 12-degree rearing temperatures. In fact, loss of gill Nka enzyme activity is known to occur between 500-900 dd which is contradicting to the current study as we find that the activity increases even after 500 dd. However, in the future more frequent samplings timepoints should be performed from 350-900 dd as to find the optimal "smolt window" and determine when substantial loss of Nka activity occurs. Satisfactory gill Nka activity levels are often related to the relative capacity of the gills to handle increased salinity and thus are important to measure before transfer to SW. However, it is not the only predictor of long-term SW growth as indicated by (Zydlewski & Zydlewski, 2012).The gills are not the only osmoregulatory organ in fish, and these studies among others (Handeland et al., 2013; Hiroi & McCormick, 2012; McCormick et al., 2009) do not consider the development of Nka activities in the kidney and intestine which individually and together

perform crucial roles in ion and water transport (Sundell and Sundh, 2012; Takvam et al., 2021b).

4.2.3.2 NKA enzyme activity in the proximal and distal intestine during smoltification and SW acclimation

The proximal and distal intestine showed an increase in Nka activity similar to that of other studies (Sundell et al., 2003; Sundell & Sundh, 2012; Sundh et al., 2014; Takvam, 2020)). As smolt develop the drinking reflex, it is key that sufficient transportation capabilities are developed, and thus this increase is central for optimal smolt quality mainly to enable concurrent water and NaCl uptake in the intestine (Sundell & Sundh, 2012). The increased fluid absorption in SW is associated with elevated NKA activity along the whole intestinal canal, and thus an increase in activity is expected during smolt development (Sundell et al., 2003; K. S. Sundell & Sundh, 2012). High Nka values are also required in FW, to facilitate absorption of ions, which are dearly needed in FW, which corresponds to our findings. Furthermore, NKA activity was measured much higher in the proximal intestine, reflected by its main function of nutrient absorption (Bakke-Mckellep et al., 2000; Bjørgen et al., 2020). This study measured Nka values are relatively high compared to previous studies, however they correspond well to that of Takvam (2020). Moreover, this study used a modified version of the micro assay method of Mccormick (1993), identical to that of Takvam (2020). This method has been further polished and may thus provide more accurate reading of intestinal Nka activity (Takvam et al 2023) than other similar studies (Sundell et al., 2003; Sundell & Sundh, 2012).

4.2.3.3 NKA enzyme activity in the kidney during smoltification and SW acclimation

The present study also aimed to investigate the changes in kidney Nka (Na+/K+-ATPase) activity during smoltification in Atlantic salmon, as they have received significantly less attention than the gills and intestine. Only a few studies have so far have measures Nka activity during smoltification (McCartney, 1976; Takvam et al., 2021a), and one has shown increase NKAa1a and NKAa1b activity (Madsen et al., 2020). This study shows an overall increase in kidney Nka activity during smoltification, which corresponds with previous studies. Takvam found kidney Nka activity to peak later than gill Nka, at 450 dd, while sufficient activity was recorded at 350 dd (Takvam et al., 2021a). This study on the contrary, did not show any significant Nka activity increase from experimental start, until 516 dd, in which activity peaked, and was significantly higher. While this suggests a higher dd for sufficient activity, this study does not have any datapoints between 350 and 518 dd. in this range, it is possible that sufficient activity could be recorded, as in former studies (Økland, 2021; Takvam et al., 2021a). The increase in Nka activity is likely a preparatory response to meet the enhanced requirements for

active ion reabsorption during smoltification, as smolts shift from reabsorption of ions (Edwards & Marshall, 2012)o secretion in SW (Edwards & Marshall, 2012). Through the kidney, Nka activity facilitates the reabsorption of approximately 95% of NaCl in FW fish, in order to maintain homeostasis (Perry et al., 2003; Tang et al., 2010), as ions are lost through diffusion. Such high reabsorption is possible due to the low water permeability of the distal and collecting ducts of the kidney, allowing high reabsorption of ions while removing excess water producing a dilute urine (Hickman & Trump, 1969, Takvam et al., 2021a). This high reabsorption is facilitated by high UFR and GFR, which coupled with reabsorption of 80% of na+ in FW and 23% in SW (Talbot et al., 1992) indicates that the relatively high Nka activity early in smoltification plays a significant role in FW homeostasis. The relatively high Nka enzyme activity levels observed in the kidney early in the smolt development is likely associated with the need to reabsorb most Na and Cl from the pre-urine. While the higher level in SW is likely related to the increased need to secrete divalent ions such as Mg, Ca and SO42- (Takvam et al., 2021a; Takvam et al., 2021b).The delayed increase in Nka enzyme activity levels in the kidney highlight that the development of the pumping capacity (e.g level of Nka enzyme activity) necessary to remove excess salts is clearly different than that of the gills. Thus, early seawater transfer may have unknown physiological consequences for the fish's ability to remove divalent ions after SW transfer. Indeed, Nka enzyme activity is crucial in establishing the transmembrane potential for secondary transport of solutes such as divalent ions. Thus, higher Nka enzyme activity levels before transfer to SW would likely aid towards and easier transition from the low salinity environment of FW to the high salinity environment of SW.

4.2.4 The effect of starvation on gill, intestinal and kidney Nka enzyme activity in smolts

There are no studies investigating the effect of short-term starvation on smolt performance, in relation to Nka activity. As short-term starvation is common industry practice to empty the gut and reduce metabolites in transfer tanks (Waagbø et al., 2017), it is important to study its effects on smolt development and secondly the effect on performance after SW transfer. All three organs appear to be affected by starvation through increased Nka enzyme activity compared to the non-starved group. This was observed in the gills at 348 dd, 516 dd in the kidney and at all three timepoints in the distal intestine (168 dd, 348 dd and 516 dd). No significant changes were observed in the proximal intestine, but clear trends could be observed. Thus, three hypotheses were formulated to explain this response.

Hypothesis 1:

increased Nka enzyme activity in the gills may be a respone to the shift in using fat as their main energy source as this have been observed in rainbow trout but using longer starvation times of 27-40 days (Jezierska et al., 1982). Although lipids are known to modulate the Nka pump (Cornelius, 2001; Cornelius et al., 2015) we can still only speculate to which this is the main cause for the increased activity levels at 348 dd, but we do know that starvation quickly changes the energy metabolism in salmonids (Hevrøy et al., 2011) which may have unknown consequences for the fish in the energy demanding period of smoltification. This same concept would also possibly explain the higher activities observed in gill (348 dd) and kidney (516 dd) where the Nka likely would be modulated by the increased circulating lipids delivered to the different tissues. One explanation why this difference appears to be more prominent in the intestine may be that intestine is known to have large fat stores (Hansen et al., 2020). Thus, fats may be more readily available in the intestine once fish are starved and switch their energy metabolism to use fat stores during the energy demanding smoltification process. The gills and kidney may not have an immediate availability which may explain the different responses in these tissues at the different transfer times. Future studies need to better understand how fats and energy metabolism changes during short and long-term starvation periods and the possible link to Nka enzyme activity levels (pumping capacity) as well as compensatory effects related to increased need for salt abortion when feed is removed. This need to be considered from the perspective of the gills but also kidney and intestine as we observed similar response in these tissues.

Hypothesis 2:

Another possible explanation could be that starved fish do not receive any salt, as their main source is through feed in FW. Commercial feed is relatively high in salts due to the use of fish meal (Økland, 2021). As fish are in low salinity environment (FW) salts are essential and as starved fish lose its main source of salts, this may result in a compensatory increase in the intestinal Nka enzyme activity to reabsorb more salts. While there are clearly differences in the proximal intestine (not significant), the response is clearly stronger in the distal intestine (significant). The distal intestine may compensate more for the loss of salts compared to the proximal intestine. A newly published paper showed that salt absorption in the distal segment of the intestine was crucial for freshwater rainbow trout (Ciavoni et al., 2023). Furthermore, the study showed that Na^+ was absorbed in the distal segment, while it was secreted in the proximal segment. $Ca2^+$ was significantly different in middle and distal segment, indicating that there is increased need for absorption of salts in a low electrolyte diet (Ciavoni et al., 2023).

Collectively, this indicate that the distal segment has a more prominent role in salt absorption (at least Na^+ and $Ca2^+$) and the increased Nka enzyme activity observed in the current study may be a strong compensatory effect associated with the complete loss of salts when starved which promptly may induce increased need for salt absorption in the salinity scarce environment of FW. As we observed the same response in the gill (348 dd) and kidney (516 dd) we cannot rule out a similar compensatory response associated with increased need for salt absorption. In the gill the increase in Nka enzyme activity at 348 dd in starved fish may be related to the increased need to absorb Na^+ and Cl^- (Nilsen et al 2007; McCormick et al 2013) and in the kidney increased need for reabsorption as the kidney in general reabsorb approximately 95% of NaCl in FW fish, in order to maintain homeostasis (Perry et al., 2003; Tang et al., 2010), this increase of activity may attempt to compensate for less NaCl retrieved through feed. Such a mechanism has been suggested in common carp (Cyprinus carpio), as a similar pattern was shown in low- feeding groups vs. high-feeding groups (Liew et al., 2015).

Hypothesis 3:

Studies have shown up-regulation of ghrelin in blood plasma during short-term fasting (Hevrøy et al., 2011) which is a known orexigenic (Abdalla, 2015; Székely et al., 2013). Ghrelin stimulates secretion of Growth hormone (GH) which is well known to stimulate hypo-osmoregulatory ability, through an increase in branchial Na+,K+-ATPase activity, and a similar increase in hypothesized in the kidney and intestine (Björnsson, 1997; Nisembaum et al., 2021). IGF-1 has also shown increased activity after 3 days fasting (Breves et al., 2016), which is well known to play a role in increasing salinity tolerance prior to SW transfer (Hoar, 1988; Mccormick, 2013; Wood et al., 2005). There is also seen a higher sensitivity for GH during starvation in the intestine, leading to increased lipid mobilization. The increased sensitivity in fatty tissue enhances the lipolytic actions of GH (Norbeck et al., 2007). Thus, it is possible that a combination of direct up-regulation of Nka activity through hormones, combined with lipids mobilized by hormones, modulating the Nka pump as discussed in hypothesis 1 are responsible for the large increase in the distal intestine. However, such a connection is not established, and further studies are needed.

4.2.5 Nka enzyme activity in gills, kidney and intestine in SW, and SW performance

It is common to use Chloride tests to examine if proper osmoregulatory capacity has been developed in Atlantic salmon (Handeland et al., 1996, 2003; Staurnes et al., 2001). This was

however not possible due to time constraints and lacking data from Skretting. The optimal range of plasma chlorine is between 130-140 mmol/l (Trond et al., 2010), and thus we speculated that the early transfer group would likely be slightly below this, while the normal and late groups would be within the range.

4.2.5.1 Nka enzyme activity in the gills after 6 weeks SW transfer

The osmoregulatory organs (gills, kidney and intestine) require a higher Nka activity in SW than FW, as they transition from hyper- to hypo-osmoregulation, to expel excess ions and prevent dehydration (Grosell, 2010; Nilsen et al., 2007; Perry et al., 2003).

The results show clear tendency of increased NKA activity in the gills, except for the late starved group. These results correspond well with established findings that show and increase following SW transfer of smolt, to ensure sufficient NaCl secretion across the gill epithelia (McCormick et al., 1989, 2013; Nilsen et al., 2007). The normal transfer group shows a much higher increase in Nka activity than the early group. This indicates that a higher performance at 348 dd, which correlates well with former findings (Handeland et al., 2004; Økland, 2021; Takvam, 2020). The late transfer group showed a trend of decreasing gill Nka activity, with a significant difference in the fed group. One possible explanation is that desmoltification has started, hence reduced Nka activity and hypo-osmoregulatory ability (Stefansson et al., 1998). Furthermore, while a peak Nka value was found at 516dd in this study, the actual peak is likely located between 346 and 516dd, corresponding with the results during smoltification.

4.2.5.2 Nka enzyme activity in the intestine after 6 weeks SW transfer

In the intestine, the results show varying degrees of increase/decrease in Nka activity based on transfer time. The proximal intestine shows a significant decrease in the early transfer group, indicating a premature SW transfer. The normal and late transfer group maintained high Nka activity levels, with higher activity in the late group. The distal intestine shows an overall decrease of activity after 6 weeks of SW exposure. The late transfer group shows higher activity, which indicates better performance at 516 dd. Combined, the results show that 516 dd is optimal for SW transfer, while 168 is premature. In general, Nka activity remained relatively high after 6 weeks of SW exposure, supporting the importance of basolateral Nka in counteracting water loss after SW transfer (Grosell, 2010; Sundell et al., 2003; Sundell & Sundh, 2012). The intestine absorbs more fluids in SW, which is associated with Nka activity. Thus, it is of high importance to maintain high Nka values for optimal performance (Sundell et al., 2003; Sundell & Sundh, 2012). Furthermore, usage of the Ussing chamber methods enable measurement of paracellular and transcellular transport in the intestine, through measurement

of electrical characteristics of its epithelia (Sundell et al., 2003). Studies using this method show that paracellular permeability of the intestinal tract is reduced in both the proximal and distal intestine (increased TER), shown in Atlantic salmon (Sundell & Sundh, 2012). This suggests a transition from para- to transcellular pathway, possibly meditated by incorporation of aquaporins into the enterocyte membranes (Sundell & Sundh, 2012), which coupled with increased high Nka activity are vital for optimal SW acclimation, as well as ion-coupled water absorption in the intestine.

4.2.5.3 Nka enzyme activity in the kidney after 6 weeks SW transfer

While there are nearly no studies on kidney Nka activity in Atlantic salmon, the kidney shows similar Nka activity levels a recent study (Takvam et al., 2021a), and further supported by increased in nka-a1a and a1b mRNA levels after SW exposure (Madsen et al., 2020) While the intestine and kidney showed similar patterns of Nka activity during smoltification, the kidney seems to closer resemble the gills in regards of Nka activity after 6 weeks of SW. little change is shown in early and late transfer group, however the normal transfer (348dd) had a highly significant increase of Nka activity, to levels much higher than that of early and late transfer groups. While this may indicate better SW performance after 348dd, it is also possible that Nka levels in the smolt kidney were insufficient, thus prompting a strong compensatory increase. Fish lose water through the gills in SW, and to avoid dehydration tubular activity is necessary, shown by reduced GFR and UFR in SW, (Madsen et al., 2020; Takvam et al., 2021b; Talbot et al., 1992). In order to acclimates to higher salinites the kidney must remove excess divalent ions such as Mg^{2+}, Ca^{2+} and SO_4^{2-} to maintain normal plasma levels(Takvam et al., 2021a). This is suggested to correlate with high Nka enzyme activity shown after 1 month in SW(Takvam et al., 2021a). While said study did not show any compensatory increase in Nka activity from 350dd after 1 month in SW, activity at 350dd was already sufficient and did not require compensation. A similar compensation is not found in the early group, which showed nigh identical Nka activity during smoltification as the normal group. This strengthens the hypothesis that at 168dd sufficient osmoregulatory function has not developed in the kidney.

4.2.5.4 The effect of starvation on SW Nka activity

There are no studies investigating the effect of short starvation during smoltification in the long term on salmonids. All organs showed retention of high Nka activity, similar to the fed group, suggesting no long-term reduction of osmoregulatory capability. However, the gills still show significantly higher Nka activity after 6 weeks of SW exposure, and the distal

intestine showed the same trend, though not significant. One possible explanation could be that activity levels are simply retained, as there is no need for down-regulation of activity. Increased Nka activity in the gills and the intestine may be very beneficial as the fish transfer to SW, as the gills are vital for expulsion of ions, especially Na^+ and Cl^-, while the intestine is vital for water absorption in SW. The distal intestine has more prominent function of water-absorption (Edwards & Marshall, 2012), which could explain the retention of high activity in the starved groups. It is evident that extensive research in needed to determine specific long-term effects, as well as their specific cause.

4.2.5.4 Comparative analysis of SW performance based on growth parameters, Nka activity and feeding treatment.

As determined earlier, the optimal transfer times for Nka activity varies between 348 and 516dd. This is well reflected in growth data. The early and group shows the lowest SGR, with a massive drop at SW transfer (168dd). Reduced SGR of smolts at SW transfer may in part be due to the energetic demands of smolt development, which include increased basal metabolic rate, increased activity, and reduced lipid content (Mccormick, 2013). This is the case for the normal group as well. The late group shows a much smaller decrease, suggesting that more energy is delegated to growth. However, Nka data suggests that desmoltification may have occurred (discussed earlier). Furthermore, the starved group showed a significantly lower SGR at SW transfer, followed by a significantly higher SGR at 6 weeks of SW. This may be due to compensatory growth, by enhanced growth hormone (GH) secretion by the pituitary gland, mobilizing fatty acids, which are used to support energy requirements (Johansen et al., 2001). This further supports our hypothesis (1) of fat mobilization, which modulates the Nka pump, increasing values in the starved groups, particularly in the distal intestine. Furthermore, Full compensatory growth has been reported in fish starved for 1-7 days (Azodi et al., 2015; Maclean & Metcalfe, 2001; Oh et al., 2008; Tian & Qin, 2003). Though our results do not show a complete catchup in weight for the early and late groups after 6 weeks of SW, a similar compensatory growth may have occurred, hence higher SGR in the starved group.

4.2.6 Application for aquaculture

As the results show, there is a delayed onset of peak Nka activity in the kidney and intestine, which require further research to determine an exact optimal transfer time. It is commonplace in the salmon farming industry to use gill Nka as an indicator of smoltification. As this study indicates that the kidney and intestine do not reach their peak until significantly later than the gills, this may be a one factor related to high mortality rates of smolt transferred to SW. a more holistic approach should be considered, in which all three organs have sufficiently

developed for SW transfer, as the intestine and kidney also play vital roles for osmoregulation. Research to determine an optimal transfer time for all organs could contribute to more robust smolt, and thus lower mortality. Starvation prior to SW transfer is beneficial to reduce stress and maintain good water quality during transfer (Hvas et al., 2020), and our findings of increased Nka activity after short term starvation indicate that it may be beneficial physiologically as well. However, more studies are clearly needed to further understand the mechanisms regulating the Nka activity increase, as well as long term effects on performance. In the starved groups, increased Nka activity is maintained in the distal intestine after SW transfer, as well as a significantly higher SGR, further corroborating it as a healthy and beneficial practice.

5. Conclusions

The findings of this study determined that gill Nka enzyme activity is insufficient in determining smolt development, as well as SW performance and osmoregulatory capacity in Atlantic salmon. The intestine and gills show a slower development of osmoregulatory capacity, measured as Nka activity and should thus be considered in assessment of smolt quality. While 516dd showed peak Nka values, our findings suggest that desmoltification may have started, and the actual optimal transfer time is located between 348-516dd. This also requires further study, to determine the optimal transfer time, considering all three osmoregulatory organs. Short-term starvation seems to have a surprisingly large effect on Nka activity, especially in the distal intestine. Some increased activity is maintained after 6 weeks of SW exposure. Thus, short-term starvation may be beneficial physiologically for Atlantic salmon. While several reasons have been hypothesized for these Nka activity increases, further studies are clearly needed to determine their mechanisms, as well as long term effects. Finally, to briefly summarize the findings in relation to the objectives of this study:

Objective 1: determine NKA enzyme activity in the three main osmoregulatory organs; the gills, kidney and intestine during smolt development (Early, Normal and late transfer)

This study demonstrated a steady increase of Nka enzyme activity in the gills from early transfer (168 dd) to normal transfer (360 dd) and late transfer (516 dd). This pattern is not reflected in the kidney and intestine, which do not show a significant increase until 516 dd. This is consistent with previous studies examining optimal transfer times of all 3 osmoregulatory organs. Thus, a later transfer time seems to be optimal for osmoregulatory capacity.

Objective 2: determine the effects of short-term starvation (4 days) on osmoregulatory capabilities across the different transfer times (early, normal and late) in gill, intestine and kidney.

Our results show that the effect of short-term starvation greatly varies in the organs, but significant differences all show an increase in Nka activity. The gills show a significant increase at normal transfer, but no changes in early or late. The kidney only shows a significant increase at late transfer (516 dd). The proximal intestine showed no increase, however large increases in Nka activity were measured in the distal intestine in starved fish. This shows that short-term starvation may contribute to better osmoregulatory capacity in smolt, and thus greater preparedness for SW transfer. However, this field is sparsely studies, and need a more data for corroboration, as well as determining the mechanisms which increase Nka activity.

Objective 3: determine the effect of different transfer times (early, normal and late) on short-term (6 weeks) SW performance.

Nka activity remained mostly high after 6 weeks of SW exposure. Our data shows that 168dd may be sufficient for the gills, as a slight increase in Nka activity is shown, while the kidney and intestine show drastic drops in activity at 168dd, clearly indicating insufficient osmoregulatory capability, which could drastically affect performance. In general, 348dd seemed to be the optimal transfer point for Nka activity in the gills and kidney. They also showed a drop at 518dd in activity, suggesting desmoltification. The intestine showed large decreases of Nka activity at 168dd, like the kidney. However, while 348dd seems to show sufficient activity at 348dd, much higher activity was shown at 518dd, particularly in the distal intestine. This reinforces our conclusion from the first hypothesis, that a later transfer time (perhaps somewhere between 348-516dd) would be the optimal transfer time, based om osmoregulatory capability.

Objective 4: determine the effect of short-term starvation (4 days) on short-term (6 weeks) SW performance.

Starvation showed mostly similar Nka values of the fed groups. However, the starved normal transfer group (348dd) of the gills and distal intestine and, as well as a noticeable, non-significant trend in the distal intestine, showed a maintained higher Nka activity which was observed in FW. It is difficult to determine what caused this. Based on our hypothesis of a compensatory mechanism, one can hypothesize that the increased in FW starved groups proved beneficial after SW transfer, and thus no decrease in activity occurred. Thus, it is possible that

short term starvation of smolt prior to SW exposure may be slightly beneficial to osmoregulatory capacity, especially in the distal intestine, which is central in water absorption in SW. While weight and SGR was generally lower in the starved group, most weight was recovered after 6 weeks SW, and SGR was higher. This suggests that compensatory growth occurred.

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7. Appendix

Appendix 1: Weight, length, condition factor and Specific growth rate

Weight

Table 3 *Weight (g) mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon fed and starved, both in FW and SW. p-values between fed/starved groups , as well as p-values between each sampling point.in FW/SW, for both fed/starved groups are included*

Transfer group (dd)	Treatment	Sampling time	Mean	SEM	Min	Max	p-value
1 (168)	Fed	1 (start)	82.8	1.00	54	127	0.178
	Starved	1 (start)	80.8	1.14	45	121	
	Fed	2 (168dd)	87.7	1.06	53	122	0.00269
	Starved	2 (168dd)	79.0	1.13	43	119	
	Fed	3 (6 weeks SW	143.0	1.71	70	222	<0.0001
	Starved	3 (6 weeks SW	133.1	2.12	79	190	
2 (348)	Fed	1 (start)	84.8	1.02	52	126	0.7175
	Starved	1 (start)	80.9	1.30	51	104	
	Fed	2 (348dd)	112.3	1.28	74	153	0.4424
	Starved	2 (348dd)	100.5	1.67	62	138	
	Fed	3 (6 weeks SW	174.4	2.43	88	244	0.8886
	Starved	3 (6 weeks SW	180.5	3.12	129	287	
3 (516)	Fed	1 (start)	81.1	0.96	51	134	0.07714
	Starved	1 (start)	78.2	1.00	46	109	
	Fed	2 (516dd)	134.0	1.61	88	214	0.1085
	Starved	2 (516dd)	117.0	1.56	64	166	
	Fed	3 (6 weeks SW	215.0	2.96	131	347	0.0038
	Starved	3 (6 weeks SW	203.1	2.83	105	325	

Transfer group (dd)	Sampling	Fed	Starved
Early(168dd)	1-2	0.1307	0.85
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001
Normal (348dd)	1-2	0.0005	<0.0001
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001
late(516dd)	1-2	<0.0001	<0.0001
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001

Length

Table 4 *Length (cm) mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon fed and starved, both in FW and SW. p-values between fed/starved groups , as well as p-values between each sampling point.in FW/SW, for both fed/starved groups are included*

Transfer group (dd)	Treatment	Sampling time	Mean	SEM	Min	Max	p-value
1 (168)	Fed	1 (start)	18.1	0.215	16.7	20.7	0.0738
	Starved	1 (start)	17.6	0.164	16.6	18.8	
	Fed	2 (168dd)	18.5	0.145	16.5	20.5	0.0898
	Starved	2 (168dd)	18.1	0.165	16.6	20.0	
	Fed	3 (6 weeks SW	22.8	0.116	20.5	24.5	<0.0001
	Starved	3 (6 weeks SW	22.0	0.164	19	24.8	
2 (348)	Fed	1 (start)	18.5	0.315	16.0	22.2	0.424
	Starved	1 (start)	18.0	0.400	16.4	20.4	
	Fed	2 (348dd)	19.8	0.142	17.5	21.5	0.03343
	Starved	2 (348dd)	20.3	0.196	18.5	22.5	
	Fed	3 (6 weeks SW	24.2	0.180	20.6	26.5	0.5304
	Starved	3 (6 weeks SW	24.1	0.203	22.1	26.5	
3 (516)	Fed	1 (start)	17.8	0.214	15.7	20.1	0.0167
	Starved	1 (start)	18.9	0.387	17.3	23.4	
	Fed	2 (516dd)	21.6	0.165	19.0	23.5	0.4153
	Starved	2 (516dd)	21.4	0.168	19.0	24.0	
	Fed	3 (6 weeks SW	26.0	0.160	23.0	29.0	0.0108
	Starved	3 (6 weeks SW	25.5	0.132	23.0	28.0	

Transfer group (dd)	Sampling	Fed	Starved
Early(168dd)	1-2	0.1837	0.0541
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001
Normal (348dd)	1-2	<0.0001	<0.0001
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001
late(516dd)	1-2	<0.0001	<0.0001
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001

Condition factor

Table 5 *Condition factor mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon fed and starved, both in FW and SW. p-values between fed/starved groups, as well as p-values between each sampling point.in FW/SW, for both fed/starved groups are included*

Transfer group (dd)	Treatment	Sampling time	Mean	SEM	Min	Max	p-value
1 (168)	Fed	1 (start)	1.40	0.031	1.08	1.62	0.3544
	Starved	1 (start)	1.44	0.024	1.26	1.59	
	Fed	2 (168dd)	1.40	0.011	1.27	1.55	0.0038
	Starved	2 (168dd)	1.34	0.017	1.20	1.49	
	Fed	3 (6 weeks SW	1.27	0.007	1.14	1.41	0.1218
	Starved	3 (6 weeks SW)	1.25	0.009	1.07	1.46	
2 (348)	Fed	1 (start)	1.37	0.047	0.900	1.79	0.4782
	Starved	1 (start)	1.42	0.049	1.05	1.60	
	Fed	2 (348dd)	1.40	0.025	1.06	2.07	<0.0001
	Starved	2 (348dd)	1.25	0.016	1.11	1.61	
	Fed	3 (6 weeks SW	1.25	0.015	1.01	1.61	0.4396
	Starved	3 (6 weeks SW	1.27	0.017	0.876	1.49	
3 (516)	Fed	1 (start)	1.46	0.046	1.05	1.83	0.1362
	Starved	1 (start)	1.34	0.064	0.82	1.65	
	Fed	2 (516dd)	1.29	0.009	1.17	1.45	0.0649
	Starved	2 (516dd)	1.24	0.032	1.09	2.03	
	Fed	3 (6 weeks SW	1.28	0.009	1.13	1.50	0.1161
	Starved	3 (6 weeks SW	1.26	0.008	1.11	1.40	

Transfer group (dd)	Sampling	Fed	Starved
Early(168dd)	1-2	0.9681	0.0019
	2-3	<0.0001	<0.0001
	1-3	0.0006	<0.0001
Normal (348dd)	1-2	0.658	0.0069
	2-3	<0.0001	0.4041
	1-3	0.0232	0.0141
late(516dd)	1-2	0.0019	0.1857
	2-3	0.589	0.4313
	1-3	0.0014	0.2687

Specific growth rate

Table 3 *SGR (% / day) mean, standard error of mean (SEM), and minimum (min) maximum (max) values, including for p-values for each sampling point in Atlantic salmon fed and starved, both in FW and SW. p-values between fed/starved groups, as well as p-values between each sampling point.in FW/SW, for both fed/starved groups are included.*

Transfer group (dd)	Treatment	Sampling time	Mean	SEM	Min	Max	p-value
1 (168)	Fed	0-1 (start)	1.20	0.015	0.612	1.90	0.0940
	Starved	0-1 (start)	1.16	0.018	0.455	1.84	
	Fed	1-2 (168dd)	0.42	0.033	-2.30	5.18	<0.0001
	Starved	1-2 (168dd)	-0.16	0.019	-0.764	0.397	
	Fed	2-3 (6 weeks SW	1.23	0.023	-0.172	2.00	0.1075
	Starved	2-3 (6 weeks SW	1.30	0.035	-0.123	2.02	
2 (348)	Fed	0-1 (start)	1.26	0.013	0.779	1.84	<0.0001
	Starved	0-1 (start)	1.15	0.018	0.517	1.56	
	Fed	1-2 (168dd)	1.01	0.031	-0.851	3.67	<0.0001
	Starved	1-2 (168dd)	0.76	0.022	-0.034	2.09	
	Fed	2-3 (6 weeks SW	1.05	0.029	-0.411	2.08	<0.0001
	Starved	2-3 (6 weeks SW	1.44	0.028	0.800	2.25	
3 (516)	Fed	0-1 (start)	1.15	0.014	0.557	1.65	<0.0001
	Starved	0-1 (start)	1.09	0.016	0.444	1.74	
	Fed	1-2 (168dd)	1.20	0.015	0.598	1.92	<0.0001
	Starved	1-2 (168dd)	0.96	0.019	0.343	2.53	
	Fed	2-3 (6 weeks SW	1.18	0.024	0.162	2.79	<0.0001
	Starved	2-3 (6 weeks SW	1.36	0.019	0.593	1.86	

Transfer group (dd)	Sampling	Fed	Starved
Early(168dd)	1-2	<0.0001	<0.0001
	2-3	<0.0001	<0.0001
	1-3	0.3142	0.0006
Normal (348dd)	1-2	<0.0001	<0.0001
	2-3	0.5038	<0.0001
	1-3	<0.0001	<0.0001
late(516dd)	1-2	<0.0001	<0.0001
	2-3	<0.0001	<0.0001
	1-3	<0.0001	<0.0001

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

Gill Nka activity

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 fed and starved, both in FW and SW. Number of individuals (N) in each group and timepoint is included. p-values between fed/starved groups in FW, as well as p-values between each sampling point.in FW/SW, for both fed/starved groups.*

Sampling (day degrees)	Group (n)	Mean (activity)	SEM (activity)	Min (activity)	Max (activity)	P-value
1 (168)	Fed (12)	8.14	1,05	4.16	15.71	
	Starved (10)	6.41	1,15	9.39	3.25	0.6811
2 (348)	Fed (12)	10.65	1,05	5.35	21.93	
	Starved (12)	14.58	1,05	6.61	21.34	0.0444
3 (516)	Fed (12)	19.66	1,05	15.61	22.28	
	Starved (12)	21.01	1,05	12.18	26.33	0.7995
SW transfer						
1 (168)	Fed (12)	13.96	0.45	12.14	16.23	
	Starved (12)	16.10	0.88	11.11	22.63	0.1806
2(348)	Fed (12)	21.76	1.23	14.64	26.75	
	Starved (12)	24.44	0.69	20.65	28.32	0.0271
3 (516)	Fed (11)	10.41	0.61	7.54	13.96	
	Starved (11)	12.05	0.54	10.09	15.49	0.0955

Sampling (FW)	Fed	Starved
1-2	0.2145	<0.0001
2-3	<0.0001	0.0001
1-3	<0.0001	<0.0001

Sampling	Treatment	Environment	P-value
1 (168)	Fed	FW	0.0001
		SW	
2 (348)		FW	<0.001
		SW	
3 (516)		FW	0.0213
		SW	

Sampling	Treatment	Environment	P-value
1 (168)	Starved	FW	<0.0001
		SW	
2 (348)		FW	<0.0001

		SW	
3 (516)		FW	0.3837
		SW	

Kidney nka activity

Table 8: *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 fed and starved, both in FW and SW. Number of individuals (N) in each group and timepoint is included. p-values between fed/starved groups in FW, as well as p-values between each sampling point.in FW/SW, for both fed/starved groups.*

Sampling (day degrees)	Group (n)	Mean (activity)	SEM (activity)	Min (activity)	Max (activity)	P-value
1 (168)	Fed (12)	8,15	0.64	6,63	12,95	
	Starved (12)	6,41	0.64	8,86	17,17	0.8546
2 (348)	Fed (12)	10,65	0.64	8,49	13,05	
	Starved (10)	14,58	0.78	7,08	17,88	0.8866
3 (516)	Fed (10)	19,67	0.64	20,57	19,89	
	Starved (11)	21,02	0.64	13,79	20,18	0.0151
SW transfer						
1 (168)	Fed (12)	13,96	0,46	9,46	15,30	
	Starved (12)	16,10	0,88	8,78	13,50	0.3143
2(348)	Fed (11)	21,77	1,23	16,33	20,16	
	Starved (11)	24,45	0,69	15,50	19,61	0.9764
3 (516)	Fed (12)	10,42	0,62	10,46	15,54	
	Starved (11)	12,05	0,55	10,13	15,17	0.6687

Sampling	Fed	Starved
1-2	0.9367	0.9625
2-3	<0.0001	<0.0001
1-3	<0.0001	<0.0001

Sampling	Treatment	Environment	P-value
1 (168)	Fed	FW	0.1546
		SW	
2 (348)		FW	<0.0001
		SW	
3 (516)		FW	0.0079
		SW	

Sampling	Treatment	Environment	P-value
1 (168)	Starved	FW	0.8874
		SW	
2 (348)		FW	0.0003
		SW	
3 (516)		FW	<0.0001
		SW	

Proximal intestine nka activity

Table 9: *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 fed and starved, both in FW and SW. Number of individuals (N) in each group and timepoint is included. p-values between fed/starved groups in FW, as well as p-values between each sampling point.in FW/SW, for both fed/starved groups.*

Sampling (day degrees)	Group (n)	Mean (activity)	SEM (activity)	Min (activity)	Max (activity)	P-value
1 (168)	Fed (12)	10,99	2.11	4,68	16,69	
	Starved (11)	12,20	2.02	7,31	18,42	0.9758
2 (348)	Fed (12)	13,91	2.02	5,20	23,69	
	Starved (9)	16,52	2.48	9,87	38,51	0.8430
3 (516)	Fed (12)	21,41	2.02	13,09	34,51	
	Starved (12)	25,10	2.02	11,30	43,68	0.5728
SW transfer						
1 (168)	Fed (12)	6,58	0,86	11,37	1,97	
	Starved (12)	6,65	0,72	10,77	2,18	0.4776
2(348)	Fed (11)	19,56	1,07	13,49	27,53	
	Starved (12)	17,58	1,04	12,50	24,04	0.6825
3 (516)	Fed (12)	24,44	1,18	12,43	34,08	
	Starved (12)	26,78	1,69	14,62	36,10	0.485

Sampling	Fed	Starved
1-2	0.5658	0.3801
2-3	0.0264	<0.0001
1-3	0.0001	0.0230

Sampling	Treatment	Environment	P-value
1 (168)	Fed	FW	0.0024
		SW	
2 (348)		FW	0.0241
		SW	
3 (516)		FW	0.0109
		SW	

Sampling	Treatment	Environment	P-value
1 (168)	Starved	FW	0.0009
		SW	
2 (348)		FW	0.0979
		SW	
3 (516)		FW	0.3274
		SW	

Distal intestine nka activity

Table 10: *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 fed and starved, both in FW and SW. Number of individuals (N) in each group and timepoint is included. p-values between fed/starved groups in FW, as well as p-values between each sampling point.in FW/SW, for both fed/starved groups.*

Sampling (day degrees)	Group (n)	Mean (activity)	SEM (activity)	Min (activity)	Max (activity)	P-value
1 (168)	Fed (12)	3,87	1.07	2,45	5,91	
	Starved (11)	10,33	1.02	4,25	15,39	0.0002
2 (348)	Fed (12)	8,15	1.02	1,86	19,45	
	Starved (9)	12,74	1.25	3,90	19,22	0.0272
3 (516)	Fed (12)	5,91	1.02	2,75	10,52	
	Starved (12)	14,78	1.02	9,81	24,81	<0.0001
SW transfer						
1 (168)	Fed (12)	1,98	0,33	0,84	4,90	
	Starved (12)	2,50	0,50	0,81	7,02	0.2913
2(348)	Fed (11)	2,25	0,36	0,89	5,12	
	Starved (11)	3,75	0,81	0,90	10,64	0.5619
3 (516)	Fed (12)	7,14	1,31	3,25	17,46	
	Starved (12)	8,23	0,46	5,23	10,63	0.0597

Sampling	Fed	Starved
1-2	0.0123	0.30009
2-3	0.2713	0.4200
1-3	0.3544	0.0073

Sampling	Treatment	Environment	P-value
1 (168)	Fed	FW	0.0003
		SW	
2 (348)		FW	0.0029
		SW	
3 (516)		FW	0.7987
		SW	

Sampling	Treatment	Environment	P-value
1 (168)	Starved	FW	<0.0001
		SW	
2 (348)		FW	0.0002
		SW	
3 (516)		FW	0.0016
		SW	