Effects of photoperiod regimes and salinity on Na+, K+-ATPase α-isoform expression in gills and kidney during smoltification in Atlantic salmon (*Salmo salar*) in recirculating aquaculture systems

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ABSTRACT

Understanding the molecular mechanisms of smoltification and seawater adaptation in Atlantic salmon (*Salmo salar*) is crucial for optimizing smolt production in aquaculture. This study investigated the expression patterns of Na+/K+-ATPase (NKA) subunits during smoltification in domesticated salmon under different light regimes and salinities in gills and kidney. The experiment was carried out in four photoperiodic treatments: 1) No Winter (NW): continuous light (24L:0D), 2) Early Winter (EW): Standard production light regime (6 weeks winter signal (12L:12D) followed by summer signal (24L:0D)), 3) Late Winter (LW): Delayed winter signal using standard production light regime (6 weeks winter signal (12L:12D) followed by summer signal (24L:0D)) and 4) Late Long Winter (LLW): Delayed and prolonged winter signal (20 weeks winter signal (12L:12D) followed by summer signal (24L:0D)). There were initially 8 tanks with freshwater (FW) (0-1 ppt) which split into brackish water (BW) (12 ppt) for all photoperiodic light regimes after summer signal was initiated. NKAa1a mRNA abundance in gills decreased during smoltification in all groups. NKAa1b mRNA abundance in gills did not increase as expected in gills, but NKAa1b protein levels increased. NKAa1c and NKAa3 isoforms had low and stable expression in the gills in FW. NKAa1c may have a compensatory function under specific conditions. In the kidney, NKAa1c was the most abundant isoform. NKAa1b mRNA abundance in the gills increased with higher salinity, supporting its role in salinity adaptation. NKAa1a mRNA expression increased in response to brackish water in the kidney, while NKAa1b showed no significant changes. NKAa1c and NKAa3 had minimal responses to salinity in the kidney.
Monitoring the decrease in NKAa1a mRNA abundance may indicate smolt readiness and seawater tolerance. The increase in NKAa1b mRNA and protein abundance is associated with salinity tolerance and can therefore be a indicator for successful smoltification.

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INTRODUCTION

1.1 NORWEGIAN AQUACULTURE

Aquaculture has experienced significant growth as a food-producing industry in recent decades, and salmon has emerged as one of the most successful species cultivated through technological and biological innovations (Asche et al., 2013; Subasinghe, Soto & Jia, 2009). Norway is one of the world's leading producer and exporter of salmonids, with Atlantic salmon (*Salmo salar*) comprising 95% of its aquaculture production (Barentswatch.com). In 2021, Norway produced approximately 1.6 million tons of salmonids, with 1.5 million tons being Atlantic salmon. The country currently operates 989 fish farms in the sea and 58 on land, divided between freshwater and seawater facilities (Sommerset et al., 2023). A key factor contributing to the success of salmon aquaculture is an improved understanding of the Atlantic salmon biology.

However, in recent years, the growth of sea-based salmon aquaculture has been limited due to environmental challenges such as sea lice infestations and disease (Sommerset et al., 2023). Typically, salmon are reared in land-based systems with low salinity water (freshwater (FW) or brackish water (BW)) from hatching until they reach the smoltification stage. Subsequently, the fish are transferred to net pens in the sea for further growth until they reach market size (Fossmark et al., 2021). The most used systems for sea-based farming are flow-through (FT) systems, although the adoption of recirculating aquaculture systems (RAS) has increased due to their lower water consumption and high productivity (Ytrestøyl et al., 2020; Ahmed & Turchini, 2021). RAS also provides a more controlled and stable environment, including better temperature regulation, which is crucial for Atlantic salmon, especially during the smoltification process (Ytrestøyl et al., 2020).

Smolt production has increasingly shifted towards RAS technology to avoid performance issues and ensure the growth, survival, and quality of smolts (Badiola et al., 2017). By producing larger post-smolts in RAS, the time spent in sea cages can be shortened, potentially reducing mortality rates (Ytrestøyl et al., 2020). Furthermore, larger post-smolts have been argued to be more resilient to the challenging conditions encountered in open sea cages and are better equipped to withstand sea lice attacks (Fossmark et al., 2021).

One of the most critical developmental stages for Atlantic salmon is the parr-smolt transformation (PST). The adoption of more intensive farming systems like RAS in recent years has raised concerns about the potential negative impacts on PST, leading to unfavorable

effects on smolt quality and survival during the seawater phase (Ytrestøyl et al., 2020). Understanding the underlying physiological mechanism involved during critical stages of PST is becoming increasingly important, particular in relation to emerging intensified production protocols used in the industry to produce larger smolts. Consequently, it has become crucial to redirect focus towards the osmoregulatory capacity and integrative role of gills, intestine, and kidneys in order to provide a more holistic view of fish's overall osmoregulatory capacity before transferring fish to seawater (SW) (Takvam et al., 2023).

1.2 PARR-SMOLT TRANSFORMATION (PST)

The Atlantic salmon have an anadromous life cycle, where juveniles migrate from FW to a SW environment where they spend most of their adult life before they migrate back to their native river (Seear et al., 2010). While still in freshwater, the salmon prepare to enter the marine environment where juveniles undergo extensive remodeling processes in the life-history process termed parr-smolt transformation (PST) or smoltification (Hoar, 1988; Björnsson et al., 2011). This process involves substantial morphological, behavioral, and physiological changes, where environmental cues (in particular circadian rythms; photoperiod) such as increasing day length during spring induce important preparatory changes in the fish (Björmsson et al., 2011; Vargas-Chacoff et al., 2018). One of the most crucial physiological changes that occur during smoltification is the adaptation to seawater tolerance. This adaptation is characterized by an increase in the activity of the Na+, K+-ATPase (NKA) enzyme which is vital for most secondary transport of solutes and water (Nisembaum et al., 2021). The gills, kidney, and intestine play vital roles in the osmoregulatory adjustments required for the salmon's acclimation to seawater (McCormick et al., 2013; Sundell and Sundh, 2012; Takvam et al., 2021a; Takvam 2021b). During smoltification, the salmon experiences a loss of body lipid reserves and undergoes increased length growth, resulting in a slimmer body shape and reduced condition factor (CF) (Hoar, 1988; Ytrestøyl et al., 2022).

In industrial salmon production, the survival and growth performance of smolts in seawater are critical indicators of successful smoltification. If smolts are prevented from transitioning to seawater, many of the preparatory changes associated with marine life are reversed, a process known as desmoltification. This natural reversion process closes the "smolt window," which is the optimal period for smolts to enter and adapt to seawater. The duration of the smolt window typically ranges from 300 to 400 days degrees (Handeland et al., 2004).

In the salmon farming industry, the decision to transfer smolts to seawater is often based on a threshold value of NKA activity. However, little consideration is given to the dynamic properties of NKA activity, such as whether it is increasing (during the smoltification phase), reaching its peak, or declining (during the desmoltification phase). Studies have shown that gill NKA activity measurements can predict performance during the initial days of acclimation to seawater, but they do not necessarily predict long-term growth and size in SW (Zydlewski and Zydlewski, 2012). However, most studies only evaluate osmoregulatory capacity in the gill, while both intestine and kidney are equally important in evaluating seawater tolerance (Sundell and Sundh, 2012; Takvam et al., 2021). Understanding the physiological changes and adaptations that occur during smoltification and the salmon's acclimation to seawater having a more holistic view on osmoregulation is crucial for better evaluation of smolt quality in the aquaculture industry (McCormick, 2013).

1.3 OSMOREGULATION AND THE IMPORTANCE OF THE NKA PUMP IN ATLANTIC SALMON

Osmoregulation is a vital process for the Atlantic salmon, enabling it to maintain the balance of water and ions within its body as it transitions between freshwater and seawater environments. The osmoregulatory mechanisms in fish involves the coordinated function of several organs, including the gills, kidney, and intestine (Marshall and Grosell, 2005). In Atlantic salmon, as with other teleosts fish the general challenge in FW is passive water load and ion loss mainly over the large surface area of the gill. Thus, they must limit oral intake of water, actively absorb ions from the environment and ingested food through the gills and intestine, while producing large volumes of dilute urine (Evans et al., 2005; Marshall and Grosell, 2005). In SW they face opposite challenges in which passive influx of ions and osmotic water loss force the fish to increase drinking rates and actively take up water through the intestine while excreting excess monovalent ions through the gills (ref). The kidney is primarily excreting divalent ions such sulfate (SO42-), magnesium (Mg2+) and calsium (Ca2+) producing low volumes of an isotonic urine (Takvam et al., 2021a). A key constituent that establishes in regulating ion and water balance in preparation for SW is the NKA pump which are found in all three osmoregulatory organs (Nilsen, McCormick & Sørensen, 2007; Tipsmark, Madsen & Seidelin, 2010; Sundh et al., 2014; Takvam et al., 2021).

1.4 NKA PUMP

The Na+, K+-ATPase (NKA) is the most studied enzyme in salt-transport and is present in all animal cells providing cellular homeostasis (Striberny et al., 2021; McCormick, Regish & Christensen, 2009). It consists of the three subunits alpha (a), beta (b) and gamma (λ). The α-subunit contains the binding sites for Na, K, ATP, and the inhibitor oubain (Nilsen et al., 2010), and is responsible for the enzyme's catalytic and ion regulatory functions (Regish et al., 2018). The β-subunit is a glycosylated polypeptide that plays a significant role in stabilizing the folding of the α-subunit (Nilsen et al., 2010), and promotes positioning of the protein into the basolateral plasma membrane (McCormick et al., 2013). The λ-subunit is often referred to as the FXYD protein and appears to modify the kinetic properties of the NKA enzyme by modulating the affinity for Na^+ and K^+ transport (Blanco and Mercer, 1998). In salmonids, five different α-isoforms (α1a, α1b, α1c, α2 and α3), each having a salmonid specific paralogue, and four β-isoforms (β1a, β1b, β2 and β3b) have been identified (Richards et al., 2003; Gharbi et al., 2004; 2005). Several studies suggest that differential expression of α-subunit isoforms may be an important mechanism for altering NKA enzyme activity as a response to different physiological requirements (Schulte, 2004; Nilsen et al., 2007; McCormick et al 2009; 2013; Sundh et al., 2014). NKA enzyme activity in the gills, intestine and kidney increases during PST in Atlantic salmon as part of the preparation for a marine life and is often associated with differential regulation of NKA-α isoforms (Nilsen et al., 2003; Sundell et al., 2003; Sundh et al., 2014; Takvam et al., 2021b). Increased NKA-α1b isoform mRNA and protein levels has largely been linked elevated enzyme activity in gills (Nilsen et al., 2007; McCormick et al 2009; 2013), while upregulation of the NKA-α1c isoform mRNA and protein appears coupled with elevated intestinal enzyme activity (Tipsmark et al 2010; Sundh et al 2014). The *nka-α1a* and *α1b* mRNA are present in Atlantic salmon (Madsen et al., 2020) and the *nka-α1b*, *α1c* and *α3* isoforms are present in rainbow trout kidney (Richards et al., 2003). Despite intestinal and kidney NKA activity increase during PST (McCartney, 1976; Sundell et al., 2003; Takvam et al., 2021b) while nothings is known about the regulation expression patterns of salmonid specific NKA-*α* isoforms in the kidney of Atlantic salmon smolts. Due to the limited knowledge in the kidney, this study will direct focus on this organ, but will include the gills for comparison in better understanding the functional role of the NKA pump in water and ion balance in Atlantic salmon.

1.5 THE INFLUENCE OF PHOTOPERIOD AND SALINITY

In Atlantic salmon, smoltification is regulated by seasonal changes in photoperiod (Imsland, Handeland & Stefansson, 2014). The natural photoperiod includes short days during winter followed by increasing day lengths in spring (Ytrestøyl et al., 2022). In the salmon farming industry, it is common to use photoperiod manipulation to get maximum growth with as short time as possible. Extended day length, or continuous light (24 light:0 dark), has shown to be the best light regime for this outcome (Imsland, Handeland & Stefansson., 2014).
In addition to photoperiod, salinity levels profoundly affect smoltification in Atlantic salmon. Exposure to increasing salinity gradients gradually induces physiological changes in the fish, including alterations in ion transport and osmotic regulation (Ytrestøyl et al., 2022). As smoltification progresses, Atlantic salmon gradually acquire the ability to balance salt and water in their bodies, enabling them to survive in seawater conditions (Ytrestøyl et al., 2022).

1.6 COMMONLY USED SMOLT MARKERS IN SALMON AQUACULTURE

Measures at the transcript level often constitute an earlier indicator of the subsequent protein expression and changes in NKA enzyme activity levels. Changes in NKA-α subunit isoform expression, or NKA-α1b/α1a ratios, provide a very sensitive indicator of smolt development (Nilsen et al 2007; Stefansson et al., 2007; Gallagher et al., 2012) and is routinely used in commercial settings (Smolt Vison or SmoltTimer). Generally, when smolts is not transferred to seawater they will start reverting back to a freshwater state, a process referred to as de-smoltification (McCormick, 2013). This process is associated with decrease in gill NKA-α1b, concurrent with increasing gill NKA-α1a expression prior to any observable changes in NKA enzyme activity (Nilsen et al., 2007), thereby providing an early warning of de-smoltification and thus give the farmer sufficient time to react. The caveat with measuring mRNA abundance is that transcripts are often very sensitive and small changes in the environment, particularly ion composition, salinity, and other water quality parameters (Nilsen et al., 2010; 2013) may result in changes in NKA-α1b/α1a ratios that not necessarily reflect a true smolt development. Additionally, the most used methods for evaluating seawater tolerance, and thus smolt status, is subjecting smolts to a standardized seawater challenge test (Blackburn and Clark, 1987). Smolts are transferred directly to full-strength seawater for 24 hours and plasma ions or osmolality is measured. High quality smolts will be able to regulate ion levels within 10% of levels normally observed freshwater smolts. Poor quality smolts are often unable to

sufficiently regulate ion levels and it is not uncommon to observe up to 30% variations in plasma ion levels. It should be noted, however, that larger fish often display greater seawater tolerance as a function by size (Parry, 1960; Bjerknes et al., 1992) and ability to ion regulate may thus not necessarily be indicative of a true physiological smolt.
In addition, new potential markers for smoltification are under development. While some alternative markers are being implemented for research purposes, the salmon industry has not yet adopted them for routine tests, mainly due to a lack of quantitative data supporting their advantages over NKA measurements alone. Ongoing research, including transcriptomics and small RNA sequencing, aims to identify new markers for smoltification (Morera et al., 202; Iversen et al., 2020; West et al., 2020).

1.7 APPLICATION TO SMOLT PRODUCTION AND SUSTAINABLE AQUACULTURE

As the aquaculture industry continues to expand and intensify production, there is a growing need for a comprehensive understanding of the physiological processes involved in smolt production in Atlantic salmon (Asche et al., 2013). Smoltification, the process by which salmon adapt to the marine environment, is crucial for their successful transition. Missing the optimal "smolt window" can result in lower marine survival rates, highlighting the importance of producing high-quality smolts. However, variations in smolt quality, such as stunted growth and high transfer mortality, remains a concern. Additionally, production disorders like nephrocalsinoses in the kidney, hemorrhagic smolt syndrome (HSS), and other growth-related issues have raised questions about their impact on smolt physiology. It is concerning that the assessment of smolt quality often overlooks the transport capacity of the kidney during and after smoltification. A more holistic approach that considers these organs is necessary to evaluate smolt quality in the industry (Byrne et al., 1998; Ytrestøyl et al., 2020), where we can look at the kidney in connection with development in the gill. Although the focus here will be on gills and kidney in response to photoperiod and salinity. Future studies should include all three organs as to better understand the overall osmoregulatory capacity such as that investigated in previous master thesis on the project (Takvam et al., 2023).
A better understanding of the Na+, K+-ATPase (NKA) pump, which plays a critical role in ion transport, in various organs, not just the gills, can provide valuable insights into the osmoregulatory capacity of salmon during their transfer to seawater (SW). Examining NKA in multiple organs can also contribute to assessing smolt quality. Given the rapidly changing

production protocols in recirculating aquaculture systems (RAS), it is essential to have better tools for evaluating smolt quality in SW (Asche et al., 2013). Improved smolt quality can lead to higher survival rates, enhanced growth, and improved fish welfare in SW, contributing to a more sustainable production process on land and at sea. However, due to limited technology and regulations, further research is needed to address the knowledge gaps in production (Asche et al., 2013). Moreover, the biological requirements of post-smolts in closed-containment systems are still poorly understood (Ytrestøyl et al., 2020). In Norway, an average of 16% of Atlantic salmon smolts transferred to sea cages are lost before reaching harvest size (Sommerset et al., 2020), with smolt quality and infections being major factors contributing to these mortalities. Increasing the size and robustness of the fish through improved smolt quality can enhance survival and growth after transfer to sea cages.
Here we investigate the NKA pump in gills and kidney on both gene and protein level and its responses to use of different photoperiodic protocols and use of salinity. The aims are to better understand the impact new rearing protocols in the industry have on smolt development where multiple organs are included (in this case gills and kidney). Hopefully this can increase our knowledge on these organs alone and together and have this may relate to smolt quality evaluation before fish are transferred to SW.

1.8 OBJECTIVES

In this study, investigate the NKA pump in gills and kidney on both gene and protein level and its responses to use of different photoperiodic protocols and use of salinity is studied. The aims are to better understand the impact new rearing protocols in the industry have on smolt development where multiple organs are included (in this case gills and kidney). Hopefully this can increase our knowledge on these organs alone and together and have this may relate to smolt quality evaluation before fish are transferred to SW.

Objective 1: Gene expression patterns of NKAα isoforms during smoltification

The first objective investigates the gene expression patterns of NKAa isoforms during the process of smoltification. Smoltification is a critical life stage in the development of salmonid fish, during which they undergo physiological and morphological changes to adapt from a freshwater to a saltwater environment. By examining the gene expression patterns of NKAa isoforms, which are known to play a crucial role in ion regulation, this objective aims to

understand how these isoforms are regulated and how they contribute to the smoltification process.

Objective 2: Protein expression patterns of NKAα isoforms during smoltification

The second objective is studying the protein expression patterns of NKAa isoforms during smoltification. While gene expression provides information about the levels of mRNA transcripts, protein expression reflects the actual presence and abundance of functional proteins. By analyzing the protein expression patterns of NKAa isoforms, this objective aims to identify any changes or variations in their abundance throughout the smoltification process.

Objective 3: How different light regimes effects smoltification

The third objective of this study is to investigate the effects of different light regimes on the process of smoltification. Light is an environmental cue that plays a crucial role in regulating various physiological processes in fish, including smoltification. This objective aims to explore how different light treatments influence the timing and progression of smoltification.

Objective 4: How different salinity effects smoltification

The fourth objective focuses on examining the effects of different salinity levels on the smoltification process. As Atlantic salmon transition from freshwater to saltwater habitats, they undergo physiological adaptations to cope with changes in osmotic balance. This objective aims to investigate how the use of brackish water influence smoltification.

MATERIAL AND METHOD

2.1 FISH STOCK

The experiment was carried out at Nofima Centre for Recirculating in Aquaculture at Sunndalsøra, Norway (Terjesen et al., 2013). Eggs from Atlantic salmon come from salmobreed (ER-121), Bolaks strain (Eikelandsosen, Norway) and was first reared in standard flow-through (FT) tank systems until parr stage. The eggs were then reared in RAS tanks during the rest of the experiment. Rearing and experimental protocols were in accordance with the guidelines provided through the approved experimental protocol given by the Norwegian Food Safety Authority (FOTS). There were approximately 9000 parr (weight: 52.5 +/- 0.64 grams, length: 16.1 +/- 0.06 cm). All fish were tagged using Passive Integrated Transponders (PIT) and randomly distributed in 16 experimental tanks (3.2 m^3, diameter: 2 m). All fish were vaccinated (ISA vaccine; Pharmaq) roughly one week before initiating the photoperiodic treatments.

2.2 EXPERIMENTAL DESIGN

The photoperiodic treatments (figure 1):

1. No winter group (NW): Continuous light (24L:0D)
2. Early winter group (EW): Standard production light regime (6 weeks winter signal (12L:12D) followed by summer signal (24L:0D))
3. Late winter group (LW): Delayed winter signal using standard production light regime (6 weeks winter signal (12L:12D) followed by summer signal (24L:0D))
4. Late long winter group: (LLW): Delayed and prolonged winter signal (20 weeks winter signal (12L:12D) followed by summer signal (24L:0D))

Tank environment and salinity treatments:

There were initially 8 tanks with FW (0-1 ppt) which split into BW (12 ppt) for all photoperiodic light regimes after summer signal was initiated. The first group (NW) was split at the same time as the second group (EW). Two RAS systems were used, where all tanks had a volume of 3.2 m^3 (water flow: 120-140 L/min), water velocity: roughly 20 cm/second, water temperature: 12 +/- 1 degree Celsius). The water currency was adjusted to the fish growth. Oxygen saturation < 80% (automatic logic controller NSJ8, Omron, Kyroto, Japan). The tanks were covered during the experiment to secure photoperiodic control and reduce unnecessary disturbance.

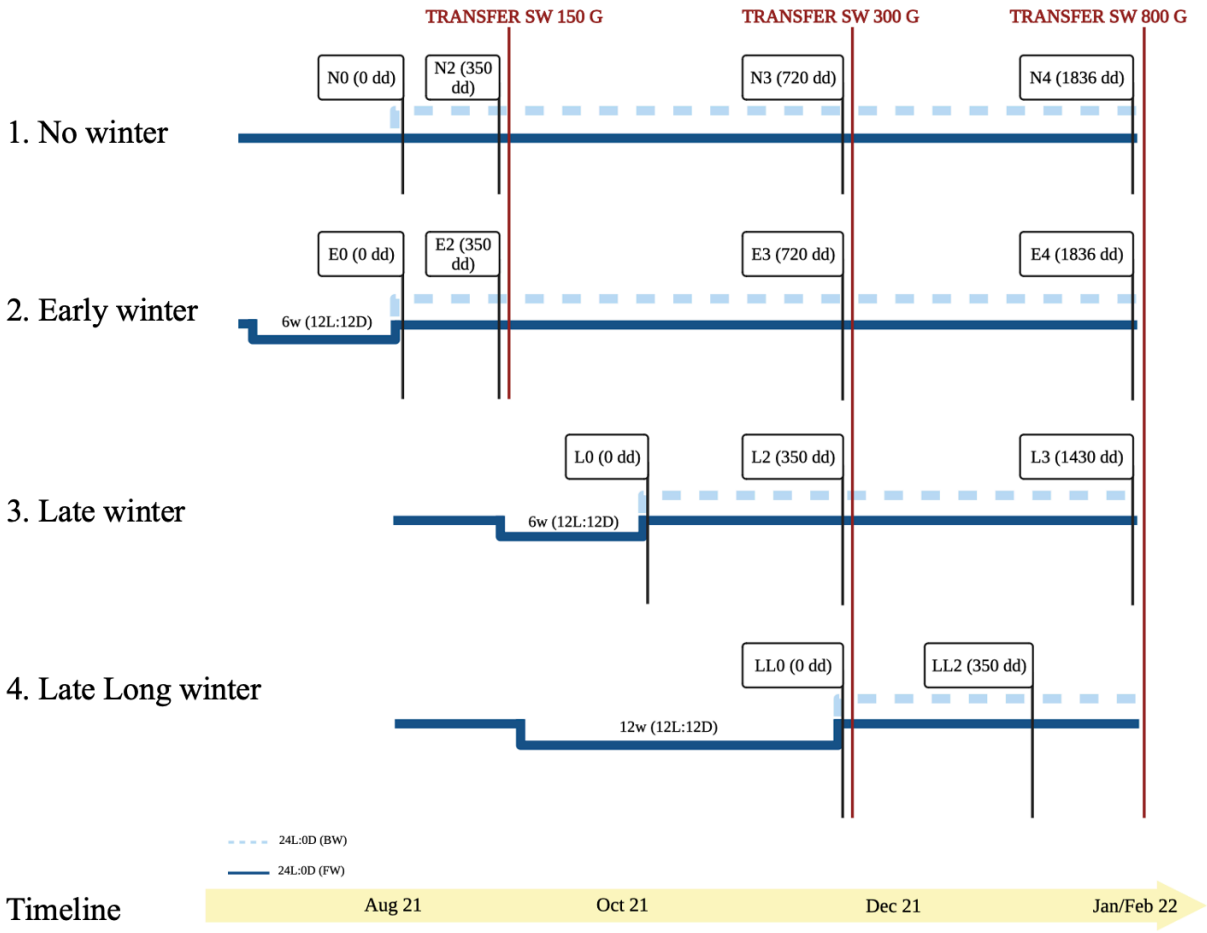

Figure 1: Experimental setup of the four different light regimes: 1) No winter, 2) Early winter, 3) Late winter and 4) Late Long winter in RAS. *The figure is created in BioRender.com.*

2.3 Sampling protocol

Light sedation was used before the fish were dip-netted from tanks (Metakain; Finquel, MS-222, 30 mg/mL). Lethal dose was used before the fish were sampled (< 100 mg/mL). Weight and fork length were measured, and blood samples were sampled from caudal vein with a vacutainer. The blood was centrifuged within two hours, and the serum was stored at -80°C. Gills and kidney were sampled for gene expression and western blot and directly frozen on dry ice. All PIT tags were collected for registration.

2.4 RNA isolation

Total RNA from kidney and gills were extracted using the QIAsymphony Robot (Qiagen) using the QIAsymphony RNA kit according to manufactures protocol (Qiagen). The samples were stored in RNAlater at -80*C and putted on ice. Approximately 20-25 mg of tissue was homogenized in 600 µl of RTL plus buffer and Reagent DX (Qiagen Qiasymphony mRNA

extraction kit) using ceramic spheres (Bertin Technologies BERT03961-1-103, distributed by VWR) and the Precellys 24 tissue homogenizer (Bertin Technologies) to disrupt cells and dissolving cell components. The isolated total RNA was diluted in 100 µl (kidney) and 50 µl (gills) of ultra-pure water and stored at -80*C.

2.4.1 QUANTIFICATION, PURITY, AND INTEGRITY OF TOTAL PROTEIN

Quantification and testing of RNA concentrations for kidney, gill and intestine tissue were accurately measured using the Invitrogen Qubit 4 fluorometer (Thermo Fisher Scientific) applying the QubitTM RNA HS Assay Kit protocol (InvitrogenTM, Thermo Fisher Scientific). The integrity of total RNA in samples was measured with an Agilent 2100 expert analyzer (Agilent technologies) using the Agilent RNA 6000 Nano kit (Agilent Technologies). Total RNA integrity was classified and assigned an RNA Integrity Number (RIN) ranging from 1 to 10, with 1 being the lowest integrity and 10 being the highest integrity (Schroeder et al., 2006).

2.4.2 COMPLIMENTARY DNA (CDNA) ANALYSIS

A total RNA amount of 1500 ng (kidney) and 500 ng (gills) was diluted in a total volume of 11 µl ultra-pure water before Oligo(dT)20 primer (2,5 µM) and dNTP mix (0,5 mM) were added, resulting in a total volume of 13 µl. After incubation at 65°C for 5 min on the C1000 Touch Thermo Cycler (Bio-Rad Laboratories), samples were placed on ice for one min to reduce formation of secondary structures. A master mix consisting of First strand Buffer ((20% (v/v) + (Tris-HCL (250 mM at pH 8,3), KCL (375 mM), MgCl2 (15 mM))), dithiothreitol (DTT; 5mM), RNaseOUTTM Recombinant RNase Inhibitor (40 U) and SuperScriptTM III Reverse Transcriptase enzyme (RT; 200 U) were added to the samples, resulting in a final volume of 20 µl. The complementary DNA (cDNA) synthesis was then carried out according to the manufacturers (Invitrogen) thermocycler protocol: first step at 50 °C for 60 min, followed by an inactivation at 70 °C for 15 min and then down to 4 °C before storage at -20 °C until Quantitative Polymerase Chain Reaction (qPCR) analysis to determine mRNA abundance.

2.5 REAL TIME QUANTITIVE POLYMERASE CHAIN REACTION (QPCR) ANALYSIS

Test qPCR were performed on a pool of cDNA for gills and kidney. This was done to test the specificity and efficiency of all primer sets, and to provide a first indication of their relative tissue distribution. The specificity was confirmed by the presence of a single melting curve. The PCR efficiency for each primer pair for each tissue was calculated with a two-fold dilution series (1:10, 1:20, 1:40, 1:80 and 1:160) of each cDNA pool to establish a dilution curve. This was also used to determine the optimal dilution of the cDNA to use for all the experimental samples. The mean Ct value from each dilution was plotted against the logarithmical cDNA diluted concentrations and the primer efficiency (E) was determined based on the slope of the regression line generated from this curve.

To determine the optimal reference gene for kidney and gills, three genes were tested on a range of samples covering the whole experiment: gapdh, ef1a and b-actin. Based on several tests incorporated in RefFinder (Xie et al., 2012): BestKeeper (Pfaffl et al., 2004), Normfinder (Andersen, Jensen and Falck Ørntoft, 2004), Genorm (Vandesompele et al., 2002), the comparative delta-Ct method (Silver et al., 2006), ef1a was used for normalization of the expression for the genes of interest. The target genes were NKAα1a1, NKAα1a2, NKAα1b1, NKAα1b2, NKAα1c1, NKAα1c2, NKAα3a and NKAα3b in all gill samples, and NKAα1a1, NKAα1b1, NKAα1b2, NKAα1c1, NKAα1c2, NKAα3a and NKAα3b in all kidney samples. For full primer list see table 1.

Real-time quantitative PCR (qPCR) was used to measure mRNA abundance of all target genes. All pipetting was automated using the Hamilton pipetting robot (Microlab STAR). The qPCR was carried out using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories) in a total volume of 12.5 µl using exon junction-spanning primers (Table 1) at a final concentration of 200 nM. A total of 420 samples, with n=210 for both kidney and gill tissue, were randomized and utilized for the measure of relative mRNA abundance of all the genes. All samples were analyzed in duplicates for all the target genes and the selected reference gene, including a control with only ultra-pure water, referred to as the non-template control (NTC), and a cDNA pool. Elongation factor (Ef1a) was used as reference gene in both gills and kidney. The reactions were run in a C1000 Touch™ Thermo cycler, CFX96™ Real-Time PCR detection System, and CFX Manager software (software version 3.1; Bio-Rad Laboratories). The thermal conditions consisted of an initial denaturation for 2 min at 95°C, followed by 37 cycles at 95°C for 15 s and 60°C for 25 s. Melt curve analysis verified that the primer sets for each qPCR assay had no primer–dimer artifacts and generated only one single product and amplification efficiency >80 %.The mean Ct value of the sample duplicates were

used for the quantification of mRNA abundance, using the reference gene(s) value(s) for normalization. The following formulas were used for calculations (Pfaffl, 2001).

I) Primer efficiency:

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

II) Relative quantification of *NKAα1a1, NKAα1a2, NKAα1b1, NKAα1b2, NKAα1c1, NKAα1c2, NKAα3a, NKAα3b*:

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

E_{target}: The qPCR efficiency of target gene (*NKAα1a1, NKAα1a2, NKAα1b1, NKAα1b2, NKAα1c1, NKAα1c2, NKAα3a, NKAα3b*)
E_{ref}: The qPCR efficiency of the reference gene (*ef1a*)
ΔCt_{mean} target: The mean Ct value of the target gene in duplicates (*NKAα1a1, NKAα1a2, NKAα1b1, NKAα1b2, NKAα1c1, NKAα1c2, NKAα3a, NKAα3b*)
ΔCt_{mean} ref: The mean Ct value of the reference gene (ef1a)

Table 1: **Target and reference genes/primers for kidney and gills qPCR**. *Overview of primer sequences used to measure mRNA abundance of NKAα1a1, NKAα1a2, NKAα1b1, NKAα1b2, NKAα1c1, NKAα1c2, NKAα3a, NKAα3b and the reference gene ef1a in the kidney and gill samples.*

Gene	Forward and reverse primer sequences (5′-3′)	mRNA reference
NKAα1a1 (gills, kidney)	CGCCGCTGTCGTGATTGCTC CCTGGGCTCCGTCGCATG	Unpublished
NKAα1a2 (gills)	AGACGGATATGGATGACCTG GCCATCGCGAAGAAGAAG	Unpublished
NKAα1b1 (gills, kidney)	GTTGACCTTGGATGAACTTCATC GCACCAATCCATAGGAGCATAG	Unpublished

NKAα1b2 (gills, kidney)	GTTGACCTTGGATGAACTTCATAG GCACCAATCCATAGGAGCATAG	Unpublished
NKAα1c1 (gills, kidney)	ACCTGGTCCCACAGCAAG CAGAGATGATTCGCAAATCAG	Unpublished
NKAα1c2 (gills, kidney)	ACCTGGTCCCACAGCAAG GGGTTGTCATTGGAGTAGTCC	Unpublished
NKAα3a (gills, kidney)	CTGCCAGCGACTGGGTGCC GACCCTCTTCCACTCCGGTGAC	Unpublished
NKAα3b (gills, kidney)	TGCCAGCGACTGGGTGCC CGACCCTCTTCTACTCCAGTAAC	Unpublished
Ef1a (gills, kindey)	CCCCTCCAGGACGTTTACAAA CACACGGCCCACAGGTACA	Olsvik et al., 2005

2.6 WESTERN BLOT (GILLS AND KIDNEY)

Extraction:

Making the RIPA-i buffer

1 mL 10X RIPA (Radioimmunoprecipitation assay) protein extraction buffer + 9 mL MQ H2O + 1 cOmplete Mini Tablet = 10 mL 1X RIPA-i buffer

A small amount of gill and kidney tissue was mechanically homogenized with 150 µl RIPA-I buffer using a pestle. When the tissue had dissolved, 100 µl RIPA-i buffer was added and incubated overnight on an orbital shaker in cold room (11-12°C).

The next day the samples were centrifuged at 2000 rpm, 4°C in 20 minutes. After the centrifugation, the supernatant was transferred into clean Eppendorf tubes. To avoid contamination, it was important that the last drop of supernatant was never in contact with the pellet.

Quantification

The proteins were quantified using the Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Massachusetts, USA). The samples were diluted 1:10 with 5 µl proteins and 45 µl RIPA-I buffer. The four standards (concentrations 0, 2, 5, 10 for gills, 0, 5, 10, 20 for kidney) were made with MilliQ H20 and BSA standard mixture (table 1, 2). Ten µl of each standard

and protein samples were pipetted in triplicates to a 96-well plate (COSTAR). The BCA Protein Assay Reagents was made with 50 parts reagent A and 1 part reagent B and 200 µl working reagent mixture was added to all triplicates and incubated at 37°C for 30 minutes. An endpoint measurement of the absorbance was read at 540 nm in the plate reader (Tecan Spark Multimode plate reader). The samples were then normalized to 2 µg/µl using RIPA-I as dilutant and aliquot/15 µl in PCR tubes where each tube correspond to one well in the gel, containing 30 µg total protein.

Table 2: Overview of the final concentrations of the standards used to quantify the proteins in all gill samples.

Concentration (µg/10 µl)	diH2O (µl)	2 mg/mL BSA std (µl)
0	200	0
2	180	20
5	150	50
10	100	100

Table 3: Overview of the final concentrations of the standards used to quantify the proteins in all kidney samples.

Concentration (µg/10 µl)	diH2O (µl)	2 mg/mL BSA std (µl)
0	100	0
2	75	25
5	50	50
10	0	100

SDS-PAGE electrophoresis:

Making the Laemmli buffer:

The Laemmli buffer must be prepared the same day as the electrophoresis is planned.

800 µl 2X Laemmli buffer + 200 µl 1M DTT = 1 mL 0.2 M Reducing Laemmli sample loading buffer

15 µl Laemmli buffer were added to each 15 µl total protein in PCR tubes. Then 0,35 µl of 1:60 diluted Unstained standards (BioRad 1610363) and 5 µl All Blue Standards (BioRad 1610373) were added to PCR tubes to make the standards. This standard mixture is visible in the gel (blue), after gel imaging, on the membrane after blotting (blue) and after chemiluminescence detection. The samples and standards were denatured at 90°C for five minutes in the ThermoCycler (REDPROTPAGE program).

The PAGE gels (pre-cast Criterion TGX Stain-Free gel) were unwrapped and placed in chamber with 1X TGS buffer (400 ml in the outside chamber, 60 ml in the gel´s own chamber). The wells were rinsed by pipetting TGS buffer into the wells. The samples were loaded in the gel and ran at 200V for 45 minutes.

After the electrophoresis, the plastic case was opened, and the gel was transferred onto the tray (ChemiDoc tray) of the ChemiDoc imager (BioRad, ChemiDoc Touch Imaging System). The stain-free component of the gel was activated by UV exposure (45 s in the ChemiDoc imager, dedicated program) and then the gel was imaged to visualize total protein content.

Blotting:

The TransBlot transfer machine and a Trans-Blot Turbo TM midi PVDF Membrane Transfer Pack was placed into the TransBlot cassette where the pack components were placed in the following order: 1. Bottom wet pad. 2. PVDF membrane. 3. Gel. 4. Top wet pad. Air bubbles were removed with a roller. The cassette was placed into the TransBlot transfer machine (Bio-Rad Trans-Blot Turbo Transfer System). Band ran with the standard program for midi TGX gels for 30 minutes. All the layers were discarded except the membrane which was rinsed with TBS-T (TBS 0,1% Tween).

Immunodetection:

The membrane incubated in blocking buffer (TBS-T-Cas) for 1h. The blocking buffer was then replaced with Ab1 (NKA1a (gills), NKA1b (gills and kidney), NKA1c (kidney)), diluted in TBS-T-Cas and incubated overnight in cold room on an orbital shaker.

The next day the membrane was flushed with TBS-T, followed by 3 x 5 min wash with TBS-T on orbital shaker. The membrane incubated 1h in Ab2 HRP-conjugated (1:20 000) in TBS-T-Cas at room temperature on orbital shaker before it was flushed with TBS-T and washed 3 x 5 min with TBS-T on orbital shaker.

Chemiluminescence:

A large plastic sleeve was cut in two and left open on the ChemiDoc tray while the ECL buffer (50:50 luminol/enhancer solution and peroxide solution) from the Clarity Kit (Clarity Western ECL Substrate, Bio-rad) was prepared. The membrane was placed on the open

plastic sleeve with a layer of ECL buffer on top before the sleeve was closed and imaged immediately using the Chemiluminescence function of the ChemiDoc. ImageJ (software) was used to quantify band staining intensity by measuring the mean.

2.7 STATISTICAL ANALYSIS

All statistical analysis was performed using RStudio (version 2023.03.0+386) utilizing the following packages: dplyr, ggplot2, nlme, readxl, writexl, stringr, emmeans, tibble and data.table. Linear models (Two-Way ANOVA) analysis was fitted between each response variable and the predictor variables (treatments) and samplings. 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. Statistical differences were determined by linear models (One-Way or Two-way ANOVA) followed by a Tukey's HSD post-hoc test. The p values lower than 0.05 ($p < 0.05$) were deemed a statistically significant datapoint and marked with asterisk (between groups) accordingly; $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Non-identical letters were used for significant difference between timepoints/samplings in each group. Results are presented as mean ± the standard error of mean (SEM).

RESULTS

3.1 RELATIVE MRNA ABUNDANCE AND PROTEIN ABUNDANCE OF NKAα ISOFORMS IN GILLS

3.1.1 NKAα1A1 AND NKAα1A2 IN GILLS FOR THE NW AND EW GROUP

The relative mRNA abundance of NKAα1a1 in gills remained relatively stable for both NW and EW group during the experimental period with no significant differences observed 0 days (0 d.d.) to 153 days (1836 d.d.) (figure 2). While for the NKAα1a2 a significant decrease ((Two-Way ANOVA; $p<0,001$) was observed from 0 days (0dd) to 29 days (350 dd) in both NW and EW group where the levels thereafter were kept stable (figure 2). No difference was observed on the transcript levels of NKAα1a1 and NKAα1a2 with the use of salinity (BW) in both NW and EW group (figure 2). The protein abundance of NKAα1a in the gills had a significant decrease (Two-Way ANOVA: $p<0,01$) from 0 days (0 d.d.) to 29 days (350 d.d.) in both groups, before significantly increase (Two-Way ANOVA: $p<0,05$) from 29 days (350 d.d.) to 153 days (1836 d.d.) (figure 2c). With the use of salinity (BW), the protein levels had a significant increase ($p<0,01$) from 29 days (350 d.d.) to 60 days (720 d.d.) (figure 2). There was a significant difference (Two-Way ANOVA: $p<0,05$) between NW (FW) and NW (BW), and NW (FW) and EW (BW).

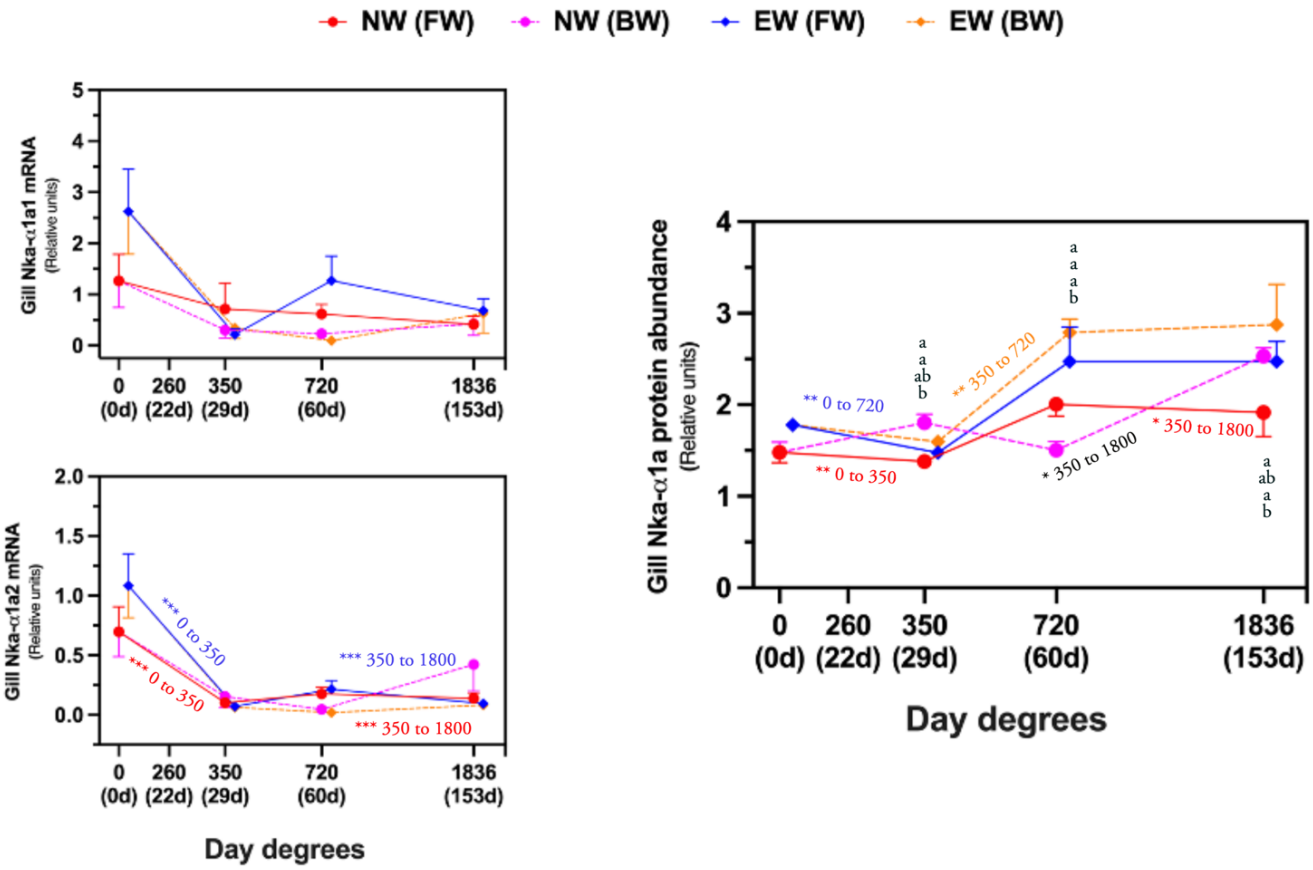

Figure 2: The relative NKAα1a1 (upper left panel) and NKAa1a2 (lower left panel) mRNA and NKAa1a protein (right panel) abundance in the gill of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) groups in freshwater (FW) and brackish water (BW). *Different lower-case letters showcase significance ($p < 0.05$) between groups at each sampling point. Asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant changes between consecutive sampling points.*

3.1.2 NKAα1A1 AND NKAα1A2 IN GILLS FOR THE LW AND LLW GROUP

The relative mRNA abundance of NKAα1a1 in gills had no significant differences from 0 days (0 d.d.) to 119 days (1430 d.d.) in both LW and LLW group (figure 3). There were no significant differences in the relative mRNA abundance of NKAα1a2 from 0 days (0 d.d.) to 119 (1430 d.d.) in both LW and LLW group (figure 3). No difference was observed on the transcript levels of NKAα1a1 and NKAα1a2 with the use of salinity (BW) in both LW and LLW group (figure 3). The relative protein abundance of NKAα1a had a significant increase

(Two-Way ANOVA: p<0,05) from 0 days (0 d.d.) to 29 days (350 d.d.) in LW group (figure 3c), and from 29 days (350 d.d.) to 119 days (1430 d.d.) (Two-Way ANOVA: p<0,001). There was a significant difference (Two-Way ANOVA: p<0,05) between LW (FW) and LLW (FW), LLW (BW) and LLW (FW), and LW (BW) and LLW (FW). There was a significant increase in the LW group from 29 days (350 d.d.) to 119 days (1430 d.d.) with the use of salinity (BW), while there were no significant differences in the LLW group (figure 3).

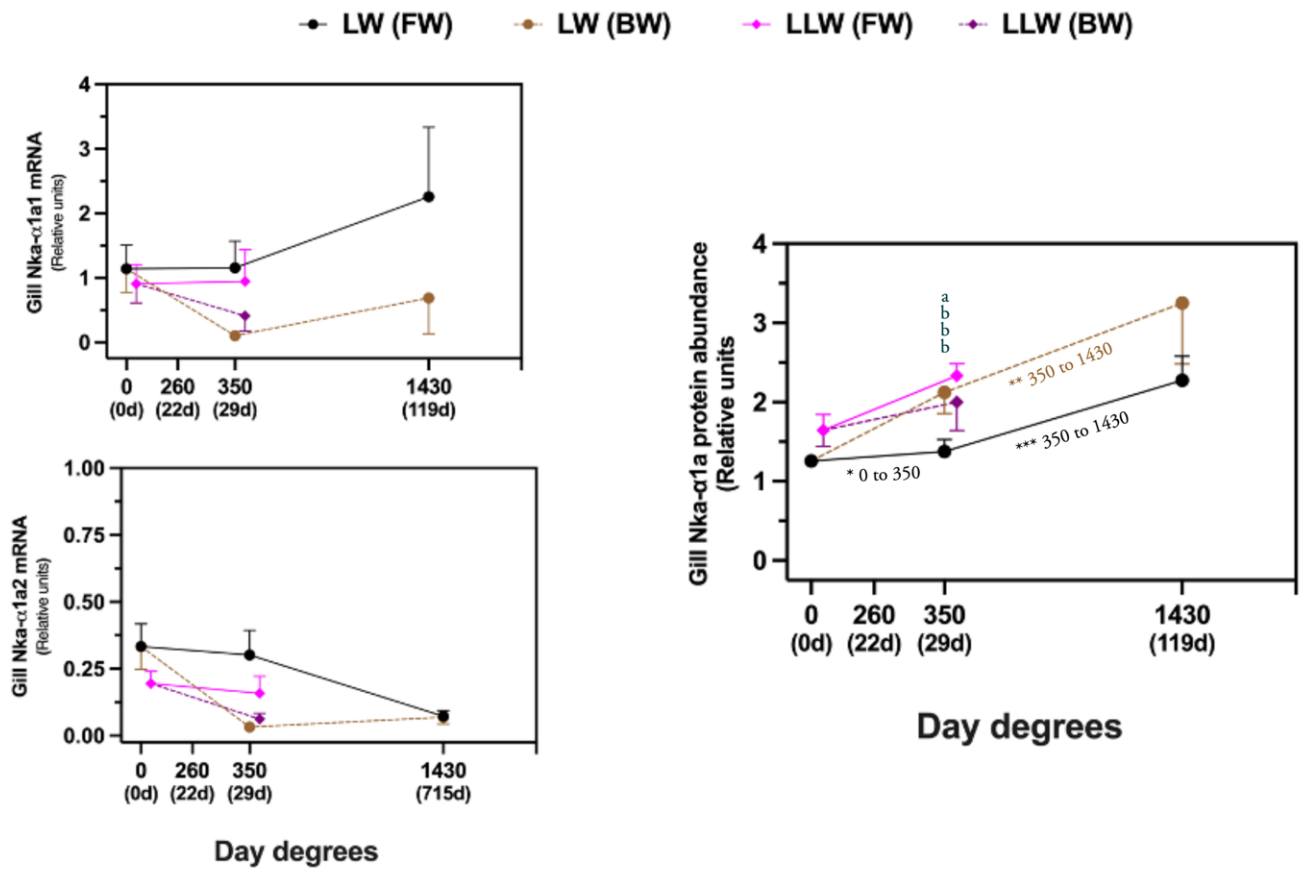

Figure 3: The relative NKAα1a1 (upper left panel) and NKAa1a2 (lower left panel) mRNA and NKAa1a protein (right panel) abundance in the gill of Atlantic salmon (Salmo salar) smolts for Late Winter (LW) and Late Long Winter (LLW) groups in freshwater (FW) and brackish water (BW). *Different lower-case letters showcase significance (p < 0.05) between groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points.*

3.1.3 NKAα1B1 AND NKAα1B2 IN GILLS FOR THE NW AND EW GROUP

The relative mRNA abundance of NKAα1b1 in gills had a significant decrease (Two-Way ANOVA: p<0,01) from 0 days (0 d.d.) to 29 days (350 d.d.) in NW group (figure 4). In EW

group there was no significant differences (figure 4). Observed differences in the abundance of NKAα1b2 in both NW and EW group from 0 days (0 d.d.) to 153 days (1836 d.d.) were not significant (figure 4). No difference was observed on the transcript levels of NKAα1b1 and NKAα1b2 with the use of salinity (BW) in both NW and EW group (figure 4). The protein abundance of NKAα1b in the gills showed no significant increase from 0 days (0 d.d.) to 29 days (350 d.d.) in both NW and EW group (figure 4). A significant difference (Two-Way ANOVA: p<0,05) was observed between NW (FW) and EW (FW).

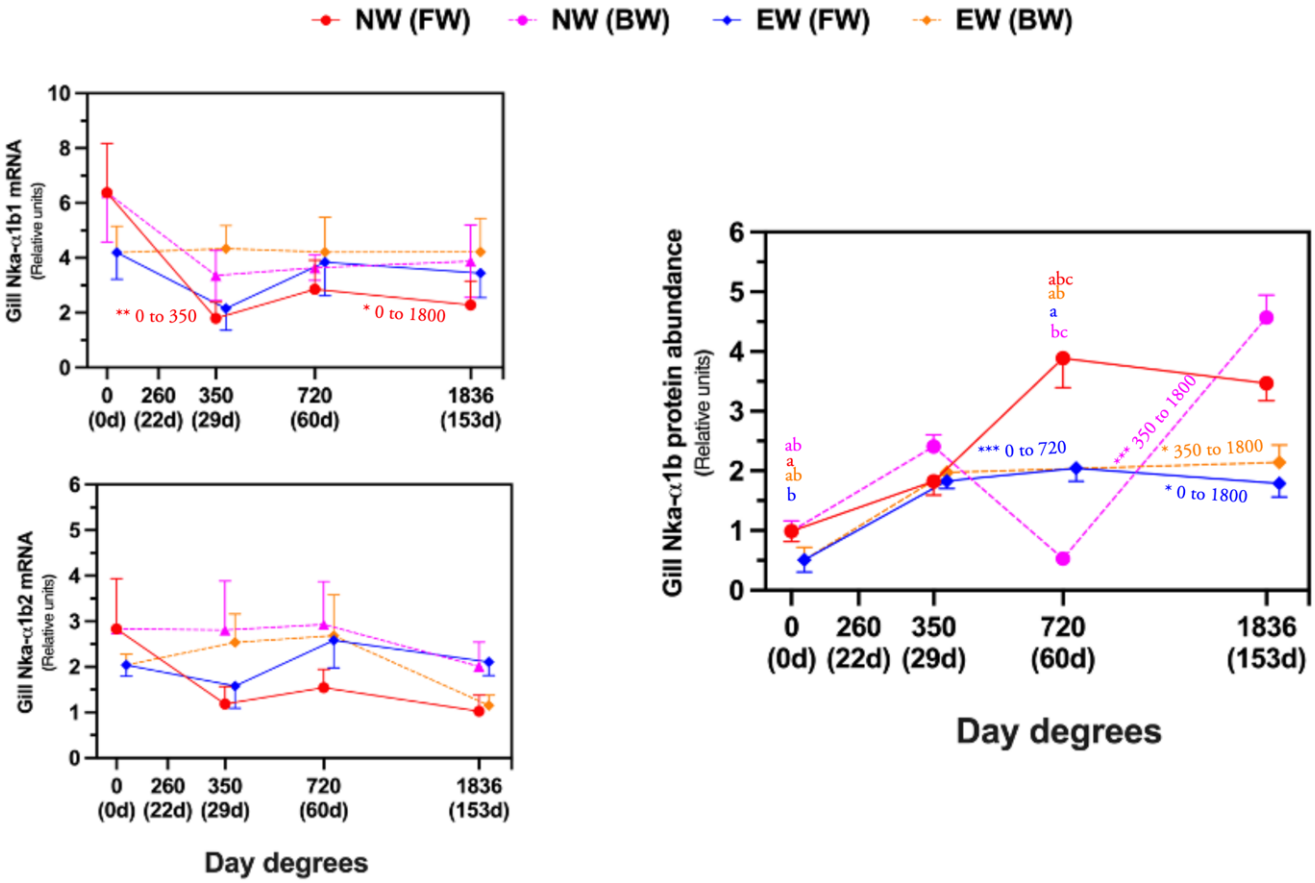

Figure 4: The relative NKAα1b1 (upper left panel) and NKAa1b1 (lower left panel) mRNA and NKAa1b protein (right panel) abundance in the gill of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) groups in freshwater (FW) and brackish water (BW). *Different lower-case letters showcase significance (p < 0.05) between groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points.*

3.1.4 NKAα1B1 AND NKAα1B2 IN GILLS FOR THE LW AND LLW GROUP

The relative mRNA abundance of NKAα1b1 in gills had no significant differences from 0 days (0 d.d.) to 119 days (1430 d.d.) in both LW and LLW group (figure 5). There was no significant difference in mRNA abundance of NKAα1b2 observed in the two groups from 0 days (0 d.d.) to 119 days (1430 d.d) (figure 5). No difference was observed on the transcript levels of NKAα1b1 and NKAα1b2 with the use of salinity (BW) in both LW and LLW group (figure 5). The protein abundance of NKAα1b in the gills showed a significant difference (Two-Way ANOVA: p<0,05) at 29 days (350 d.d.) between LLW (FW) and LLW (BW), LLW (FW) and LW (BW), and LLW (FW) and LW (FW) (figure 5).

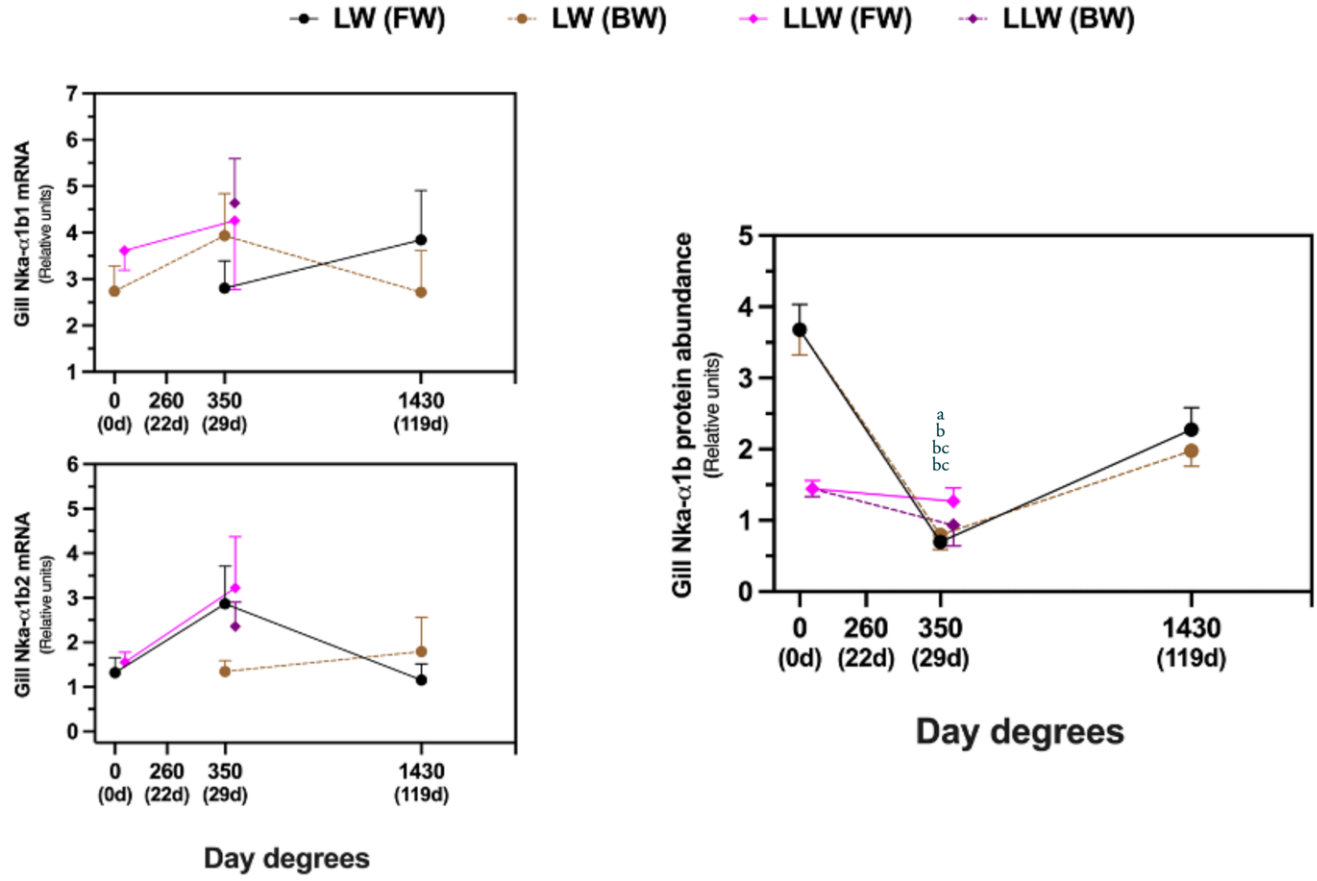

Figure 5: The relative NKAα1b1 (upper left panel) and NKAa1b2 (lower left panel) mRNA and NKAa1b protein (right panel) abundance in the gill of Atlantic salmon (Salmo salar) smolts for Late Winter (LW) and Late Long Winter (LW) groups in freshwater (FW) and brackish water (BW). *Different lower-case letters showcase significance (p < 0.05) between groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points.*

3.1.5 NKAα1C1 NKAα1C1 IN GILLS FOR THE NW AND EW GROUP

The relative mRNA abundance of both NKAα1c1 in gills remained relatively stable for both NW and EW group during the experimental period. There was a significant difference (Two-Way ANOVA: p<0,001) from 0 days (0 d.d.) to 29 days (350 d.d.) in EW group, and in NW group (Two-Way ANOVA: p<0,05) (figure 6). No significant difference was observed in the abundance of NKAα1c2, and no differences in the transcript levels of NKAα1c1 and NKAα1c2 with the use of salinity (BW) in both NW and EW group (figure 6).

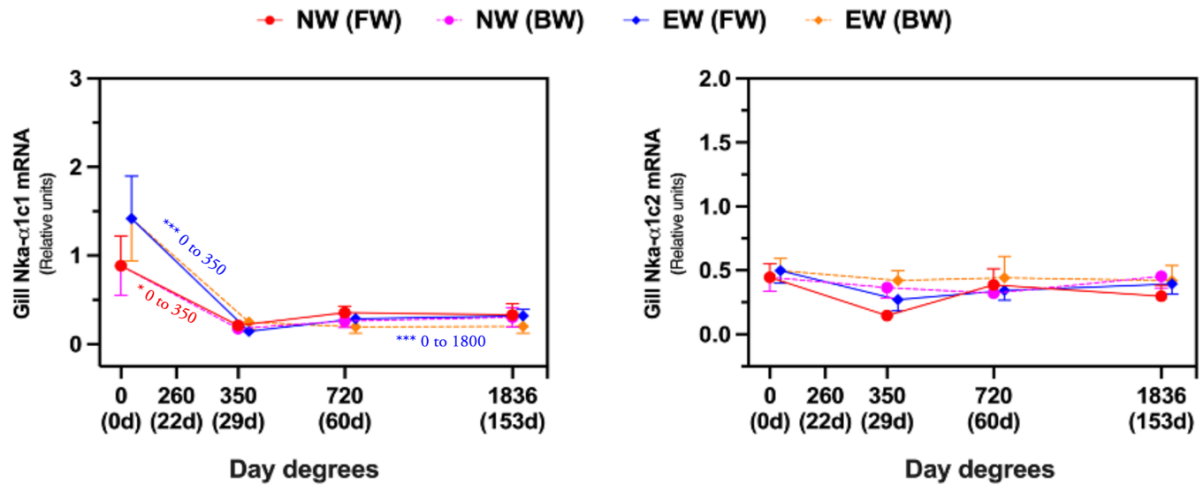

Figure 6: The relative NKAα1c1 (upper left panel) and NKAa1a2 (lower left panel) mRNA and NKAa1 protein (right panel) abundance in the gill of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) groups in freshwater (FW) and brackish water (BW). Different lower-case letters showcase significance (p < 0.05) between groups at each sampling point. Asterisk (* p < 0.05; ** p < 0.01; *** p < 0.001) indicate significant changes between consecutive sampling points.

3.1.6 NKAα1C1 AND NKAα1C2 IN GILLS FOR THE LW AND LLW GROUP

The relative mRNA abundance of both NKAα1c1 and NKAα1c2 in gills remained relatively stable for both LW and LLW group during the experimental period with no significant differences observed 0 days (0 d.d.) to 119 days (1430 d.d.) (figure 7). No significant difference was observed on the transcript levels of NKAα1c1 and NKAα1c2 with the use of salinity (BW) in both LW and LLW group (figure 7).

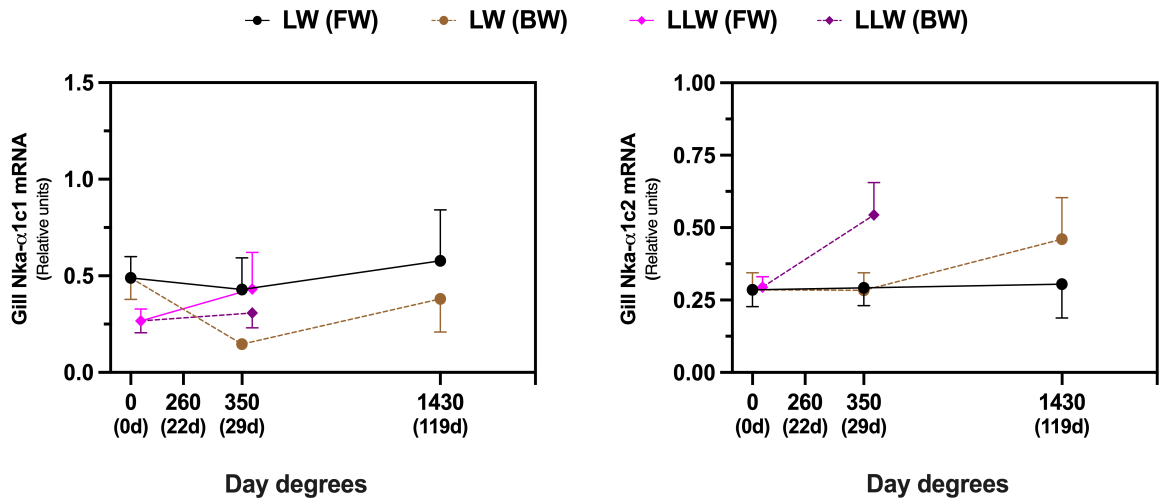

Figure 7: The relative mRNA abundance of a. NKAα1c1 and b. NKAα1c2 in the gill of Atlantic salmon (Salmo salar) smolts for Late winter (LW) and Late Long Winter (LLW) in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.1.7 NKAα3A AND NKAα3B IN GILLS FOR THE NW AND EW GROUP

The relative mRNA abundance of both NKAα3a and NKAα3b in gills remained relatively stable for both NW and EW group during the experimental period with no significant differences observed 0 days (0 d.d.) to 153 days (1836 d.d.) (figure 8). No difference was observed on the transcript levels of NKAα3a and NKAα3b with the use of salinity (BW) in both NW and EW group (figure 8).

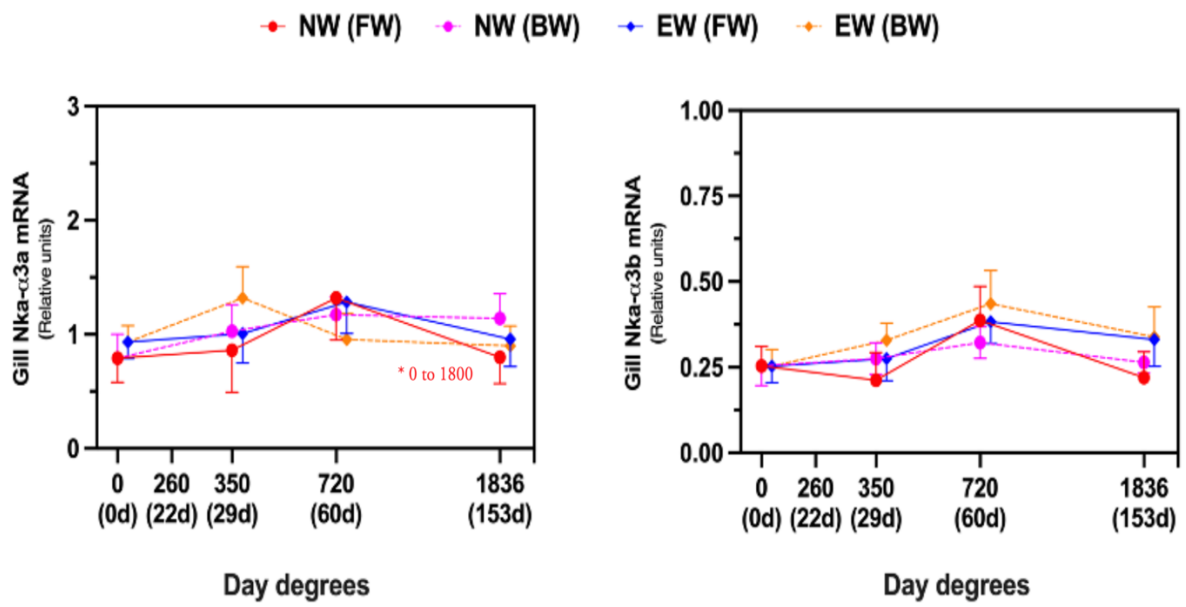

FIGURE 8: The relative mRNA abundance of a. NKAα3A and b. NKAα3B in the gill of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) group in freshwater (FW) and brackish water (BW). Different lower-case letters showcase significance ($p < 0.05$) between groups at each sampling point. Asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant changes between consecutive sampling points.

3.1.8 NKAα3A AND NKAα3B IN GILLS FOR THE LW AND LLW GROUP

The relative mRNA abundance of both NKAα3a and NKAα3b in gills in both LW and LLW group showed no significant differences from 0 days (0 d.d.) to 119 days (1430 d.d.) (figure 9). No significant difference was observed on the transcript levels of NKAα3a and NKAα3b with the use of salinity (BW) in both LW and LLW group (figure 9).

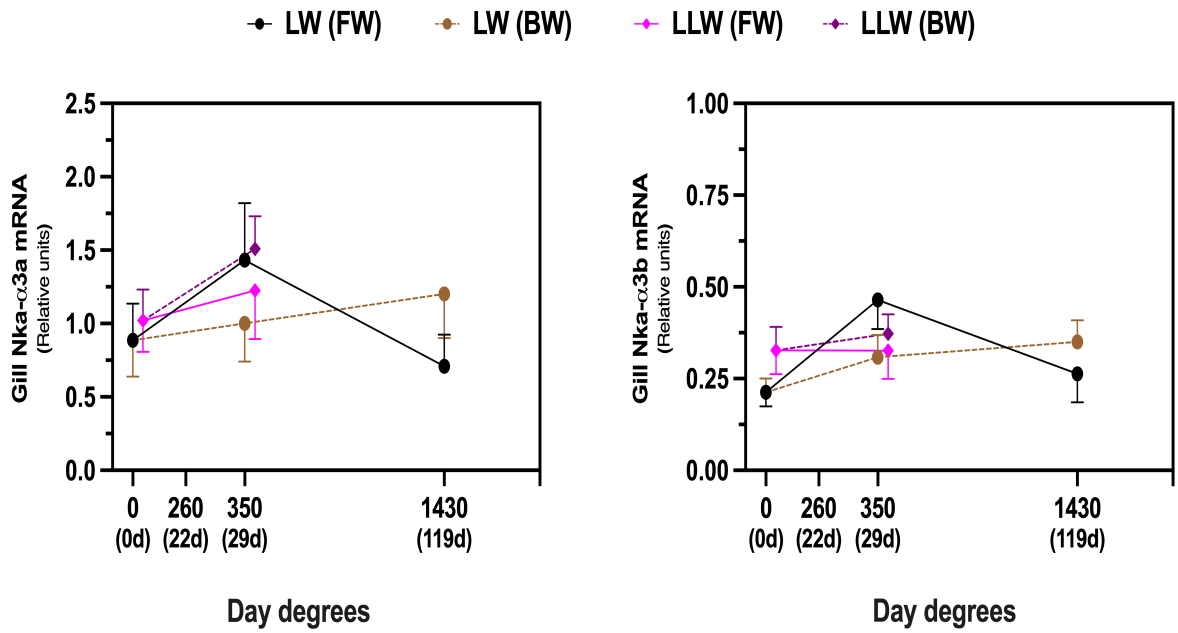

Figure 9: The relative mRNA abundance of a. NKAα3A and b. NKAα3B in the gill of Atlantic salmon (Salmo salar) smolts for Late winter (LW) and Late Long Winter (LLW) in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.2 RELATIVE MRNA OF NKAα ISOFORMS IN THE KIDNEY

3.1.1 NKAα1A1 AND NKAα1A2 IN KIDNEY FOR THE NW AND EW GROUP

The relative mRNA abundance of NKAα1a1 in kidney in both NW and EW group showed no significant differences from 0 days (0 d.d.) to 153 days (1836 d.d.) (figure 10). No difference was observed on the transcript levels with the use of salinity (BW) in both NW and EW group (figure 10).

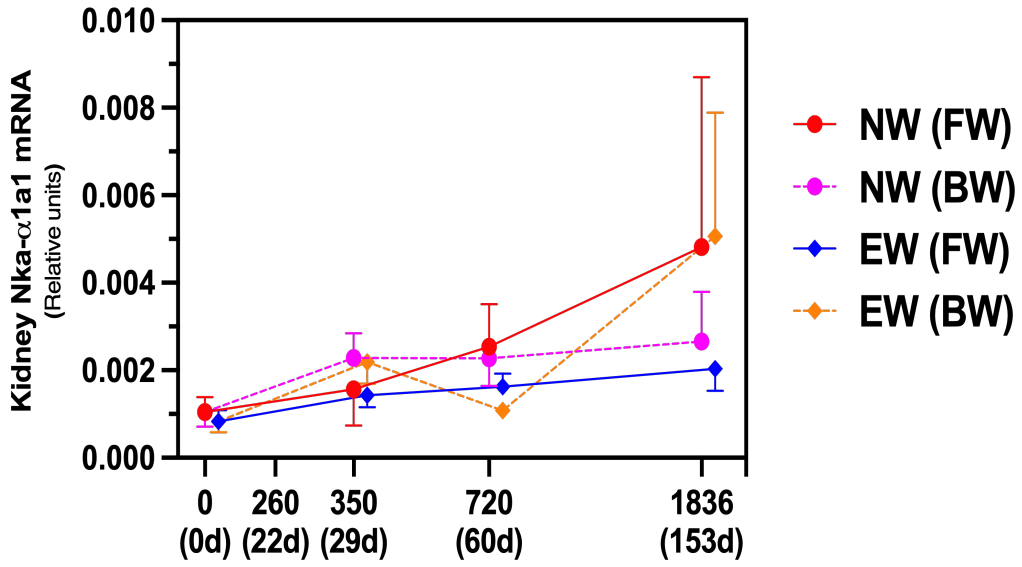

Figure 10: The relative mRNA abundance of NKAα1a1 in the KIDNEY of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) group in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.1.2 NKAα1A1 IN KIDNEY FOR THE LW AND LLW GROUP

The relative mRNA abundance of NKAα1a1 kidney in both LW and LLW group showed no significant differences from 0 days (0 d.d.) to 119 days (1430 d.d.) (figure 11). No difference was observed on the transcript levels with the use of salinity (BW) in both LW and LLW group (figure 11).

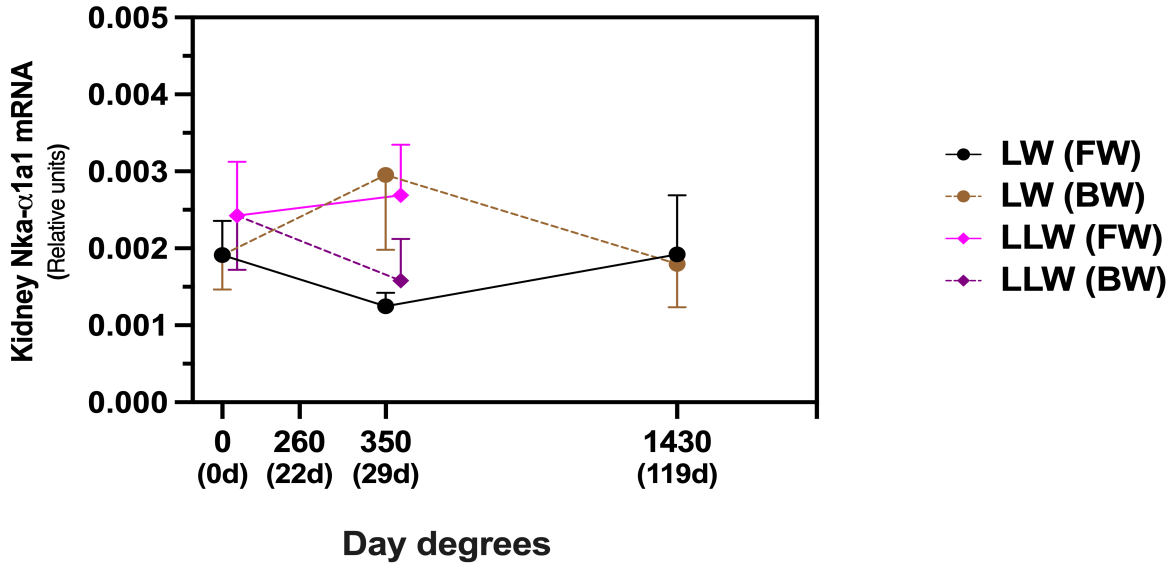

Figure 11: The relative mRNA abundance of NKAα1a1 in the KIDNEY of Atlantic salmon (Salmo salar) smolts for Late winter (LW) and Late Long Winter (LLW) in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.1.3 NKAα1B1 AND NKα1B2 IN KIDNEY FOR THE NW AND EW GROUP

The relative mRNA abundance of NKAα1b1 and NKAα1b2 in kidney in both NW and EW group showed no significant differences from 0 days (0 d.d.) to 153 days (1836 d.d.) (figure 12). No difference was observed on the transcript levels with the use of salinity (BW) in both NW and EW group (figure 12).

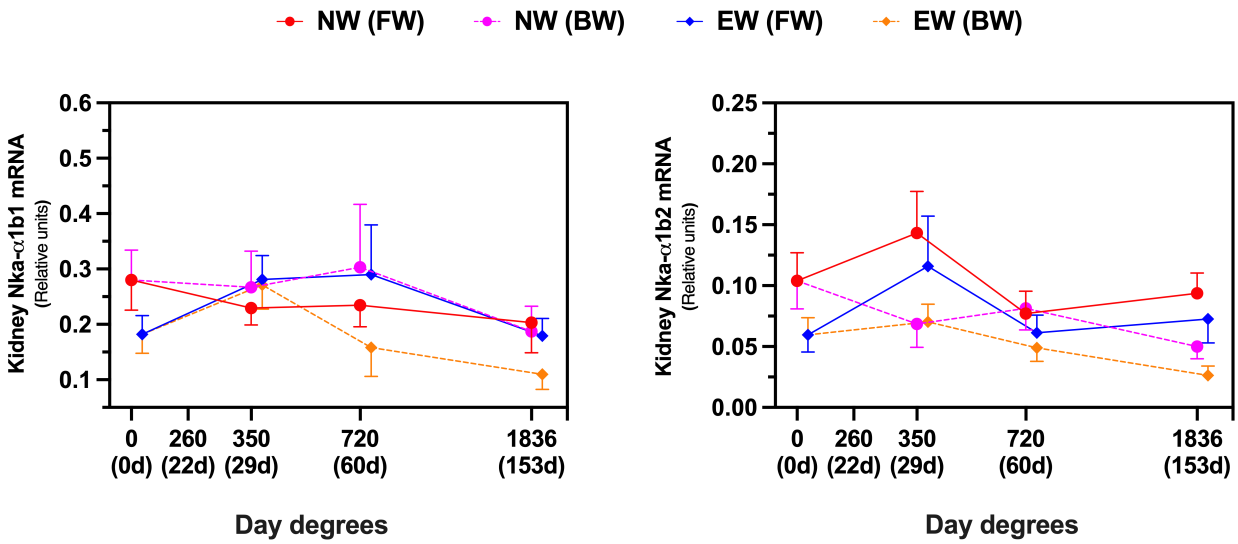

Figure 12: The relative mRNA abundance of NKAα1B1 and NKAα1aB2 in the KIDNEY of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) group in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.1.4 NKAα1B1 AND NKα1B2 IN KIDNEY FOR THE LW AND LLW GROUP

The relative mRNA abundance of NKAα1b1 and NKAα1b2 in kidney in both LW and LLW group showed no significant differences from 0 days (0 d.d.) to 119 days (1430 d.d.) (figure 13). No difference was observed on the transcript levels with the use of salinity (BW) in both LW and LLW group (figure 13).

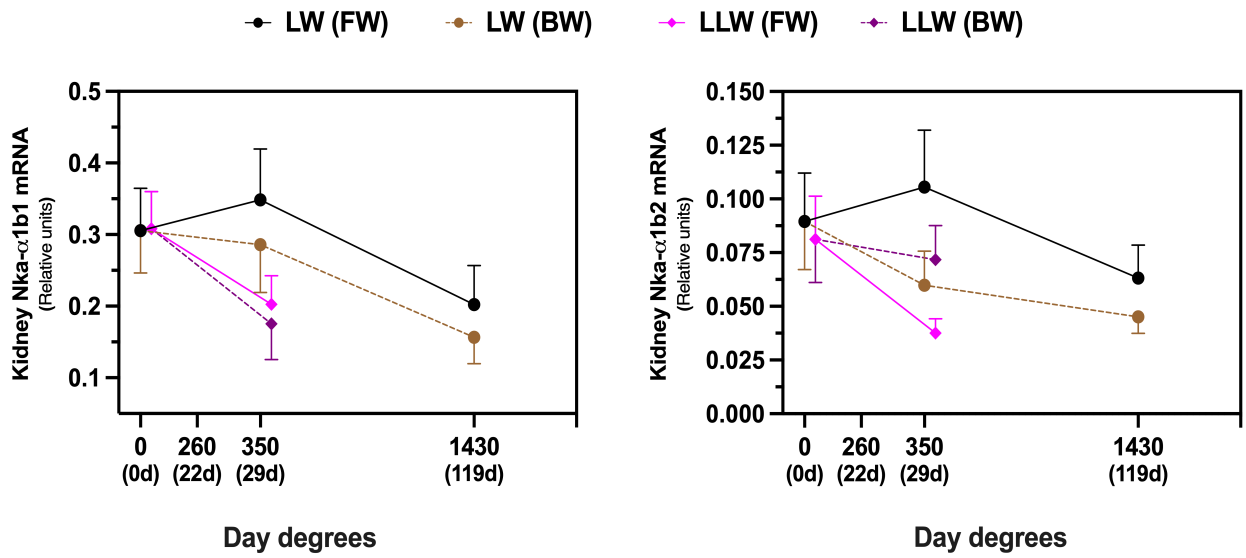

Figure 13: The relative mRNA and protein abundance of a. NKAα1B1 and b. NKAα1B2 (protein; NKAα1B) in the KIDNEY of Atlantic salmon (Salmo salar) smolts for Late winter (LW) and Late Long Winter (LLW) in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.1.5 NKAα1C1 AND NKAα1C2 IN KIDNEY FOR THE NW AND EW GROUP

The relative mRNA abundance of NKAα1c1 in kidney increased significantly from 0 days (0 d.d.) to 29 days (350 d.d.) in both NW (Two Way ANOVA: $p<0,05$) and EW (Two Way ANOVA: $p<0,001$) groups (figure 14a). A significant increase (Two Way ANOVA: $p<0,01$) in the abundance of NKAα1c2 occurred from 0 days (d.d.) to 29 days (350 d.d.) in EW group (figure 14c). No difference was observed on the transcript levels of NKAα1C1 and NKAα1C2 with the use of salinity (BW) in both NW and EW groups (figure 14). The protein abundance of NKAα1C in the gills showed a significant difference (Two Way ANOVA: $p<0,001$) from 0 days (0 d.d.) to 29 days (350 d.d in EW group. A significant decrease (Two Way ANOVA: $p<0,001$) was shown in NW group with the use of salinity (BW) from 29 days (350 d.d.) to 60 days (720 d.d.) (figure 14c).

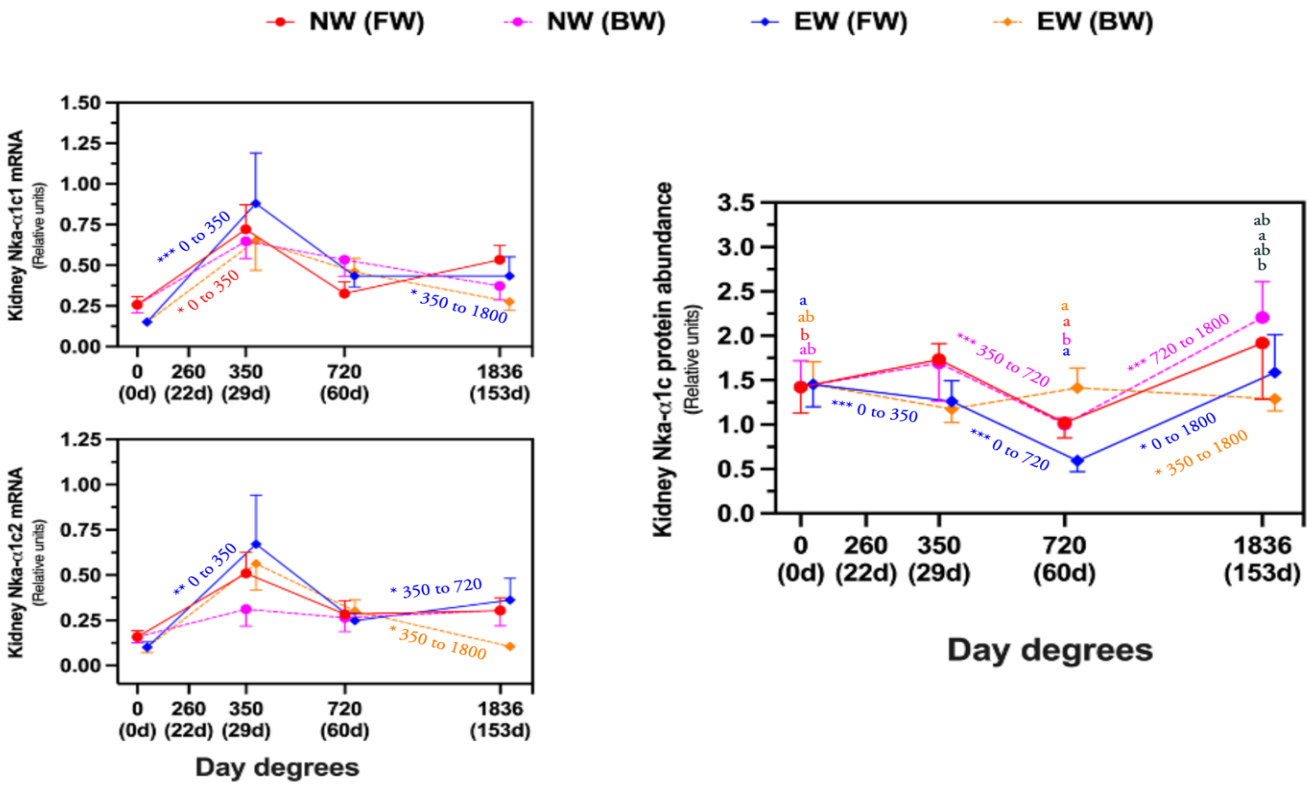

Figure 14: The relative mRNA AND PROTEIN abundance of a. NKAα1C1 and b. NKAα1C2 (c. protein: NKAα1c) in the kidney of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) group in freshwater (FW) and brackish water (BW). Different lower-case letters showcase significance ($p < 0.05$) between groups at each sampling point. Asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant changes between consecutive sampling points.

3.1.6 NKAα1C1 AND NKAα1C2 IN KIDNEY FOR THE LW AND LLW GROUP

The relative mRNA abundance of both NKAα1c1 and NKAα1c2 in kidney remained relatively stable for both LW and LLW group during the experimental period with no significant differences observed from 0 days (0 d.d.) to 119 DAYS (1430 d.d.) (figure 15a, 15b). No difference was observed on the transcript levels of NKAα1C2 and NKAα1C2 with the use of salinity (BW) in both LW and LLW group (figure 15). The protein abundance of NKAα1C in the gills showed a significant decrease (Two Way ANOVA: $p<0,01$) from 0 days (0 d.d.) to 29 days (350 d.d.) in both LW group (figure 15c). There was a significant

difference between LLW (FW) and LLW (BW), LLW (FW) and LW (FW), and LLW (FW) and LW (BW).

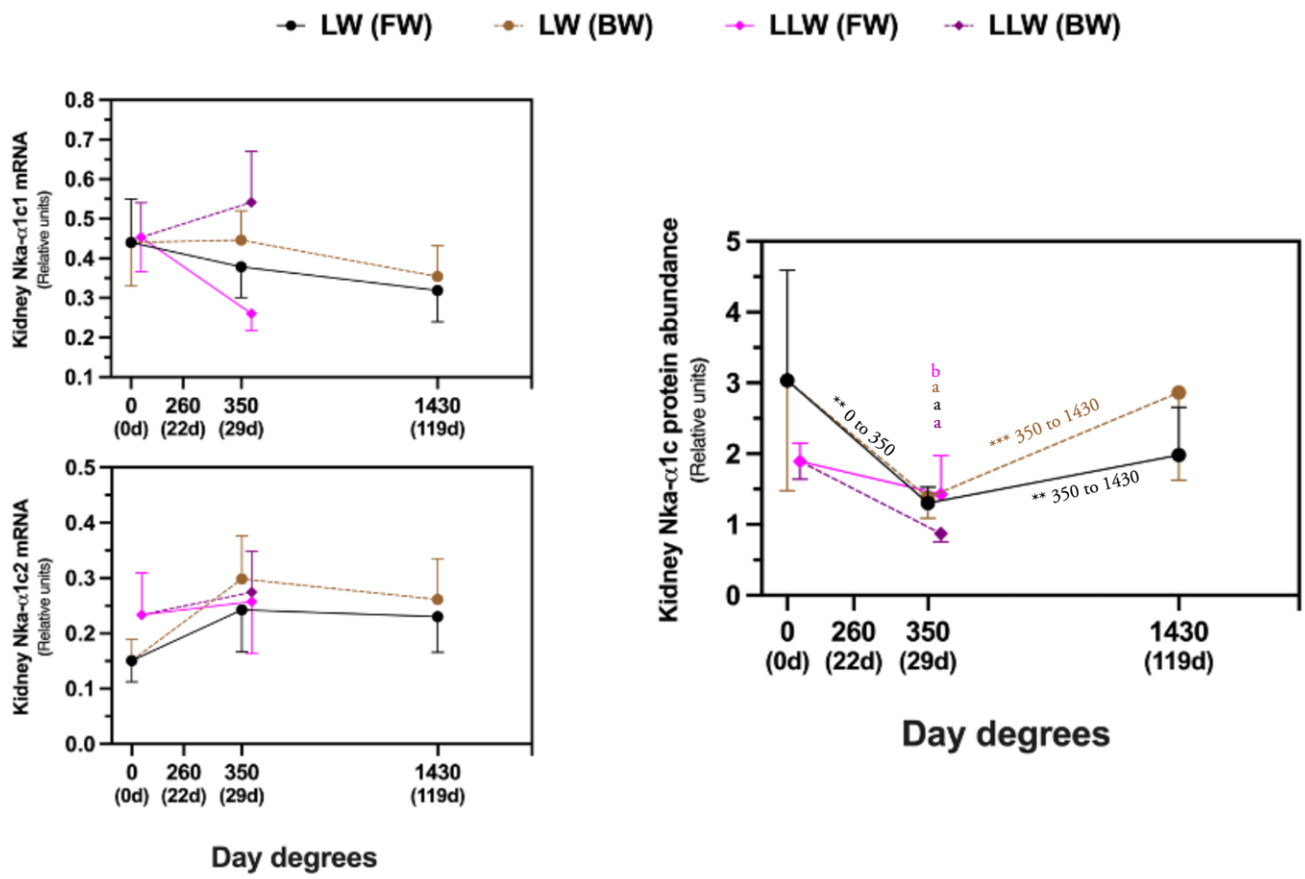

Figure 15: The relative mRNA AND PROTEIN abundance of a. NKAα1C1 and b. NKAα1C2 (C. PROTEIN; NKAA1C) in the KIDNEY of Atlantic salmon (Salmo salar) smolts for Late winter (LW) and Late Long Winter (LLW) in freshwater (FW) and brackish water (BW). Different lower-case letters showcase significance ($p < 0.05$) between groups at each sampling point. Asterisk (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) indicate significant changes between consecutive sampling points.

3.1.7 NKAα3A AND NKAα3B IN KIDNEY FOR THE NW AND EW GROUP

The relative mRNA abundance of NKAα1b1 and NKAα1b2 in kidney in both NW and EW group showed no significant differences from 0 days (0 d.d.) to 153 days (1836 d.d.) (figure 16). No difference was observed on the transcript levels with the use of salinity (BW) in both NW and EW group (figure 16).

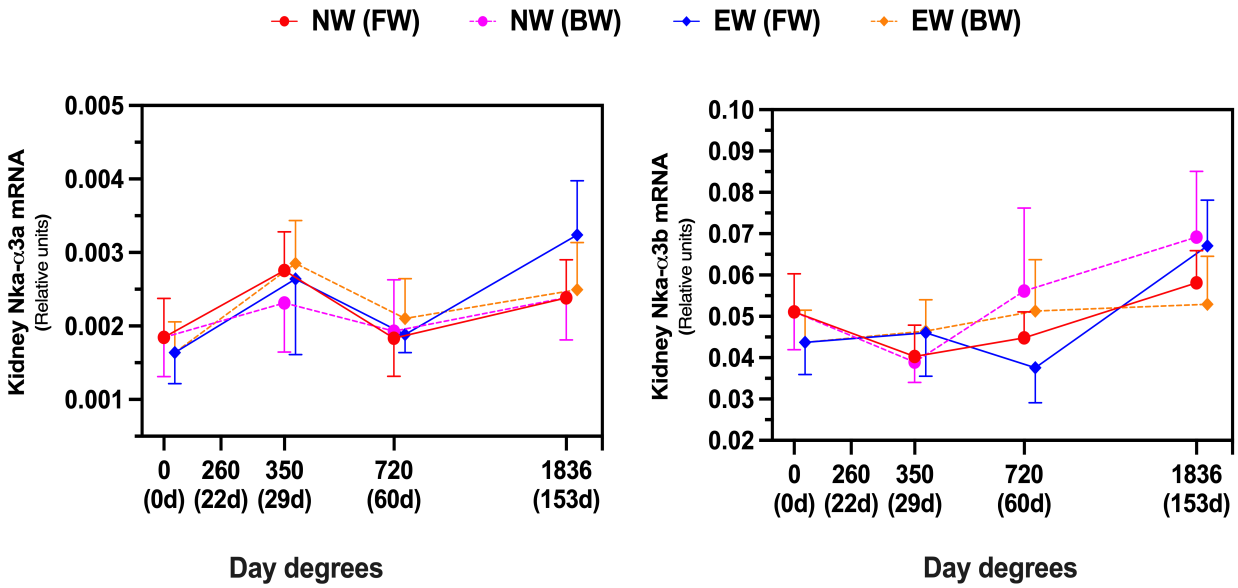

Figure 16: The relative mRNA abundance of a. NKAα3A and b. NKAα3B in the kidney of Atlantic salmon (Salmo salar) smolts for No Winter (NW) and Early Winter (EW) group in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

3.1.8 NKAα3A AND NKAα3B IN KIDNEY FOR THE LW AND LLW GROUP

The relative mRNA abundance of NKAα3A and NKAα3B in kidney in both LW and LLW group showed no significant differences from 0 days (0 d.d.) to 119 days (1430 d.d.) (figure 17). No difference was observed on the transcript levels with the use of salinity (BW) in both LW and LLW group (figure 17).

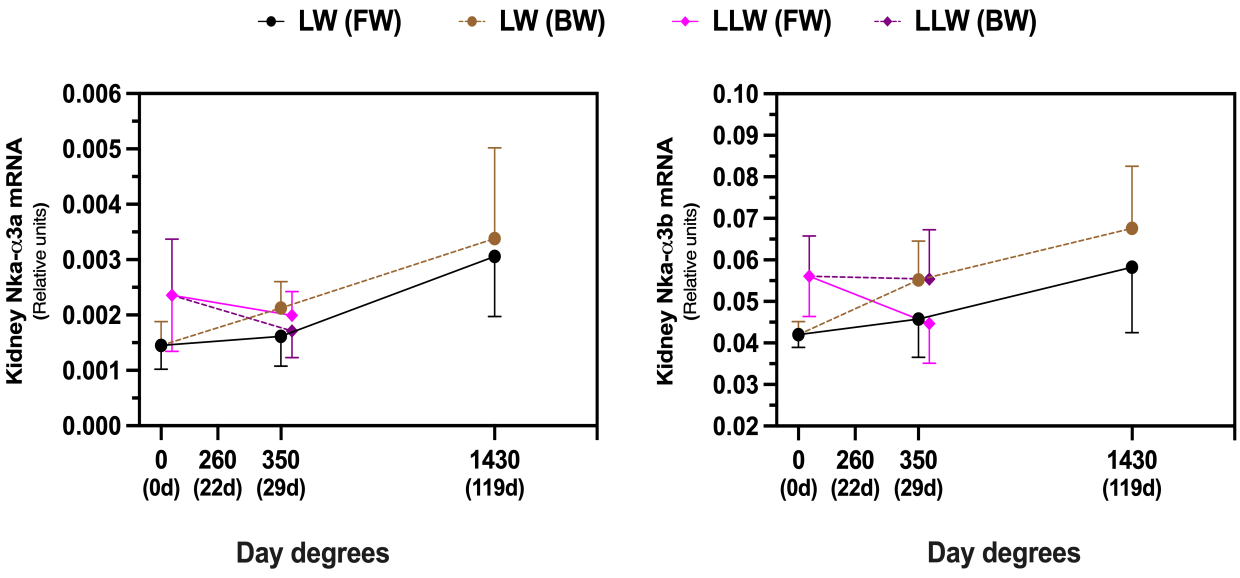

Figure 17: The relative mRNA abundance of a. NKAα3A and b. NKAα3B in the kidney of Atlantic salmon (Salmo salar) smolts for Late winter (LW) and Late Long Winter (LLW) in freshwater (FW) and brackish water (BW). *No significant differences were observed.*

DISCUSSION

4.1 CONSIDERATIONS MATERIAL AND METHODS

The experimental design in this study involved triplicate tanks for each treatment group to minimize the potential effects of tank variability on the growth data. While growth was not the main focus of this thesis, replicates were also beneficial for physiological data to mitigate the risks associated with individual tank-level artifacts or the loss of an entire group or timepoint. Previous studies conducted at the RAS facility at Sunndalsøra have reported minimal variation and no tank effects on the measured parameters during smoltification (Kolarevic et al., 2014; Ytrestøyl et al., 2020; 2022). Water quality parameters such as oxygen, ammonia, nitrogen, carbon dioxide, and temperature were maintained within accepted ranges based on previous RAS studies (Kolarevic et al., 2014). Thus, it can be concluded that water quality and temperature had no adverse physiological perturbations on the fish used in this study.

Increased salinity is commonly employed in commercial production of large smolt and post-smolts, although the specific salinity levels and timing of introduction during the production cycle may vary (Brauner and Richards, 2020). From a growth perspective, it has been found that salmon smolts and post-smolts reared at 12, 22, and 33 ppt, showed the best growth at 12 ppt (Ytresøyl et al., 2023). However, from an osmoregulatory standpoint, salinity levels above 12 ppt are necessary to maintain a true hypo-osmoregulatory capacity (Ytrestøyl et al., 2020; 2022). In this study, a salinity of 12 ppt was chosen based on growth considerations and the focus on measuring osmoregulatory capacity at the gill level. The aim of this thesis was to address the knowledge gap regarding osmoregulatory status in post-smolts at intermediate salinity levels (Brauner and Richards, 2020).

The extraction of total RNA is a critical step in obtaining gene expression results (Bustin, 2002). In this study, an RNA isolation robot in conjunction with the Fast Prep Homogenization System was used to effectively isolate RNA from a large number of samples. This automated approach not only provided high-capacity and efficient extraction of high-quality RNA but also eliminated the need for hazardous compounds. The principles behind different RNA extraction methods are beyond the scope of this thesis. The purity and integrity of RNA are crucial for downstream analysis in real-time qPCR. The A260/A280 ratio is an indicator of RNA-to-protein ratio, with a low value suggesting impurities. In this study, all RNA samples were within the recommended range, indicating acceptable purity for

subsequent cDNA synthesis. RNA integrity was assessed using electrophoresis (Agilent 2100 Bioanalyzer), which measures the integrity of RNA. The RNA Integrity Number (RIN) ranges from 1 to 10, with higher values indicating higher RNA integrity. A subset of RNA samples in this study exhibited RIN values between 8.0 and 10.0, indicating a high degree of RNA integrity, which was assumed to be representative of all samples.

The reverse transcription (RT) step, which involves the synthesis of complementary DNA (cDNA) from RNA, is a major source of variability in RT-qPCR experiments (Plaffl, 2004). To minimize variability, all reagents from the SuperScript® III kit (Invitrogen™, USA) used in this study were added to Master Mixes (MM), and the Hamilton pipetting robot was employed for accurate and reproducible dispensing. The SuperScript III kit, known for its excellent results, was chosen for cDNA synthesis. Real-time qPCR is a widely used method for measuring mRNA levels. The endogenous reference gene Ef1a was selected based on previous screenings for suitable reference genes and its stability (Olsvik et al., 2005). Non-template controls (NTC) were included in all plates to ensure no contamination. The primers used in this study were designed to span exon-exon boundaries to avoid detecting potential residual genomic DNA contamination. Primer specificity was confirmed through restriction enzyme analysis of PCR products conducted by Dr. Elsa Denker.

Western blot analysis was employed to measure the protein abundance of NKA- α1a, α1b, and α1c in this study. SDS page gel electrophoresis and blotting to cellulose membranes facilitated size separation of proteins. Polyclonal antibodies against isoform-specific sequences in salmon were used to visualize the NKA- α1a, α1b, and α1c proteins (McCormick et al., 2009; Sundh et al., 2014). Protein quantification was performed relative to the abundance of β-actin, a widely studied reference protein for western blot analysis and a structural housekeeping protein in cells (Amini et al., 2014).

4.2 INFLUENCE OF PHOTOPERIODS

In this study, salmon was produced in RAS using four different protocols: 1) continuous light during the whole period in RAS (NW), 2) using a normal winter signal (6 weeks) at size 40 g to induce smoltification (EW), 3) using a normal winter signal (6 weeks) at size 120 g to induce smoltification at a later stage (LW) or 4) using a longer winter signal (12 weeks) at size 120 g to induce smoltification (LLW). The main argument for use of continues light is that has a clear growth promoting effect (Imsland, Handeland & Stefansson., 2014), which is important in aquaculture. However, the use of a classic square light regime, a period with a

short-day length (usual regime is 12 h light:12 h dark) followed by a period of 24 h light is based on the salmon's biology in its natural environment and is necessary for stimulating smoltification, particularly endocrine changes and development of sea water tolerance. (Stefansson et al., 1991; 2007; Ebbesson et al., 2007). One important marker for smolt development has been to measure gill NKA enzyme activity in gills (McCormick, 2013). It has also been shown that enzyme activity increase in the intestine (Sundell, et al., 2003) and Kidney (Takvam et al., 2021). Indeed, two master students, Erik Hemidal and August Brage Engberg Sindre, analyzed and reported NKA activity in the gills, intestine, and kidney of all the experimental groups in this study. Figure 18 shows a modified version of the NKA activity in gills and kidney are included here to show the increase of NKA activity in all groups. Interestingly, it appears as gill NKA activity remains during experimental period and that gills display an earlier increase in NKA activity than in the kidney.

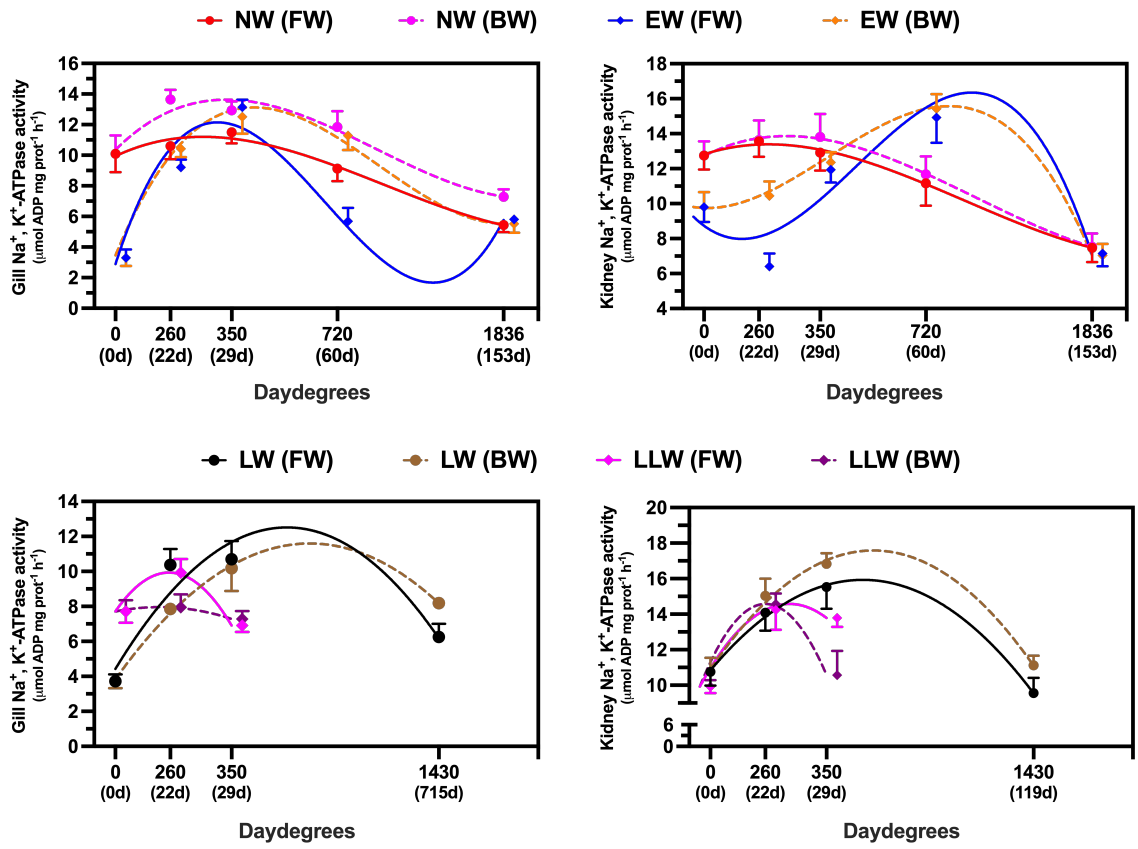

Figure 18: Modified graphs showing gill NKA activity (upper left panel) and Kidney NKA activity (upper right panel) in No Winter (NW) and Early Winter (EW) groups in both freshwater (FW) and brackish water (BW) in RAS. Gill NKA activity (lower left panel) and kidney (lower right panel) in Late Winter (LW) and Late Long Winter (LLW) groups in freshwater (FW) and brackish water (BW) in RAS.

Although the studies reporting subunit expression in gills during smoltification is increasing, nor one has looked at the mRNA expression of paralogues (NKa1a1, a1a2, NKAa1b1, a1b2 and NKAa1c1, a1c2) in the gills, nor has anyone reported expression of NKA subunits in the kidney during smoltification. Because of this, it was difficult to consistently conclude regarding differences in expression patterns of NKA paralogues at the mRNA level. Hence, in the following discussion of expression patterns as general expression trend for all paralogues will be referred to as NKa1a some places instead of discussing NKAa1a1 and a1a2 separately. The same applies for both NKAa1b and NKAa1c.

4.2 No Winter vs. Early Winter (EW)

NKAa1a and NKAa1b are the main subunit that is differentially regulated in the gills of salmonids (Richards, et al., 2003; Bystriansky et al., 2006; 2007; Nilsen et al., 2007; McCormick et al., 2013). Gill NKAa1a is shown in earlier studies to be important for hyperosmoregulation and is associated with expression in freshwater (FW) and ion uptake (McCormick et al, 2013; Khaw et al., 2021). During smolt development in the NW and EW groups in the present study, the relative mRNA abundance of NKAa1a1 and NKAa1a2 decreased and were stable at a low level after smolting. This is an expected result due to previous studies that have suggest a decrease of the overall NKAa1a subunits during smoltification (Nilsen et al., 2007; Madsen et al., 2009). NKAa1a protein abundance had a distinct decrease during smoltification in both groups, which is supported by McCormick et al., (2009; 2013). From earlier studies it is shown that during smoltification the NKAa1a subunit decrease, while the NKAa1b is increasing (Nilsen et al., 2007; Stefansson et al., 2007). In this thesis, NKAa1b was the highest expressed subunit in the gills of both NW and EW out of all analyzed, although it did not increase as expected. In fact, it decreased during smolting before increasing after. One explanation could be that expression patterns of NKAa1b in this experiment is conducted in domesticated salmon and these may thus display a different pattern than reported when using offspring from wild strains and a simulated natural and lower temperatures (Nilsen et la., 2007; Stefansson et al., 2007; McCormick et al., 2013). NKAa1b is known to be the highest expressed subunit during smoltification and is directly linked to the increase in salinity tolerance (McCormick et al., 2013). On the other hand, NKAa1b protein abundance increased during smolting. This result is also found in Christensen et al. (2018), were NKAa1a mRNA abundance decreased and NKAa1b protein increased. This could suggest that decrease of NKAa1a mRNA abundance is a secure

indicator of SW tolerance, while NKAa1b gene expression and protein may not correlate (Christensen et al., 2018). Nevertheless, the overall increase in NKAa1b mRNA and protein abundance suggest that there also may be a time lag between expression of mRNA and protein levels. It should also be noted that if mRNA levels peaks earlier than the protein and NKA enzyme activity, it's possible that this could occur at the 260-day degree timepoint. Moreover, it is not uncommon to see more variation in mRNA and protein abundance under intensive production of larger smolt and post-smolts (Takvam et al., 2023). It is interesting to note that the expression patterns of mRNA and protein in the NW group do not differ much from the EW, despite high NKA activity was sees in the NW group. Hence, a similar drop in gill NKA activity levels observed at 0dC for the EW groups do not occur at mRNA and protein levels and should be of interest for future studies. The classic increase in gill NKA activity to peak smolt levels, followed by a loss of NKA activity at 720 dC is in line with previous studies reporting loss of NKA activity levels after 500 dC (Stefansson et al., 1998; McCormick et al., 1999; Handeland et al., 2004). Our findings suggest that similar clear patterns in mRNA and protein abundance as NKA enzyme activity during smoltification and de-smotification in domesticated salmon subjected to an out of season square wave photoperiod regime is not present in our finding. This may be due to rapid transcriptional and/or translational changes, or that smolts under intensive production protocols display a more asynchronous smolt development appears to occur at the mRNA level (Khaw et al., 2021).

NKAa1c and NKa3 do not seems to play an important part in SW adaption in gills (McCormick et al., 2013) because of the low and stable levels during the whole period. This is backed in Nilsen et al. (2007) and McCormick et al. (2013) where NKAa1c and NKAa3 were expressed, but at a much lower level than NKAa1a and NKAa1b, consistent with the proposed housekeeping function of NKa1c and that NKAa3 is not as important for ionocytes as NKa1a and a1b (Nilsen et al., 2007). However, it should be noted that NKAa1c in gills of smolt that were exposed to low pH and high aluminum showed an increase in gill NKAa1c mRNA expression when NKAa1b was downregulated, suggesting not only a housekeeping function, but also a compensatory role of NKAa1c in gills if needed (Nilsen et al., 2010).

In the kidney, on the other hand, the relative mRNA abundance for all genes, except NKAa1c1 and NKAa1c2, were remarkably low in both NW and EW. Further, the expression pattern of NKAa1c mRNA in the kidney is consistent with this isoform not serving a

housekeeping function in the kidney. The NKAa1c increased in the intestine during smoltification and showed a significant upregulation after transfer to SW (Sundh et al., 2014), and the authors suggested that α1c to be the predominant isoform in SW intestines. NKAa1c is also found to be abundantly expressed in kidneys of rainbow trout (Richards, et al., 2003). It is interesting to note that a1c is the predominant isoform expressed in both kidney and intestine of salmon. Both organs often have a high basal NKA activity in both freshwater and seawater acclimated animals, which probably reflect their additional role in ensuring either nutrient uptake and/or reabsorption of ions (Takvam et al., 2023). The a1c protein abundance not changing to same extent as the mRNA levels in present study. The relatively small changes in NKAa1c mRNA and protein abundance in the kidney also reflect the smaller fold change in NKA activity in the EW than observed for the ills and intestine. It may be that the relative high enzyme activity in the kidney results in a constant and steady high expression of a1c mRNA and protein. Such an interpretation can be supported by the fact that there is no clear smolt related expression pattern of NKAa1c in the kidney. The lower expression levels of NKAa1b and a3 suggest these are not the most important NKA isoform in the kidney, with a1b being more important in the gill (McCormick et al., 2013) and the a3 is more important in muscle (Richards et al 2003).

4.2.2 Late Winter (LW) vs. Late Long Winter (LLW)

In wild Atlantic Salmon, the smoltification is triggered by short days during winter followed by increasing day lengths in spring (McCormick, 2013; Ytrestøyl et al., 2022). Both LW and LLW include a winter signal followed by continuous light, where the winter signal in LLW has double the length of LW. If the fish goes through smoltification, the expected outcome would be increased NKAa1b values and decreased NKAa1a values in the gills (Nilsen et al., 2007). The relative mRNA abundance of NKAa1a1 in gills decreased from 0 to 350 d.d. in both groups, but then increased rapidly after 350 d.d in LW group. This can indicate desmoltification in the LW group because a1a is associated with expression in FW and ion uptake (Khaw et al., 2021), and when the smolt window closes and/or the fish do not transfer to SW, the smoltification will be reversed or not happen (Stefansson et al., 1998; McCormick et al., 1999). In this case, it may well be argued that they went through smoltification, because of the decrease in NKAa1a mRNA abundance, and started desmolting afterwards. Alternatively, it could also be, as mentioned above, a result of transcriptional and translational

changes. Based on changes in gill NKA enzyme activity the smolt window is observed to be around 300-400 d.d. (Handeland et al., 2004), and an increase in NKAa1a1 is likely because of the not transfer to SW and closed smolt window. The abundance of NKAa1a2 on the other hand is decreasing during the whole experimental period in both LW and LLW, which consistent with previous studies (Nilsen et al., 2007, Stefansson et al., 2007). For the NKAa1b1 and NKAa1b2, the relative mRNA abundance increased for both groups, before LW group decreased from 350 to 1430 d.d for NKAa1b2. The increase is in line with earlier findings, as well as the two previous light regimes discussed above. The decrease could also mean the salmon is desmolting (Handleand et al., 2004)), which supports the increase of NKAa1a1, and is in alignment with the decreasing NKA activity. For NKAa1c1 there was a slight increase in the LWW group and a decrease in the LW group. The levels are still relatively low, something that is expected due to what is seen in NW and EW groups discussed above, and other studies (Nilsen et al., 2007; Nilsen et al., 2010; Stefansson et al., 2007). For NKAa1c2, there was no changes in both groups and overall low abundance for NKAa1c and NKAa3. This is something that is expected in relation to other studies (Nilsen et al., 2007).

In the kidney, the relative NKAa1a1 mRNA abundance decrease, while the NKAa1b1 and NKAa1b2 increases in the LW group, which is reminiscent of changes observed in gills. Expression levels of NKAa1a1 were very low, suggesting a minor osmoregulatory role of this subunit in kidney. The same can be postulated for the NKAa3a and NKAa3b subunits due to very low abundance in the kidney, though the a3b was the most abundant of these two subunits. Nevertheless, NKAa1c was also in these groups the most abundant of all subunit measured in the kidney, with a1c mRNA levels showing a somewhat stable expression patterns in both groups, increasing slightly up to the first seawater transfer at 350 d.d. Interestingly, Kidney NKA enzyme activity in the LLW do not differ from the LW group at 0dC as observed for the gill NKA enzyme activity where the LLW group do not display a reduced gill enzyme activity at 0dC as for all the EW and LW groups. Despite such differences in enzyme activity are not reflected by similar differences in mRNA and protein abundance of NKA subunits, it interesting to note the lack of decrease in NKA activity. This could either be due to the length of the winter signal in the LLW group, or that the fish is becoming so big that it does not need to alter NKA activity levels in response to photoperiod. It is well known that larger fish have an inherent great sweater tolerance just due their size alone (Parry et al., 1962).

4.3 INFLUENCE OF SALINITY

In this study, salmon were produced in RAS with two different salinities: 1) freshwater (0-1 ppt) or 2) brackish water (12 ppt). All four photoperiodic light regimes groups were split into FW and BW when summer signal was initiated. An earlier study suggest that the use of BW in RAS is beneficial in terms of improved growth, survival, and fish welfare (Ytrestøyl et al., 2020; 2022). As mentioned, previous studies have shown that NKAa1a is important for hyperosmoregulation and is associated with ion uptake in freshwater environments (McCormick et al., 2013). Additionally, it is expected that NKAa1b will increase in expression during smoltification in brackish water conditions (Stefansson et al., 2007; McCormick et al., 2013).

In this study, BW did not have an effect on gill NKAa1a mRNA and protein abundance in NW and EW, but it influences LW and LWW having a lower abundance than in FW. On the other hand, NKAa1b mRNA abundance had a higher level in both NW and EW in response to higher salinity, which support the finding of upregulated expression in McCormick et al. (2013). Gill NKAa1c1 increases in response to salinity in both LW and LLW, whereas it did not affect the abundance in NW and EW group. NKA activity were at an overall higher level in BW than in FW, indicating a response of higher salinity. This is also seen in the study of Vargas-Chacoff et al. (2018) where the NKA activity increased in gills of SW fish compared to FW fish.

Moving on to the kidney, there was no changes in EW in brackish water. The isoform NKAa1a increased in expression in LW and a decrease in LLW. NKAa1b showed no significant overall effect in response to BW, and if any, there was a reduction in their expression. Similarly, little to no effect of BW was observed on the NKAa1c isoform in the kidney. Additionally, the isoforms NKAa3a and NKAa3b displayed minimal changes in response to BW, indicating a limited influence of salinity on these isoforms in the kidney (McCormick et al., 2013).

5. CONCLUSION AND FURTHER PERSPECTIVES

The findings from this study reveal important insights into the expression patterns of NKA subunits during smoltification and desmoltification in domesticated salmon under different photoperiodic light regimes and salinities. The expected increase in NKAa1b mRNA abundance did not occur, possibly due to the use of domesticated salmon in intensive production settings. Nevertheless, NKAa1b protein abundance increased during

smoltification, indicating a time lag between mRNA and protein levels. These findings suggest that a decrease in NKAa1a mRNA abundance may be a reliable indicator of seawater tolerance, while NKAa1b gene expression and protein levels may not directly correlate. Secondly, NKAa1c and NKAa3 do not appear to play a crucial role in gill ion regulation and seawater adaptation. Their expression levels remained low and stable throughout the study, indicating a lesser importance compared to NKAa1a and NKAa1b. However, NKAa1c may have a compensatory role in the gills under specific conditions, such as exposure to low pH and high aluminum.

In the kidney, NKAa1c was the most abundant isoform, suggesting its role in ion reabsorption. NKAa1a and NKAa1b also showed some expression in the kidney, but at lower levels. The changes in mRNA abundance and protein levels of NKA subunits in the kidney were relatively small compared to the gills, reflecting a smaller fold change in NKA activity. Regarding the influence of salinity, NKAa1b mRNA abundance in gills increased with higher salinity, supporting its role in salinity adaptation. In the kidney, NKAa1a mRNA expression increased in LW response to brackish water, while NKAa1b showed no significant changes. NKAa1c and NKAa3 isoforms had minimal responses to salinity in the kidney.

Based on these findings, it is important to consider the expression patterns of NKA subunits, particularly NKAa1a and NKAa1b, in future smolt production. Monitoring the decrease in NKAa1a mRNA abundance may serve as an indicator of smolt readiness and seawater tolerance. Additionally, the increase in NKAa1b mRNA and protein abundance is associated with increased salinity tolerance. The use of brackish water in recirculating aquaculture systems (RAS) may have beneficial effects on growth, survival, and fish welfare, as suggested by previous studies. However, further research is needed to fully understand the complex mechanisms underlying NKA expression and ion regulation in different environmental conditions.

Based on the NKA expression data, it is difficult to determine if any specific group is better suited for smolt production based solely on NKAa expression. Further studies considering other factors, such as growth performance, survival rates, and welfare indicators, would be necessary to make a comprehensive assessment of the suitability of different groups for smolt production.

Objective 1: Gene expression patterns of NKAα isoforms during smoltification

The relative mRNA abundance of NKAa1a and NKAa1a2 decreased during smoltification, while NKAa1b showed the highest expression levels in the gills. These findings suggest that NKAa1a may play a role in freshwater adaptation and ion uptake, while NKAa1b is associated with increased salinity tolerance. However, it should be noted that the expression patterns observed in domesticated salmon under intensive production conditions may differ from those in wild strains. Further research is needed to understand the precise roles of NKAa isoforms during smoltification.

Objective 2: Protein expression patterns of NKAα isoforms during smoltification

distinct decrease in NKAa1a protein abundance, consistent with previous studies. Surprisingly, NKAa1b protein abundance increased during smoltification, contrary to the expected decrease based on mRNA levels. This discrepancy suggests a time lag between mRNA expression and protein synthesis. Additionally, the overall increase in NKAa1b protein abundance indicates its importance for salinity tolerance. These findings highlight the complexity of NKA regulation during smoltification and emphasize the need for further investigation into the relationship between mRNA and protein levels in the context of smolt development.

Objective 3: How different light regimes effects smoltification

The overall expression patterns of NKA subunits in the kidney were not significantly affected by the different photoperiodic light regimes. This study aimed to understand how photoperiod influences the process. It found that the expression patterns of NKAa isoforms in the gills did not differ significantly between different light regimes, despite variations in NKA enzyme activity. Future studies should explore the specific mechanisms by which photoperiod influences smoltification.

Objective 4: How different salinity effects smoltification

This study observed that brackish water (BW) had distinct influences on NKAa isoform expression in the gills. NKAa1a mRNA and protein abundance were lower in BW compared to freshwater (FW) in the LLW group, while NKAa1b mRNA abundance increased in response to higher salinity. These findings support the notion that NKAa isoforms play important roles in ion regulation and salinity adaptation during smoltification. Additionally,

the kidney showed minimal changes in NKA isoform expression in response to salinity during these light regimes, suggesting a more limited influence of salinity on the kidney compared to the gills. Further research is necessary to elucidate the specific mechanisms by which different salinity levels affect smoltification**.**

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APPENDIX

APPENDIX 1. GENE EXPRESSION INFORMATION INCLUDING PRIMER DESIGN, RELATIVE MRNA ABUNDANCE.

APPENDIX 1A: PRIMER DESIGN

Table 1: *Overview of all forward (F) and reverse (R) primers designed for NKA subunits in the Atlantic salmon. The table includes gene name, primers set, sequence (5` to 3` direction), Amplicon size (nucleotides), position (exon (ex)), primer length (nucleotides), GC content (%), melting temperature (Tm; basic, salt adjusted, nearest neighbor).*

Gene	Primer name	Sequence (5´- 3´)	Position	Primer length	GC %	Oligo calc			Amplicon size
						Tm basic	Tm salt adjusted	Tm nearest neighbor	
NKAα1a1	**NKAα1a1 F2**	CGCCGCTGTCGT GATTGCTC	Ex 18	20	65	57,9	64,6	59,98	276
	NKAα1a1 R2	CCTGGGCTCCGT CGCATG	Ex 20	18	72	57,2	62,9	57,19	
NKAα1a2	**NKAα1a2 F1**	AGACGGATATGG ATGACCTG	Ex 2	20	50	51,8	58,4	50,99	143
	NKAα1a2 R1	GCCATCGCGAAG AAGAAG	Ex 4	18	56	50,3	56,3	52,4	
NKAα1b1	**NKAα1b1F1**	GTTGACCTTGGA TGAACTTCATC	Ex 3	23	43	53,5	60,9	51,93	190
	NKAα1b1+2 R1	GCACCAATCCAT AGGAGCATAG	Ex 4	22	50	54,8	62,1	54,27	
NKAα1b2	**NKAα1b2 F1**	GTTGACCTTGGA TGAACTTCATAG	Ex 3	24	42	54	62	52,85	190
	NKAα1b1+2 R1 (see above)		Ex 4						
NKAα1c1	**NKAα1c1 F1**	ACCTGGTCCCAC AGCAAG	Ex 5/6	18	61	52,6	58,4	51,59	135
	NKAα1c1 R2	CAGAGATGATTC GCAAATCAG	Ex 6	21	43	50,5	57,5	52,21	
NKAα1c2	**NKAα1c1 F1** (see above)		Ex 5/6						220
	NKAα1c2 R1	GGGTTGTCATTG GAGTAGTCC	Ex 7	212	52	54,4	61,2	50,94	
NKAα3a	**NKAα3a F1**	CTGCCAGCGACT GGGTGCC	Ex 14/15	19	74	59,7	66,1	59,14	186
	NKAα3a R1	GACCCTCTTCCA CTCCGGTGAC	Ex 15	22	64	60,4	67,9	56,89	
NKAα3b	**NKAα3b F1**	TGCCAGCGACTG GGTGCC	Ex 14/15	18	72	57,2	62,9	57,35	185
	NKAα3b R1	CGACCCTCTTCT ACTCCAGTAAC	Ex 15	23	52	57,1	64,6	55,08	

APPENDIX 1B. STATISTICAL ANALYSIS RELATIVE MRNA ABUNDANCE OF NKAα ISOFORMS

NKAα1a1:

Table 2: *NKAα1a1 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	3,9991	0,0079	2,8490	2,2572
	LLW (FW)	0,9092	0,0101	1,0343	0,2973
	LW (FW)	1,1401	0,0067	0,0896	0,4479
0	NW (FW)	3,9586	0,0070	21,4004	1,7945
	EW (BW)	0,2094	0,0490	1,8880	0,1069
	EW (FW)	0,2048	0,0026	0,7307	0,1140
	LLW (BW)	0,4541	0,0289	4,3184	0,2589
	LLW (FW)	0,9946	0,0500	2,3883	0,5491
	LW (BW)	0,1094	0,0158	2,7717	0,0500
	LW (FW)	0,7526	0,0065	4,9050	0,2826
	NW (BW)	5,9663	0,0000	3,9991	5,6454
350	NW (FW)	0,5567	0,0155	0,4698	0,4465
	EW (BW)	0,0306	0,0672	3,5527	0,0089
	EW (FW)	1,3418	0,0150	5,6717	0,5837
	NW (BW)	0,2461	0,0321	2,5648	0,0987
720	NW (FW)	0,4843	0,0445	2,3233	0,2064
	LW (BW)	0,6705	0,0035	45,4692	0,5554
1420	LW (FW)	1,1143	0,0506	0,9215	0,7732
	EW (BW)	0,5521	0,0777	13,6196	0,3426
	EW (FW)	0,7583	0,0520	1,2309	0,2456
	NW (BW)	0,4233	0,0069	3,2253	0,2231
1800	NW (FW)	0,5308	0,0617	1,8535	0,1792

Table 3: *NKAα1a1 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	3,9991	0,0003	0,0028	2,2572
	LLW (FW)	0,9092	0,0007	0,0075	0,2973
	LW (FW)	1,1401	0,0006	0,0044	0,4479
0	NW (FW)	3,9586	0,0002	0,0028	1,7945
	EW (BW)	0,2094	0,0004	0,0045	0,1069
	EW (FW)	0,2048	0,0005	0,0027	0,1140
	LLW (BW)	0,4541	0,0003	0,0054	0,2589
	LLW (FW)	0,9946	0,0005	0,0056	0,5491
	LW (BW)	0,1094	0,0005	0,0101	0,0500
	LW (FW)	0,7526	0,0005	0,0021	0,2826
	NW (BW)	5,9663	0,0004	0,0052	5,6454
350	NW (FW)	0,5567	0,0003	0,0081	0,4465
	EW (BW)	0,0306	0,0007	0,0016	0,0089
	EW (FW)	1,3418	0,0006	0,0031	0,5837
	NW (BW)	0,2461	0,0005	0,0052	0,0987
720	NW (FW)	0,4843	0,0006	0,0091	0,2064
	LW (BW)	0,6705	0,0005	0,0053	0,5554
1420	LW (FW)	1,1143	0,0003	0,0026	0,7732
	EW (BW)	0,5521	0,0004	0,0242	0,3426
	EW (FW)	0,7583	0,0005	0,0051	0,2456
	NW (BW)	0,4233	0,0002	0,0121	0,2231
1800	NW (FW)	0,5308	0,0004	0,0358	0,1792

NKAα1a2:

Table 4: *NKAα1a2 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,8951	0,0025	0,2595	0,2797
	LLW (FW)	0,1832	0,0056	0,4402	0,0414
	LW (FW)	0,3401	0,0009	0,0203	0,1051
0	NW (FW)	0,7560	0,0019	2,0636	0,2249
	EW (BW)	0,0802	0,0115	0,2052	0,0474
	EW (FW)	0,1157	0,0006	0,3549	0,0581
	LLW (BW)	0,0841	0,0032	0,7281	0,0181
	LLW (FW)	0,0912	0,0198	0,1567	0,0536
	LW (BW)	0,0348	0,0833	0,4140	0,0185
	LW (FW)	0,2069	0,0009	0,4946	0,0638
	NW (BW)	0,1699	3,557E-09	0,5485	0,0998
350	NW (FW)	0,0609	0,0021	0,1687	0,0392
	EW (BW)	0,0062	0,0089	0,8714	0,0024
	EW (FW)	0,2151	0,0000	0,4332	0,0850
	NW (BW)	0,0487	0,0086	0,6148	0,0200
720	NW (FW)	0,1608	0,0063	0,9081	0,0683
	LW (BW)	0,1120	0,0007	0,8437	0,0732
1420	LW (FW)	0,1524	0,0054	0,2031	0,0583
	EW (BW)	0,0944	0,0166	1,8745	0,0321
	EW (FW)	0,0946	0,0031	0,4367	0,0223
	NW (BW)	0,1834	0,0008	0,2798	0,0875
1800	NW (FW)	0,1050	0,0177	0,6004	0,0584

NKAα1b1:

Table 5: *NKAα1b1 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	4,1918	0,3581	9,4724	0,9691
	LLW (FW)	3,6120	0,2961	8,3574	0,4203
	LW (FW)	2,9399	0,1636	8,8762	0,6754
0	NW (FW)	5,0400	0,6059	10,3082	1,3662
	EW (BW)	4,2818	0,7381	10,0561	0,9872
	EW (FW)	2,6179	0,1255	6,8189	0,9805
	LLW (BW)	4,6395	0,1648	11,8172	0,9576
	LLW (FW)	4,6348	1,1929	10,5362	1,6075
	LW (BW)	2,9435	0,9236	5,4823	0,6379
	LW (FW)	3,8929	0,2052	15,1674	1,0080
	NW (BW)	3,4089	0,0000	7,2596	1,0324
350	NW (FW)	1,5365	0,5027	6,6771	0,6761
	EW (BW)	3,6239	0,3354	5,7791	1,2754
	EW (FW)	3,9298	0,1440	5,5169	1,3815
	NW (BW)	3,6481	0,2844	10,4239	0,5189
720	NW (FW)	2,8871	0,3652	11,7647	1,1976
	LW (BW)	3,4667	0,4587	7,4288	1,1939
1420	LW (FW)	1,7742	2,0845	7,1435	0,7860
	EW (BW)	4,2157	0,1292	10,5929	1,2249
	EW (FW)	3,5029	0,1475	8,1496	1,0007
	NW (BW)	3,8765	0,0946	4,8920	1,3231
1800	NW (FW)	2,5818	0,1485	9,3198	1,0923

Table 6: *NKAα1b1 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,1917	0,0447	0,3448	0,0364
	LLW (FW)	0,3289	0,1528	0,6563	0,0531
	LW (FW)	0,3057	0,0867	0,6087	0,0591
0	NW (FW)	0,2800	0,1093	0,6652	0,0540
	EW (BW)	0,2908	0,0551	0,4358	0,0439
	EW (FW)	0,2410	0,0925	0,3850	0,0467
	LLW (BW)	0,1755	0,0450	0,5349	0,0500
	LLW (FW)	0,2027	0,0406	0,3860	0,0400
	LW (BW)	0,3486	0,0184	0,6640	0,0711
	LW (FW)	0,2858	0,0478	0,6446	0,0668
	NW (BW)	0,2827	0,0380	0,5610	0,0716
350	NW (FW)	0,2149	0,1022	0,3569	0,0366
	EW (BW)	0,1693	0,0419	0,5221	0,0580
	EW (FW)	0,2900	0,0527	0,8427	0,0899
	NW (BW)	0,3030	0,0970	1,1569	0,1135
720	NW (FW)	*0,2345*	0,0820	0,4030	0,0386
	LW (BW)	0,2404	0,0727	0,5189	0,0630
1420	LW (FW)	0,1509	0,0390	0,4003	0,0455
	EW (BW)	0,1087	0,0092	0,2479	0,0308
	EW (FW)	0,1539	0,0673	0,2427	0,0199
	NW (BW)	0,1871	0,0231	0,4235	0,0457
1800	NW (FW)	0,1946	0,0898	0,6123	0,0706

NKAα1b2:

Table 7: *NKAα1b2 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	2,1231	0,5742	2,1483	0,2539
	LLW (FW)	1,5578	0,2806	6,5817	0,2207
	LW (FW)	1,0631	0,2381	6,3096	0,1756
0	NW (FW)	1,7652	0,8299	3,1906	0,4155
	EW (BW)	2,4223	1,0329	4,3435	0,6631
	EW (FW)	1,8321	0,2643	4,4255	0,6012
	LLW (BW)	2,4175	0,1938	5,7665	0,6010
	LLW (FW)	3,4258	0,8566	6,4983	1,2589
	LW (BW)	1,2687	0,7729	2,7293	0,2544
	LW (FW)	2,0459	0,2417	10,7330	0,5843
	NW (BW)	1,8399	0,0000	6,2694	0,5352
350	NW (FW)	1,2085	0,5698	2,8958	0,4469
	EW (BW)	2,0158	0,4401	1,7128	0,6706
	EW (FW)	2,8295	0,2184	2,8527	0,6855
	NW (BW)	2,8661	0,4094	5,3034	1,0500
720	NW (FW)	1,1718	0,3873	5,8853	0,3950
	LW (BW)	1,6844	0,3295	4,7042	0,8003
1420	LW (FW)	1,0428	0,9842	10,9296	0,3967
	EW (BW)	1,1553	0,1971	3,6094	0,2240
	EW (FW)	2,1141	0,1335	3,0569	0,3295
	NW (BW)	2,0073	0,1478	3,2244	0,5350
1800	NW (FW)	1,1983	0,3015	3,6723	0,4316

Table 8: *NKAα1b2 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,0643	0,0101	0,1238	0,0148
	LLW (FW)	0,0877	0,0255	0,2270	0,0214
	LW (FW)	0,0896	0,0146	0,1903	0,0225
0	NW (FW)	0,1039	0,0423	0,2270	0,0230
	EW (BW)	0,0751	0,0066	0,1425	0,0152
	EW (FW)	0,1061	0,0188	0,3510	0,0510
	LLW (BW)	0,0717	0,0059	0,1302	0,0159
	LLW (FW)	0,0376	0,0126	0,0776	0,0066
	LW (BW)	0,0599	0,0089	0,1638	0,0158
	LW (FW)	0,0898	0,0149	0,2255	0,0230
	NW (BW)	0,0714	0,0218	0,2120	0,0215
350	NW (FW)	0,1388	0,0485	0,3270	0,0373
	EW (BW)	0,0507	0,0130	0,1239	0,0123
	EW (FW)	0,0614	0,0227	0,1507	0,0144
	NW (BW)	0,0814	0,0183	0,1738	0,0178
720	NW (FW)	0,0772	0,0238	0,1518	0,0184
	LW (BW)	0,0351	0,0178	0,0724	0,0072
1420	LW (FW)	0,0695	0,0217	0,1501	0,0191
	EW (BW)	0,0263	0,0030	0,0780	0,0088
	EW (FW)	0,0754	0,0133	0,2190	0,0215
	NW (BW)	0,0501	0,0031	0,1033	0,0101
1800	NW (FW)	0,0954	0,0267	0,1623	0,0197

NKAα1c1:

Table 9: *NKAα1c1 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	1,2621	0,0263	0,6356	0,5071
	LLW (FW)	0,2669	0,0651	0,2910	0,0613
	LW (FW)	0,4947	0,0216	0,2373	0,1359
0	NW (FW)	0,8687	0,0713	4,9002	0,3731
	EW (BW)	0,1711	0,0702	0,8173	0,0243
	EW (FW)	0,1607	0,0590	0,3617	0,0450
	LLW (BW)	0,3090	0,0595	0,4715	0,0858
	LLW (FW)	0,4621	0,0480	0,8778	0,2093
	LW (BW)	0,1495	0,0912	0,6634	0,0331
	LW (FW)	0,3146	0,0253	1,9440	0,1303
	NW (BW)	0,1438	0,0000	1,3919	0,0317
350	NW (FW)	0,2053	0,0399	0,3661	0,0523
	EW (BW)	0,1030	0,0697	1,0537	0,0266
	EW (FW)	0,2810	0,0434	0,8229	0,0539
	NW (BW)	0,2495	0,0636	1,2687	0,0815
720	NW (FW)	0,3442	0,0197	1,0084	0,0938
	LW (BW)	0,3450	0,0409	0,2862	0,1799
1420	LW (FW)	0,2354	0,0643	0,8085	0,1045
	EW (BW)	0,2013	0,0435	3,6757	0,0796
	EW (FW)	0,3466	0,0685	0,9731	0,0755
	NW (BW)	0,3026	0,0665	0,4773	0,1097
1800	NW (FW)	0,2895	0,0446	0,8431	0,1209

Table 10: *NKAα1c1 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,1555	0,0865	0,2673	0,0215
	LLW (FW)	0,4987	0,1527	0,8171	0,0831
	LW (FW)	0,4403	0,1415	1,0854	0,1100
0	NW (FW)	0,2577	0,1161	0,5992	0,0501
	EW (BW)	0,6987	0,0858	2,1010	0,1964
	EW (FW)	0,7101	0,2047	1,8809	0,2739
	LLW (BW)	0,5419	0,0537	0,9835	0,1280
	LLW (FW)	0,2800	0,1336	0,5212	0,0431
	LW (BW)	0,4460	0,1714	0,9306	0,0744
	LW (FW)	0,3783	0,0830	0,7516	0,0771
	NW (BW)	0,6909	0,4340	1,4079	0,1105
350	NW (FW)	0,7267	0,3196	1,6652	0,1696
	EW (BW)	0,4706	0,1916	1,0340	0,0935
	EW (FW)	0,4344	0,1947	0,7099	0,0666
	NW (BW)	0,5222	0,1125	0,9095	0,1084
720	NW (FW)	0,3267	0,1653	0,7456	0,0725
	LW (BW)	0,2740	0,1433	0,6746	0,0691
1420	LW (FW)	0,3375	0,1073	0,9034	0,1026
	EW (BW)	0,2785	0,1515	0,6705	0,0612
	EW (FW)	0,4570	0,1185	1,3938	0,1289
	NW (BW)	0,3729	0,0572	0,8175	0,0854
1800	NW (FW)	0,5312	0,1805	0,8310	0,0934

NKAα1c2:

Table 11: *NKAα1c2 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,5246	0,0395	0,9057	0,1045
	LLW (FW)	0,2944	0,0393	0,8023	0,0365
	LW (FW)	0,2455	0,0215	0,5396	0,0606
0	NW (FW)	0,4182	0,0924	1,0565	0,1179
	EW (BW)	0,3593	0,0422	0,9464	0,0724
	EW (FW)	0,2928	0,0297	0,6467	0,1082
	LLW (BW)	0,5751	0,0131	0,6940	0,1194
	LLW (FW)	0,4706	0,0488	1,2229	0,1295
	LW (BW)	0,2803	0,0795	0,4311	0,0681
	LW (FW)	0,2525	0,0234	1,0650	0,0529
	NW (BW)	0,3411	0,0000	1,1055	0,0864
350	NW (FW)	0,1426	0,0392	0,6639	0,0478
	EW (BW)	0,2684	0,0567	0,5683	0,0704
	EW (FW)	0,3969	0,0228	0,3963	0,0814
	NW (BW)	0,3276	0,0843	0,5379	0,0326
720	NW (FW)	0,4196	0,0450	0,9067	0,1591
	LW (BW)	0,4563	0,0449	0,7281	0,1740
1420	LW (FW)	0,1601	0,1834	0,4492	0,0587
	EW (BW)	0,4199	0,0156	0,9793	0,1178
	EW (FW)	0,3892	0,0420	0,4985	0,0894
	NW (BW)	0,4528	0,0247	0,3262	0,0912
1800	NW (FW)	0,2243	0,0261	1,3707	0,0743

Table 12: *NKAα1c2 mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,0999	0,0185	0,3422	0,0334
	LLW (FW)	0,2563	0,0520	0,7625	0,0810
	LW (FW)	0,1508	0,0434	0,3303	0,0386
0	NW (FW)	0,1592	0,0512	0,3802	0,0334
	EW (BW)	0,5354	0,0755	1,1808	0,1593
	EW (FW)	0,6590	0,1170	2,1880	0,3230
	LLW (BW)	0,2743	0,0641	0,7367	0,0743
	LLW (FW)	0,2700	0,0349	0,9160	0,1035
	LW (BW)	0,2983	0,0294	0,7103	0,0779
	LW (FW)	0,2425	0,0450	0,6364	0,0760
	NW (BW)	0,2279	0,0833	0,4929	0,0457
350	NW (FW)	0,5479	0,0711	1,0649	0,1406
	EW (BW)	0,2859	0,0760	0,5916	0,0665
	EW (FW)	0,2480	0,0966	0,3696	0,0403
	NW (BW)	0,2640	0,0459	0,5434	0,0752
720	NW (FW)	0,2843	0,0548	0,6033	0,0749
	LW (BW)	0,1977	0,0411	0,4388	0,0626
1420	LW (FW)	0,2698	0,0607	0,6615	0,0773
	EW (BW)	0,1151	0,0453	0,2375	0,0234
	EW (FW)	0,3748	0,0278	1,3455	0,1333
	NW (BW)	0,3048	0,0370	0,7971	0,0851
1800	NW (FW)	0,2749	0,0262	0,4918	0,0587

NKAα3a:

Table 13: *NKAα3a mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,9233	0,1116	1,3087	0,1606
	LLW (FW)	1,0201	0,2002	2,9640	0,2128
	LW (FW)	0,6995	0,0948	1,2308	0,1652
0	NW (FW)	0,5584	0,1209	1,6231	0,1429
	EW (BW)	1,1945	0,0614	1,7641	0,3250
	EW (FW)	1,0189	0,1447	1,7824	0,2638
	LLW (BW)	1,4863	0,0901	3,1079	0,2485
	LLW (FW)	1,2369	0,0829	2,3346	0,3698
	LW (BW)	0,7849	0,2102	2,2922	0,1599
	LW (FW)	1,1124	0,0515	3,2158	0,2431
	NW (BW)	0,8968	0,0000	2,2522	0,2160
350	NW (FW)	0,5752	0,1491	1,4785	0,2665
	EW (BW)	0,6652	0,1408	1,3942	0,1449
	EW (FW)	1,2945	0,0695	1,8631	0,3390
	NW (BW)	1,1026	0,1534	2,1983	0,1532
720	NW (FW)	1,0631	0,1267	2,1049	0,4161
	LW (BW)	1,1218	0,1395	1,6133	0,3387
1420	LW (FW)	0,6334	0,3497	1,6806	0,2763
	EW (BW)	0,8998	0,0399	1,2779	0,1741
	EW (FW)	0,7697	0,0835	1,7850	0,1666
	NW (BW)	1,1398	0,0622	1,7530	0,2192
1800	NW (FW)	0,8124	0,0430	3,3395	0,2689

Table 14: *NKAα3a mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,0014	0,0006	0,0040	0,0004
	LLW (FW)	0,0025	0,0004	0,0107	0,0011
	LW (FW)	0,0015	0,0002	0,0035	0,0004
0	NW (FW)	0,0018	0,0003	0,0050	0,0005
	EW (BW)	0,0027	0,0004	0,0058	0,0006
	EW (FW)	0,0030	0,0003	0,0103	0,0015
	LLW (BW)	0,0017	0,0002	0,0046	0,0005
	LLW (FW)	0,0020	0,0004	0,0049	0,0005
	LW (BW)	0,0021	0,0003	0,0050	0,0005
	LW (FW)	0,0016	0,0003	0,0055	0,0005
	NW (BW)	0,0022	0,0007	0,0073	0,0008
350	NW (FW)	0,0030	0,0010	0,0052	0,0007
	EW (BW)	0,0022	0,0002	0,0059	0,0006
	EW (FW)	0,0019	0,0012	0,0032	0,0002
	NW (BW)	0,0019	0,0003	0,0071	0,0007
720	NW (FW)	0,0018	0,0003	0,0046	0,0005
	LW (BW)	0,0016	0,0004	0,0045	0,0005
1420	LW (FW)	0,0038	0,0019	0,0114	0,0013
	EW (BW)	0,0027	0,0005	0,0068	0,0007
	EW (FW)	0,0035	0,0002	0,0077	0,0008
	NW (BW)	0,0024	0,0002	0,0062	0,0006
1800	NW (FW)	0,0023	0,0010	0,0043	0,0005

NKAα3b:

Table 15: *NKAα3b mRNA abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,2587	0,0690	0,7239	0,0540
	LLW (FW)	0,3271	0,0563	0,5877	0,0644
	LW (FW)	0,1779	0,0671	0,9025	0,0361
0	NW (FW)	0,2103	0,0443	0,5705	0,0450
	EW (BW)	0,3283	0,0604	0,7442	0,0592
	EW (FW)	0,2809	0,0498	0,4906	0,0768
	LLW (BW)	0,3570	0,0273	0,7803	0,0565
	LLW (FW)	0,3472	0,0577	0,5531	0,0826
	LW (BW)	0,2679	0,1212	0,7519	0,0505
	LW (FW)	0,4204	0,0407	0,6577	0,0737
	NW (BW)	0,2568	2,83E-09	0,5756	0,0480
350	NW (FW)	0,1817	0,0544	0,4763	0,0864
	EW (BW)	0,3548	0,0574	0,3695	0,0966
	EW (FW)	0,3858	0,0484	0,5987	0,0803
	NW (BW)	0,2938	0,0914	0,7328	0,0414
720	NW (FW)	0,4173	0,0619	0,4281	0,1468
	LW (BW)	0,3095	0,0607	0,4834	0,0845
1420	LW (FW)	0,2461	0,1357	0,5806	0,0937
	EW (BW)	0,3387	0,0187	0,4486	0,0878
	EW (FW)	0,2882	0,0295	0,7383	0,0726
	NW (BW)	0,2644	0,0236	0,6665	0,0316
1800	NW (FW)	0,2522	0,0170	1,2412	0,0930

Table 16: *NKAα3b mRNA abundance, mean, minimum (min) maximum (max) values and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	0,0456	0,0026	0,0808	0,0085
	LLW (FW)	0,0612	0,0342	0,0987	0,0092
	LW (FW)	0,0420	0,0235	0,0522	0,0031
0	NW (FW)	0,0511	0,0104	0,0984	0,0092
	EW (BW)	0,0425	0,0049	0,0764	0,0072
	EW (FW)	0,0481	0,0040	0,1054	0,0153
	LLW (BW)	0,0554	0,0170	0,1294	0,0119
	LLW (FW)	0,0452	0,0104	0,1136	0,0107
	LW (BW)	0,0552	0,0251	0,1060	0,0094
	LW (FW)	0,0413	0,0057	0,0976	0,0093
	NW (BW)	0,0358	0,0212	0,0526	0,0043
350	NW (FW)	0,0441	0,0210	0,0884	0,0085
	EW (BW)	0,0495	0,0071	0,1198	0,0139
	EW (FW)	0,0375	0,0040	0,0868	0,0084
	NW (BW)	0,0558	0,0014	0,2058	0,0201
720	NW (FW)	0,0448	0,0217	0,0723	0,0063
	LW (BW)	0,0611	0,0091	0,0971	0,0143
1420	LW (FW)	0,0656	0,0188	0,1763	0,0195
	EW (BW)	0,0565	0,0189	0,1270	0,0125
	EW (FW)	0,0636	0,0274	0,1477	0,0117
	NW (BW)	0,0692	0,0245	0,1799	0,0159
1800	NW (FW)	0,0555	0,0228	0,0767	0,0086

APPENDIX 2B. TWO-WAY ANOVA (TUKEY'S HSD POST-HOC TEST) - GENE EXPRESSION

NKAα1a1:

Table 17: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1a1 in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,6814
	EW (FW) - LW (FW)	0,7295
	EW (FW) - NW (FW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	0,8554
	LW (FW) - NW (FW)	0,8911
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	1,0000
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	1,0000
	EW (BW) - LW (FW)	1,0000
	EW (BW) - NW (BW)	0,1920
	EW (BW) - NW (FW)	0,6900
	EW (FW) - LLW (BW)	1,0000
	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	0,9999
	EW (FW) - NW (BW)	0,2461
	EW (FW) - NW (FW)	0,7204
	LLW (BW) - LLW (FW)	1,0000
	LLW (BW) - LW (BW)	1,0000
	LLW (BW) - LW (FW)	1,0000
	LLW (BW) - NW (BW)	0,1939
	LLW (BW) - NW (FW)	0,6929
	LLW (FW) - LW (BW)	0,9998
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	0,3293
	LLW (FW) - NW (FW)	0,8478
	LW (BW) - LW (FW)	0,9997
	LW (BW) - NW (BW)	0,1361
	LW (BW) - NW (FW)	0,5871
	LW (FW) - NW (BW)	0,3597
	LW (FW) - NW (FW)	0,8710
	NW (BW) - NW (FW)	0,9926
720	EW (BW) - EW (FW)	0,9985
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	0,9994
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000
1430	LW (BW) - LW (FW)	0,9907
1836	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	1,0000
	LW (FW) - NW (FW)	1,0000
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	0,5579
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	1,0000
	EW (BW) - LW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,3248
	EW (FW) - LLW (BW)	0,5173
	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,2970
	LLW (BW) - LLW (FW)	0,6413
	LLW (BW) - LW (BW)	0,6667
	LLW (BW) - LW (FW)	0,4990
	LLW (BW) - NW (BW)	0,6014
	LLW (BW) - NW (FW)	1,0000
	LLW (FW) - LW (BW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	1,0000
	LLW (FW) - NW (FW)	0,4048
	LW (BW) - LW (FW)	1,0000
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	0,4293
	LW (FW) - NW (BW)	1,0000
	LW (FW) - NW (FW)	0,2826
	NW (BW) - NW (FW)	0,3681
720	EW (BW) - EW (FW)	0,9985
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	0,9994
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000
1430	LW (BW) - LW (FW)	1,0000
1836	EW (BW) - EW (FW)	0,9999
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,9999
	NW (BW) - NW (FW)	1,0000

NKAα1a2:

Table 18: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1a2 in gills of in Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	<,0001
	EW (FW) - LW (FW)	<,0001
	EW (FW) - NW (FW)	0,3424
	LLW (FW) - LW (FW)	0,8791
	LLW (FW) - NW (FW)	0,0008
	LW (FW) - NW (FW)	0,0737
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	1,0000
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	0,9998
	EW (BW) - LW (FW)	0,8273
	EW (BW) - NW (BW)	0,9997
	EW (BW) - NW (FW)	1,0000
	EW (FW) - LLW (BW)	1,0000
	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (BW)	0,9999
	EW (FW) - LW (FW)	0,8172
	EW (FW) - NW (BW)	0,9994
	EW (FW) - NW (FW)	1,0000
	LLW (BW) - LLW (FW)	1,0000
	LLW (BW) - LW (BW)	0,9996
	LLW (BW) - LW (FW)	0,8491
	LLW (BW) - NW (BW)	0,9998
	LLW (BW) - NW (FW)	1,0000
	LLW (FW) - LW (BW)	0,9996
	LLW (FW) - LW (FW)	0,8524
	LLW (FW) - NW (BW)	0,9998
	LLW (FW) - NW (FW)	1,0000
	LW (BW) - LW (FW)	0,5339
	LW (BW) - NW (BW)	0,9803
	LW (BW) - NW (FW)	0,9998
	LW (FW) - NW (BW)	0,9835
	LW (FW) - NW (FW)	0,8488
	NW (BW) - NW (FW)	0,9997
720	EW (BW) - EW (FW)	0,7694
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9129
	EW (FW) - NW (BW)	0,8723
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	0,9650
1430	LW (BW) - LW (FW)	1,0000
1836	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	0,9968
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	0,9971
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	0,9981

NKAα1b1:

Table 19: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1b1 in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value	Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9999	0	EW (FW) - LLW (FW)	0,6888
	EW (FW) - LW (FW)	0,9717		EW (FW) - LW (FW)	0,7676
	EW (FW) - NW (FW)	0,7981		EW (FW) - NW (FW)	0,9035
	LLW (FW) - LW (FW)	0,9987		LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	0,5388		LLW (FW) - NW (FW)	1,0000
	LW (FW) - NW (FW)	0,1625		LW (FW) - NW (FW)	1,0000
350	EW (BW) - EW (FW)	0,8530	350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	1,0000		EW (BW) - LLW (BW)	0,9134
	EW (BW) - LLW (FW)	1,0000		EW (BW) - LLW (FW)	0,9497
	EW (BW) - LW (BW)	0,9705		EW (BW) - LW (BW)	0,9723
	EW (BW) - LW (FW)	1,0000		EW (BW) - LW (FW)	1,0000
	EW (BW) - NW (BW)	0,9987		EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,5985		EW (BW) - NW (FW)	0,9993
	EW (FW) - LLW (BW)	0,7244		EW (FW) - LLW (BW)	0,8801
	EW (FW) - LLW (FW)	0,8451		EW (FW) - LLW (FW)	0,9244
	EW (FW) - LW (BW)	0,9998		EW (FW) - LW (BW)	0,9887
	EW (FW) - LW (FW)	0,9290		EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (BW)	0,9932		EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000		EW (FW) - NW (FW)	0,9979
	LLW (BW) - LLW (FW)	1,0000		LLW (BW) - LLW (FW)	1,0000
	LLW (BW) - LW (BW)	0,9058		LLW (BW) - LW (BW)	0,3508
	LLW (BW) - LW (FW)	0,9997		LLW (BW) - LW (FW)	0,8528
	LLW (BW) - NW (BW)	0,9881		LLW (BW) - NW (BW)	0,9401
	LLW (BW) - NW (FW)	0,4431		LLW (BW) - NW (FW)	0,9973
	LLW (FW) - LW (BW)	0,9676		LLW (FW) - LW (BW)	0,4128
	LLW (FW) - LW (FW)	1,0000		LLW (FW) - LW (FW)	0,9029
	LLW (FW) - NW (BW)	0,9984		LLW (FW) - NW (BW)	0,9677
	LLW (FW) - NW (FW)	0,5867		LLW (FW) - NW (FW)	0,9994
	LW (BW) - LW (FW)	0,9924		LW (BW) - LW (FW)	0,9928
	LW (BW) - NW (BW)	0,9999		LW (BW) - NW (BW)	0,9671
	LW (BW) - NW (FW)	0,9907		LW (BW) - NW (FW)	0,7938
	LW (FW) - NW (BW)	0,9999		LW (FW) - NW (BW)	1,0000
	LW (FW) - NW (FW)	0,7338		LW (FW) - NW (FW)	0,9963
	NW (BW) - NW (FW)	0,9339		NW (BW) - NW (FW)	0,9997
720	EW (BW) - EW (FW)	1,0000	720	EW (BW) - EW (FW)	0,7317
	EW (BW) - NW (BW)	1,0000		EW (BW) - NW (BW)	0,5897
	EW (BW) - NW (FW)	0,9971		EW (BW) - NW (FW)	0,9814
	EW (FW) - NW (BW)	1,0000		EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,9998		EW (FW) - NW (FW)	0,9977
	NW (BW) - NW (FW)	0,9994		NW (BW) - NW (FW)	0,9899
1430	LW (BW) - LW (FW)	0,9999	1430	LW (BW) - LW (FW)	0,9990
1836	EW (BW) - EW (FW)	0,9995	1836	EW (BW) - EW (FW)	0,9845
	EW (BW) - NW (BW)	1,0000		EW (BW) - NW (BW)	0,9720
	EW (BW) - NW (FW)	0,9063		EW (BW) - NW (FW)	0,9339
	EW (FW) - NW (BW)	1,0000		EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,9923		EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	0,9531		NW (BW) - NW (FW)	1,0000

NKAα1b2:

Table 20: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1b2 in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9995
	EW (FW) - LW (FW)	0,9929
	EW (FW) - NW (FW)	0,9888
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	0,8732
	LW (FW) - NW (FW)	0,7092
350	EW (BW) - EW (FW)	0,9887
	EW (BW) - LLW (BW)	1,0000
	EW (BW) - LLW (FW)	0,9883
	EW (BW) - LW (BW)	0,9411
	EW (BW) - LW (FW)	1,0000
	EW (BW) - NW (BW)	0,9999
	EW (BW) - NW (FW)	0,9083
	EW (FW) - LLW (BW)	0,9916
	EW (FW) - LLW (FW)	0,6683
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	0,9533
	EW (FW) - NW (BW)	0,9125
	EW (FW) - NW (FW)	0,9999
	LLW (BW) - LLW (FW)	0,9793
	LLW (BW) - LW (BW)	0,9497
	LLW (BW) - LW (FW)	1,0000
	LLW (BW) - NW (BW)	0,9997
	LLW (BW) - NW (FW)	0,9190
	LLW (FW) - LW (BW)	0,4257
	LLW (FW) - LW (FW)	0,9980
	LLW (FW) - NW (BW)	0,9998
	LLW (FW) - NW (FW)	0,3903
	LW (BW) - LW (FW)	0,8394
	LW (BW) - NW (BW)	0,7615
	LW (BW) - NW (FW)	1,0000
	LW (FW) - NW (BW)	1,0000
	LW (FW) - NW (FW)	0,7909
	NW (BW) - NW (FW)	0,7096
720	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9198
	EW (FW) - NW (BW)	0,9999
	EW (FW) - NW (FW)	0,9433
	NW (BW) - NW (FW)	0,7835
1430	LW (BW) - LW (FW)	0,9999
1836	EW (BW) - EW (FW)	0,9744
	EW (BW) - NW (BW)	0,9862
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,9401
	NW (BW) - NW (FW)	0,9637

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9910
	EW (FW) - LW (FW)	0,9582
	EW (FW) - NW (FW)	0,7097
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	0,9895
	LW (FW) - NW (FW)	0,9996
350	EW (BW) - EW (FW)	0,6788
	EW (BW) - LLW (BW)	1,0000
	EW (BW) - LLW (FW)	0,8716
	EW (BW) - LW (BW)	0,9999
	EW (BW) - LW (FW)	0,9959
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,1178
	EW (FW) - LLW (BW)	0,7365
	EW (FW) - LLW (FW)	0,0536
	EW (FW) - LW (BW)	0,4490
	EW (FW) - LW (FW)	0,9797
	EW (FW) - NW (BW)	0,6639
	EW (FW) - NW (FW)	0,9739
	LLW (BW) - LLW (FW)	0,8661
	LLW (BW) - LW (BW)	0,9999
	LLW (BW) - LW (FW)	0,9978
	LLW (BW) - NW (BW)	1,0000
	LLW (BW) - NW (FW)	0,1549
	LLW (FW) - LW (BW)	0,9822
	LLW (FW) - LW (FW)	0,4447
	LLW (FW) - NW (BW)	0,9117
	LLW (FW) - NW (FW)	0,0018
	LW (BW) - LW (FW)	0,9568
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	0,0519
	LW (FW) - NW (BW)	0,9940
	LW (FW) - NW (FW)	0,5138
	NW (BW) - NW (FW)	0,1188
720	EW (BW) - EW (FW)	0,9998
	EW (BW) - NW (BW)	0,9346
	EW (BW) - NW (FW)	0,9735
	EW (FW) - NW (BW)	0,9967
	EW (FW) - NW (FW)	0,9994
	NW (BW) - NW (FW)	1,0000
1430	LW (BW) - LW (FW)	0,9955
1836	EW (BW) - EW (FW)	0,6640
	EW (BW) - NW (BW)	0,9865
	EW (BW) - NW (FW)	0,2164
	EW (FW) - NW (BW)	0,9886
	EW (FW) - NW (FW)	0,9932
	NW (BW) - NW (FW)	0,7257

NKAα1c1:

Table 21: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1c1 in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,0001
	EW (FW) - LW (FW)	0,0022
	EW (FW) - NW (FW)	0,3016
	LLW (FW) - LW (FW)	0,9824
	LLW (FW) - NW (FW)	0,1617
	LW (FW) - NW (FW)	0,6780
350	EW (BW) - EW (FW)	0,9999
	EW (BW) - LLW (BW)	1,0000
	EW (BW) - LLW (FW)	0,9935
	EW (BW) - LW (BW)	0,9999
	EW (BW) - LW (FW)	0,9939
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - LLW (BW)	0,9978
	EW (FW) - LLW (FW)	0,9395
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	0,9419
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (BW) - LLW (FW)	0,9994
	LLW (BW) - LW (BW)	0,9971
	LLW (BW) - LW (FW)	0,9995
	LLW (BW) - NW (BW)	0,9993
	LLW (BW) - NW (FW)	0,9999
	LLW (FW) - LW (BW)	0,9226
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	0,9604
	LLW (FW) - NW (FW)	0,9831
	LW (BW) - LW (FW)	0,9256
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	1,0000
	LW (FW) - NW (BW)	0,9622
	LW (FW) - NW (FW)	0,9841
	NW (BW) - NW (FW)	1,0000
720	EW (BW) - EW (FW)	0,9999
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9972
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	0,9999
1430	LW (BW) - LW (FW)	0,9482
1836	EW (BW) - EW (FW)	0,9997
	EW (BW) - NW (BW)	0,9999
	EW (BW) - NW (FW)	0,9995
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,4982
	EW (FW) - LW (FW)	0,7853
	EW (FW) - NW (FW)	0,9969
	LLW (FW) - LW (FW)	0,9999
	LLW (FW) - NW (FW)	0,9234
	LW (FW) - NW (FW)	0,9921
350	EW (BW) - EW (FW)	0,8405
	EW (BW) - LLW (BW)	0,9973
	EW (BW) - LLW (FW)	0,1961
	EW (BW) - LW (BW)	0,9027
	EW (BW) - LW (FW)	0,6805
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9999
	EW (FW) - LLW (BW)	0,4411
	EW (FW) - LLW (FW)	0,0036
	EW (FW) - LW (BW)	0,1448
	EW (FW) - LW (FW)	0,0504
	EW (FW) - NW (BW)	0,8501
	EW (FW) - NW (FW)	0,9780
	LLW (BW) - LLW (FW)	0,6468
	LLW (BW) - LW (BW)	0,9990
	LLW (BW) - LW (FW)	0,9742
	LLW (BW) - NW (BW)	0,9980
	LLW (BW) - NW (FW)	0,9571
	LLW (FW) - LW (BW)	0,9418
	LLW (FW) - LW (FW)	0,9957
	LLW (FW) - NW (BW)	0,2330
	LLW (FW) - NW (FW)	0,0817
	LW (BW) - LW (FW)	0,9999
	LW (BW) - NW (BW)	0,9194
	LW (BW) - NW (FW)	0,6996
	LW (FW) - NW (BW)	0,7186
	LW (FW) - NW (FW)	0,4229
	NW (BW) - NW (FW)	0,9998
720	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	0,9999
	EW (BW) - NW (FW)	0,9934
	EW (FW) - NW (BW)	0,9995
	EW (FW) - NW (FW)	0,9986
	NW (BW) - NW (FW)	0,9427
1430	LW (BW) - LW (FW)	1,0000
1836	EW (BW) - EW (FW)	0,9751
	EW (BW) - NW (BW)	0,9988
	EW (BW) - NW (FW)	0,7619
	EW (FW) - NW (BW)	0,9999
	EW (FW) - NW (FW)	0,9984
	NW (BW) - NW (FW)	0,9718

NKAα1c2:

Table 22: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1c2 in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,8136
	EW (FW) - LW (FW)	0,7545
	EW (FW) - NW (FW)	0,9999
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	0,9551
	LW (FW) - NW (FW)	0,9312
350	EW (BW) - EW (FW)	0,9778
	EW (BW) - LLW (BW)	0,9688
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	0,9826
	EW (BW) - LW (FW)	0,9886
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,5440
	EW (FW) - LLW (BW)	0,5213
	EW (FW) - LLW (FW)	0,9220
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (BW)	0,9979
	EW (FW) - NW (FW)	0,9891
	LLW (BW) - LLW (FW)	0,9949
	LLW (BW) - LW (BW)	0,5088
	LLW (BW) - LW (FW)	0,5525
	LLW (BW) - NW (BW)	0,8939
	LLW (BW) - NW (FW)	0,0747
	LLW (FW) - LW (BW)	0,9297
	LLW (FW) - LW (FW)	0,9468
	LLW (FW) - NW (BW)	0,9992
	LLW (FW) - NW (FW)	0,3693
	LW (BW) - LW (FW)	1,0000
	LW (BW) - NW (BW)	0,9988
	LW (BW) - NW (FW)	0,9731
	LW (FW) - NW (BW)	0,9994
	LW (FW) - NW (FW)	0,9625
	NW (BW) - NW (FW)	0,7696
720	EW (BW) - EW (FW)	0,9950
	EW (BW) - NW (BW)	0,9846
	EW (BW) - NW (FW)	0,9999
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	0,9997
1430	LW (BW) - LW (FW)	0,9956
1836	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9889
	EW (FW) - NW (BW)	0,9998
	EW (FW) - NW (FW)	0,9964
	NW (BW) - NW (FW)	0,9411

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9669
	EW (FW) - LW (FW)	0,9999
	EW (FW) - NW (FW)	0,9998
	LLW (FW) - LW (FW)	0,9990
	LLW (FW) - NW (FW)	0,9994
	LW (FW) - NW (FW)	1,0000
350	EW (BW) - EW (FW)	0,9931
	EW (BW) - LLW (BW)	0,4037
	EW (BW) - LLW (FW)	0,2934
	EW (BW) - LW (BW)	0,5205
	EW (BW) - LW (FW)	0,2687
	EW (BW) - NW (BW)	0,5877
	EW (BW) - NW (FW)	0,9999
	EW (FW) - LLW (BW)	0,0902
	EW (FW) - LLW (FW)	0,0530
	EW (FW) - LW (BW)	0,1373
	EW (FW) - LW (FW)	0,0489
	EW (FW) - NW (BW)	0,1706
	EW (FW) - NW (FW)	0,9420
	LLW (BW) - LLW (FW)	1,0000
	LLW (BW) - LW (BW)	1,0000
	LLW (BW) - LW (FW)	1,0000
	LLW (BW) - NW (BW)	1,0000
	LLW (BW) - NW (FW)	0,6957
	LLW (FW) - LW (BW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	0,9999
	LLW (FW) - NW (FW)	0,5826
	LW (BW) - LW (FW)	0,9999
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	0,7986
	LW (FW) - NW (BW)	0,9997
	LW (FW) - NW (FW)	0,5423
	NW (BW) - NW (FW)	0,8475
720	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000
1430	LW (BW) - LW (FW)	1,0000
1836	EW (BW) - EW (FW)	0,5637
	EW (BW) - NW (BW)	0,8265
	EW (BW) - NW (FW)	0,8502
	EW (FW) - NW (BW)	0,9999
	EW (FW) - NW (FW)	0,9999
	NW (BW) - NW (FW)	1,0000

NKAα3a:

Table 23: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα3a in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (FW)	0,9998
	LW (FW) - NW (FW)	1,0000
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	0,9999
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	0,9999
	EW (BW) - LW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9992
	EW (FW) - LLW (BW)	0,9957
	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	0,9984
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (BW) - LLW (FW)	0,9998
	LLW (BW) - LW (BW)	0,9933
	LLW (BW) - LW (FW)	1,0000
	LLW (BW) - NW (BW)	0,9960
	LLW (BW) - NW (FW)	0,9800
	LLW (FW) - LW (BW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	1,0000
	LLW (FW) - NW (FW)	0,9994
	LW (BW) - LW (FW)	0,9975
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	1,0000
	LW (FW) - NW (BW)	0,9986
	LW (FW) - NW (FW)	0,9903
	NW (BW) - NW (FW)	1,0000
720	EW (BW) - EW (FW)	0,9998
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9993
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000
1430	LW (BW) - LW (FW)	0,9687
1836	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,2827
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,2571
	NW (BW) - NW (FW)	0,4210

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9918
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (FW) - LW (FW)	0,9940
	LLW (FW) - NW (FW)	0,9998
	LW (FW) - NW (FW)	1,0000
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	0,9724
	EW (BW) - LLW (FW)	0,9938
	EW (BW) - LW (BW)	0,9982
	EW (BW) - LW (FW)	0,9569
	EW (BW) - NW (BW)	0,9997
	EW (BW) - NW (FW)	0,8609
	EW (FW) - LLW (BW)	0,9929
	EW (FW) - LLW (FW)	0,9991
	EW (FW) - LW (BW)	0,9998
	EW (FW) - LW (FW)	0,9871
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,7838
	LLW (BW) - LLW (FW)	1,0000
	LLW (BW) - LW (BW)	1,0000
	LLW (BW) - LW (FW)	1,0000
	LLW (BW) - NW (BW)	0,9995
	LLW (BW) - NW (FW)	0,2660
	LLW (FW) - LW (BW)	1,0000
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	1,0000
	LLW (FW) - NW (FW)	0,3735
	LW (BW) - LW (FW)	0,9998
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	0,4880
	LW (FW) - NW (BW)	0,9988
	LW (FW) - NW (FW)	0,2246
	NW (BW) - NW (FW)	0,6016
720	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000
1430	LW (BW) - LW (FW)	1,0000
1836	EW (BW) - EW (FW)	0,9978
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	0,9940
	EW (FW) - NW (FW)	0,9949
	NW (BW) - NW (FW)	1,0000

NKAα3b:

Table 24: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα3b in gills (to the left) and kidney (to the right) of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9984
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (FW) - LW (FW)	0,9774
	LLW (FW) - NW (FW)	0,9983
	LW (FW) - NW (FW)	1,0000
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	0,9998
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	1,0000
	EW (BW) - LW (FW)	0,9114
	EW (BW) - NW (BW)	0,9999
	EW (BW) - NW (FW)	0,9654
	EW (FW) - LLW (BW)	0,9931
	EW (FW) - LLW (FW)	0,9999
	EW (FW) - LW (BW)	1,0000
	EW (FW) - LW (FW)	0,7732
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,9984
	LLW (BW) - LLW (FW)	0,9999
	LLW (BW) - LW (BW)	0,9993
	LLW (BW) - LW (FW)	0,9924
	LLW (BW) - NW (BW)	0,9916
	LLW (BW) - NW (FW)	0,8102
	LLW (FW) - LW (BW)	1,0000
	LLW (FW) - LW (FW)	0,9273
	LLW (FW) - NW (BW)	0,9999
	LLW (FW) - NW (FW)	0,9560
	LW (BW) - LW (FW)	0,8703
	LW (BW) - NW (BW)	1,0000
	LW (BW) - NW (FW)	0,9806
	LW (FW) - NW (BW)	0,7432
	LW (FW) - NW (FW)	0,3094
	NW (BW) - NW (FW)	0,9980
720	EW (BW) - EW (FW)	0,9988
	EW (BW) - NW (BW)	0,9760
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	0,9999
	EW (FW) - NW (FW)	0,9832
	NW (BW) - NW (FW)	0,8947
1430	LW (BW) - LW (FW)	0,9999
1836	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	0,9987
	EW (BW) - NW (FW)	0,9973
	EW (FW) - NW (BW)	0,9991
	EW (FW) - NW (FW)	0,9930
	NW (BW) - NW (FW)	0,8666

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,9837
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (FW)	0,9992
	LLW (FW) - LW (FW)	0,9867
	LLW (FW) - NW (FW)	1,0000
	LW (FW) - NW (FW)	0,9993
350	EW (BW) - EW (FW)	1,0000
	EW (BW) - LLW (BW)	0,9991
	EW (BW) - LLW (FW)	1,0000
	EW (BW) - LW (BW)	0,9992
	EW (BW) - LW (FW)	1,0000
	EW (BW) - NW (BW)	0,9997
	EW (BW) - NW (FW)	0,9999
	EW (FW) - LLW (BW)	0,9989
	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (BW)	0,9991
	EW (FW) - LW (FW)	1,0000
	EW (FW) - NW (BW)	0,9998
	EW (FW) - NW (FW)	1,0000
	LLW (BW) - LLW (FW)	0,9969
	LLW (BW) - LW (BW)	1,0000
	LLW (BW) - LW (FW)	0,9863
	LLW (BW) - NW (BW)	0,9666
	LLW (BW) - NW (FW)	0,9793
	LLW (FW) - LW (BW)	0,9972
	LLW (FW) - LW (FW)	1,0000
	LLW (FW) - NW (BW)	1,0000
	LLW (FW) - NW (FW)	1,0000
	LW (BW) - LW (FW)	0,9873
	LW (BW) - NW (BW)	0,9685
	LW (BW) - NW (FW)	0,9806
	LW (FW) - NW (BW)	1,0000
	LW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	1,0000
720	EW (BW) - EW (FW)	0,9900
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,9999
	EW (FW) - NW (BW)	0,9501
	EW (FW) - NW (FW)	0,9999
	NW (BW) - NW (FW)	0,9975
1430	LW (BW) - LW (FW)	0,9989
1836	EW (BW) - EW (FW)	0,9839
	EW (BW) - NW (BW)	0,9642
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,9991
	NW (BW) - NW (FW)	0,9962

APPENDIX 2. PROTEIN EXPRESSION INFORMATION INCLUDING RELATIVE PROTEIN ABUNDANCE.

APPENDIX 2A. STATISTICAL ANALYSIS RELATIVE PROTEIN ABUNDANCE OF NKAα ISOFORMS

NKAα1a:

Table 25: *NKAα1a protein abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
0	EW (FW)	236,01	220,18	244,59	3,89
	LLW (FW)	185,84	165,50	213,27	6,57
	LW (FW)	194,96	165,64	232,74	8,95
	NW (FW)	247,69	238,50	258,50	3,07
350	EW (BW)	246,27	241,01	250,07	1,41
	EW (FW)	227,07	214,53	240,34	3,73
	LLW (BW)	224,23	205,34	241,90	6,00
	LLW (FW)	174,29	148,95	201,04	8,69
	LW (BW)	232,76	216,45	242,31	3,82
	LW (FW)	215,51	204,44	227,11	3,56
	NW (BW)	243,33	226,19	256,83	4,37
	NW (FW)	201,97	170,50	218,74	7,18
720	EW (BW)	196,43	190,21	201,09	1,75
	EW (FW)	188,37	140,98	240,31	18,62
	NW (BW)	235,96	230,23	241,34	1,86
	NW (FW)	187,90	162,81	211,56	7,43
1420	LW (BW)	184,35	143,89	219,71	10,03
	LW (FW)	154,20	109,25	203,92	13,84
1800	EW (BW)	227,75	203,09	260,15	10,39
	EW (FW)	218,76	127,30	252,96	19,92
	NW (BW)	203,61	183,20	210,29	4,19
	NW (FW)	166,82	108,68	188,83	12,15

NKAα1b:

Table 26: *NKAα1b protein abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in gills of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	62,42	4,26	142,16	24,17
	LLW (FW)	153,81	116,33	276,95	25,40
	LW (FW)	197,85	158,35	236,92	13,60
0	NW (FW)	161,74	49,26	199,45	22,73
	EW (BW)	204,00	193,55	230,12	5,50
	EW (FW)	182,00	152,37	205,09	7,99
	LLW (BW)	99,85	77,98	139,53	11,52
	LLW (FW)	180,38	35,66	272,36	42,11
	LW (BW)	74,53	40,53	118,22	12,40
	LW (FW)	103,16	72,81	195,32	18,87
	NW (BW)	176,64	161,21	198,55	5,56
350	NW (FW)	186,68	166,20	214,93	7,70
	EW (BW)	190,61	148,75	230,75	13,37
	EW (FW)	193,22	109,39	251,35	20,42
	NW (BW)	57,93	21,43	89,71	10,01
720	NW (FW)	195,91	166,84	221,16	9,29
	LW (BW)	223,46	204,23	241,88	6,40
1420	LW (FW)	216,77	172,08	261,30	14,08
	EW (BW)	132,76	87,42	179,74	13,43
	EW (FW)	135,51	95,17	239,86	21,25
	NW (BW)	180,83	140,35	205,61	10,14
1800	NW (FW)	197,79	176,99	210,44	4,80

NKAα1c:

Table 27: *NKAα1c protein abundance, mean, minimum (min), maximum (max) and standard error of mean (SEM) for each timepoint (day degrees) in kidney of Atlantic salmon smolts.*

Day degrees	Group	Mean	Min	Max	SEM
	EW (FW)	105,05	45,21	162,95	16,36
	LLW (FW)	212,39	140,77	256,87	19,43
	LW (FW)	89,02	19,26	140,25	21,29
0	NW (FW)	103,28	43,42	183,88	23,32
	EW (BW)	87,47	67,43	100,59	5,58
	EW (FW)	102,69	44,49	195,52	22,59
	LLW (BW)	*NA*	79,76	149,66	*NA*
	LLW (FW)	211,57	131,65	265,76	22,86
	LW (BW)	151,15	82,10	217,18	21,22
	LW (FW)	131,99	51,68	190,05	19,49
	NW (BW)	120,78	31,04	185,30	25,85
350	NW (FW)	162,25	125,64	200,81	11,49
	EW (BW)	139,62	84,48	159,33	11,37
	EW (FW)	38,10	18,08	60,57	8,04
	NW (BW)	126,82	80,00	169,73	11,71
720	NW (FW)	107,45	46,35	171,51	16,57
	LW (BW)	209,97	98,73	260,67	26,88
1420	LW (FW)	181,08	38,82	272,39	35,77
	EW (BW)	243,71	209,27	268,96	9,88
	EW (FW)	189,85	85,01	242,34	25,45
	NW (BW)	194,01	150,23	232,65	13,25
1800	NW (FW)	145,33	41,22	237,99	34,36

APPENDIX 2B. TWO-WAY ANOVA (TUKEY'S HSD POST-HOC TEST) – PROTEIN EXPRESSION

NKAα1a:

Table 28: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1a in gills of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,0030
	EW (FW) - LW (FW)	0,0309
	EW (FW) - NW (FW)	0,9827
	LLW (FW) - LW (FW)	0,9961
	LLW (FW) - NW (FW)	0,0001
	LW (FW) - NW (FW)	0,0014
350	EW (BW) - EW (FW)	0,7922
	EW (BW) - LLW (BW)	0,6541
	EW (BW) - LLW (FW)	<.0001
	EW (BW) - LW (BW)	0,9610
	EW (BW) - LW (FW)	0,2314
	EW (BW) - NW (BW)	1,0000
	EW (BW) - NW (FW)	0,0141
	EW (FW) - LLW (BW)	1,0000
	EW (FW) - LLW (FW)	0,0014
	EW (FW) - LW (BW)	0,9998
	EW (FW) - LW (FW)	0,9837
	EW (FW) - NW (BW)	0,9002
	EW (FW) - NW (FW)	0,4910
	LLW (BW) - LLW (FW)	0,0032
	LLW (BW) - LW (BW)	0,9974
	LLW (BW) - LW (FW)	0,9970
	LLW (BW) - NW (BW)	0,7967
	LLW (BW) - NW (FW)	0,6429
	LLW (FW) - LW (BW)	0,0002
	LLW (FW) - LW (FW)	0,0297
	LLW (FW) - NW (BW)	<,0001
	LLW (FW) - NW (FW)	0,3606
	LW (BW) - LW (FW)	0,8687
	LW (BW) - NW (BW)	0,9904
	LW (BW) - NW (FW)	0,2305
	LW (FW) - NW (BW)	0,3543
	LW (FW) - NW (FW)	0,9606
	NW (BW) - NW (FW)	0,0288
720	EW (BW) - EW (FW)	0,9982
	EW (BW) - NW (BW)	0,0436
	EW (BW) - NW (FW)	0,9974
	EW (FW) - NW (BW)	0,0060
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	0,0053
1430	LW (BW) - LW (FW)	0,2542
1836	EW (BW) - EW (FW)	0,9964
	EW (BW) - NW (BW)	0,5422
	EW (BW) - NW (FW)	0,0001
	EW (FW) - NW (BW)	0,9293
	EW (FW) - NW (FW)	0,0018
	NW (BW) - NW (FW)	0,0778

NKAα1b:

Table 29: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1b in gills of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,0053
	EW (FW) - LW (FW)	<,0001
	EW (FW) - NW (FW)	0,0017
	LLW (FW) - LW (FW)	0,5953
	LLW (FW) - NW (FW)	1,0000
	LW (FW) - NW (FW)	0,8018
350	EW (BW) - EW (FW)	0,9837
	EW (BW) - LLW (BW)	0,0008
	EW (BW) - LLW (FW)	0,9755
	EW (BW) - LW (BW)	<,0001
	EW (BW) - LW (FW)	0,0013
	EW (BW) - NW (BW)	0,9458
	EW (BW) - NW (FW)	0,9961
	EW (FW) - LLW (BW)	0,0186
	EW (FW) - LLW (FW)	1,0000
	EW (FW) - LW (BW)	0,0005
	EW (FW) - LW (FW)	0,0282
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	1,0000
	LLW (BW) - LLW (FW)	0,0229
	LLW (BW) - LW (BW)	0,9641
	LLW (BW) - LW (FW)	1,0000
	LLW (BW) - NW (BW)	0,0362
	LLW (BW) - NW (FW)	0,0101
	LLW (FW) - LW (BW)	0,0006
	LLW (FW) - LW (FW)	0,0343
	LLW (FW) - NW (BW)	1,0000
	LLW (FW) - NW (FW)	1,0000
	LW (BW) - LW (FW)	0,9317
	LW (BW) - NW (BW)	0,0011
	LW (BW) - NW (FW)	0,0002
	LW (FW) - NW (BW)	0,0531
	LW (FW) - NW (FW)	0,0156
	NW (BW) - NW (FW)	0,9999
720	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	<,0001
	EW (BW) - NW (FW)	1,0000
	EW (FW) - NW (BW)	<,0001
	EW (FW) - NW (FW)	1,0000
	NW (BW) - NW (FW)	<,0001
1430	LW (BW) - LW (FW)	1,0000
1836	EW (BW) - EW (FW)	1,0000
	EW (BW) - NW (BW)	0,4820
	EW (BW) - NW (FW)	0,1291
	EW (FW) - NW (BW)	0,5590
	EW (FW) - NW (FW)	0,1670
	NW (BW) - NW (FW)	0,9966

NKAα1c:

Table 30: *P-values between each group for each day degree (sampling point) for mRNA abundance of NKAα1c in kidney of Atlantic salmon smolts. Significant values are marked in red.*

Day degrees	Group	P-value
0	EW (FW) - LLW (FW)	0,0092
	EW (FW) - LW (FW)	0,9994
	EW (FW) - NW (FW)	1,0000
	LLW (FW) - LW (FW)	0,0014
	LLW (FW) - NW (FW)	0,0075
	LW (FW) - NW (FW)	0,9997
350	EW (BW) - EW (FW)	0,9995
	EW (BW) - LLW (BW)	0,9979
	EW (BW) - LLW (FW)	0,0013
	EW (BW) - LW (BW)	0,3781
	EW (BW) - LW (FW)	0,7965
	EW (BW) - NW (BW)	0,9475
	EW (BW) - NW (FW)	0,1871
	EW (FW) - LLW (BW)	1,0000
	EW (FW) - LLW (FW)	0,0077
	EW (FW) - LW (BW)	0,7179
	EW (FW) - LW (FW)	0,9737
	EW (FW) - NW (BW)	0,9986
	EW (FW) - NW (FW)	0,4670
	LLW (BW) - LLW (FW)	0,2853
	LLW (BW) - LW (BW)	0,9874
	LLW (BW) - LW (FW)	0,9999
	LLW (BW) - NW (BW)	1,0000
	LLW (BW) - NW (FW)	0,9449
	LLW (FW) - LW (BW)	0,4480
	LLW (FW) - LW (FW)	0,1305
	LLW (FW) - NW (BW)	0,0498
	LLW (FW) - NW (FW)	0,6996
	LW (BW) - LW (FW)	0,9980
	LW (BW) - NW (BW)	0,9680
	LW (BW) - NW (FW)	0,9999
	LW (FW) - NW (BW)	0,9999
	LW (FW) - NW (FW)	0,9686
	NW (BW) - NW (FW)	0,8491
720	EW (BW) - EW (FW)	0,0172
	EW (BW) - NW (BW)	0,9999
	EW (BW) - NW (FW)	0,9563
	EW (FW) - NW (BW)	0,0602
	EW (FW) - NW (FW)	0,2704
	NW (BW) - NW (FW)	0,9978
1430	LW (BW) - LW (FW)	0,9757
1836	EW (BW) - EW (FW)	0,5977
	EW (BW) - NW (BW)	0,6914
	EW (BW) - NW (FW)	0,0238
	EW (FW) - NW (BW)	1,0000
	EW (FW) - NW (FW)	0,7965
	NW (BW) - NW (FW)	0,7131