Neuroendocrine factors involved in appetite control and feed intake in Atlantic salmon (*Salmo salar*) reared in Recirculating Aquaculture Systems (RAS).

Thesis for the degree

Master of Science in Aquaculture Biology

Gunnar Merckoll Berg

Department of Biological Sciences

University of Bergen

October 2018

Content

Acknowledgements

I would like to express my deepest gratitude to my supervisors Ivar Rønnestad and Sigurd Handeland for excellent supervision and support during this project. I also give a big thanks to Tharmini Kalananthan, Floriana Lai and Koji Murashita for fantastic help in the laboratory and inputs on this project. Furthermore, I would like to thank Hardingsmolt for letting me use their facilities and their fish. Lastly, I would like to thank my partner Lisa Hagevik for putting up with me during the last months when all my time has been spent at the university.

Bergen, October 1st, 2018

Gunnar Merckoll Berg

Abstract

The control of appetite in vertebrates is a complex process that depends on a range of signals from peripheral tissues and neuro- and endocrine signals originating in the brain. The hypothalamus is believed to be the main site for signal integration and control of appetite and feed intake. Previous studies on involvement of neuropeptides in appetite regulation in Atlantic salmon (*Salmo salar*) has been based on analysis of whole brain. No studies have involved dissection of the brain to quantify the spatial and temporal expression of the neuropeptides involved in appetite regulation in different parts of the brain.

Neuropeptide Y (NPY), cocaine- amphetamine related transcript (CART), agouti-related protein 1 (AgRP-1) and proopiomelanocortin- a2s (POMCa2s) are neuropeptides believed to be involved in appetite regulation in Atlantic salmon. In the present study the mRNA concentration of these neuropeptides was assessed by qPCR in 6 parts of the brain. There was a significantly higher mRNA concentration of NPY in the forebrain compared to all the other tissues of the brain. POMCa2s showed a significant higher mRNA concentration in the pituitary than in all other parts of the brain. CART had a significantly higher expression in the forebrain than in cerebellum and pituitary while AgRP-1 showed no significant differences in mRNA concentration among the different brain tissues. As part of this study the fish went through a vaccination period that involved fasting, and the only significant differences found were in the forebrain for POMCa2s and in the midbrain for NPY. The extensive expression of neuropeptides involved in appetite control in different parts of the brain suggest other roles than appetite control for these neuropeptides, and/or the appetite control is being supplemented from other parts of the brain than the hypothalamus. This study shows that further research on the involvement of the neuropeptides in control of appetite cannot be based on analysis of whole brain and needs to focus on specific regions and signaling pathways.

1 Introduction

1.1 Background

The aquaculture industry worldwide is rapidly increasing and in 2014 the amount of produced fish surpassed the wild fisheries in volume produced/caught annually. Aquaculture has the potential to continue to grow while the wild fisheries does not permit much additional sustainable growth based on the stocks currently exploited (FAO 2016, Gutierrez-Wing and Malone, 2006). In Norway the aquaculture industry is mainly focused on Atlantic salmon (*Salmo salar*) and to a small degree other species e.g. Rainbow trout (*Oncorhynchus mykiss*), Halibut (*Hippoglossus hippoglossus*) and cleaner fish used for salmon farming such as Lumpfish (*Cyclopterus lumpus*) and Ballan wrasse (*Labrus bergylta*). This thesis will however focus on salmon. As the Atlantic salmon is an anadromous species, the first period of the life cycle is in freshwater, and the latter part of its life in saltwater. This complicates the process of farming this species, as the fish needs to be successfully transferred from freshwater to saltwater after the smoltification. The salmon farming is a relatively new industry and has developed immensely since the start in the 1960s when some pioneer farmers started with one or a few small cages with a few hundred fish to supplement their income, to the present day billion-dollar industry with thousands of employees. During the 1970s a transformation took place in smolt rearing and development of dry feed for salmon, which contributed massively to the rapid expansion of the industry (Liu et al., 2011).

With the expansion of the aquaculture industry many problems have arisen, such as different diseases and parasites as well as challenges with environmental impact. Many of the diseases that emerged as problems could be treated with vaccines from the early 90s, however parasites and in particular salmon lice (*Lepeophteirus salmonis*) have continued to be a huge problem for the industry. The salmon lice are costing the industry an enormous amount of resources and is in addition a problem for wild stocks of salmon (Costello et al., 2009). Keeping the salmon in sea cages for a shorter amount of time can be beneficial to reduce the probability of diseases and also salmon lice levels and reduce the time until salmon reaches slaughter weight (Kverneland, unpublished, 2018). One of the approaches to this is to produce larger smolts in Recirculating Aquaculture System (RAS) where the fish is kept in closed systems on land.

1.2 Recirculating Aquaculture Systems, RAS

In a regular flow-through system the water will just flow through the facility and there is normally no need for oxygenating or apply methods to get rid of ammonia, CO_2 and particles, given sufficient amount of water to the biomass. The water flow into the tank is the same as the flow at the outlet (Figure 1.1). This is in many ways the classical approach in smolt production, but in the later years RAS has become a more viable choice for many farmers with the increased knowledge and improvements in technology.

Figure 1.1: The basic principle of a flow-through system. Water flows through the facility and is discharged out of the facility. The water is not recirculated and there will be no need to treat the water at the outlet (Ole Gabriel Kverneland, Akva Group).

In a RAS facility the concept is to recirculate and rinse the water through an extensive water treatment and filter system which only require 1% fresh water, basically to dilute the concentration of NO_3. This significantly conserve water and energy and is more economic and environmentally friendly, as well as reduce the risk of infectious pathogens (Gutierrez-Wing and Malone, 2006). If the water is not properly treated, a decline in water quality, with accumulation of CO_2, NO_2, NH_4, NO_3 and lower levels of oxygen will occur. Oxygen is pumped into the water to counteract the decline in oxygen in the tanks and also to prevent accumulation of CO_2 by aeration. In mature and well-run RAS systems, the ammonia excreted from the salmon, will be converted to nitrite and nitrate in the biofilters. Good surveillance is important to keep control of all the factors regarding water quality. A schematic overview of a basic RAS facility is shown in figure 2.1.

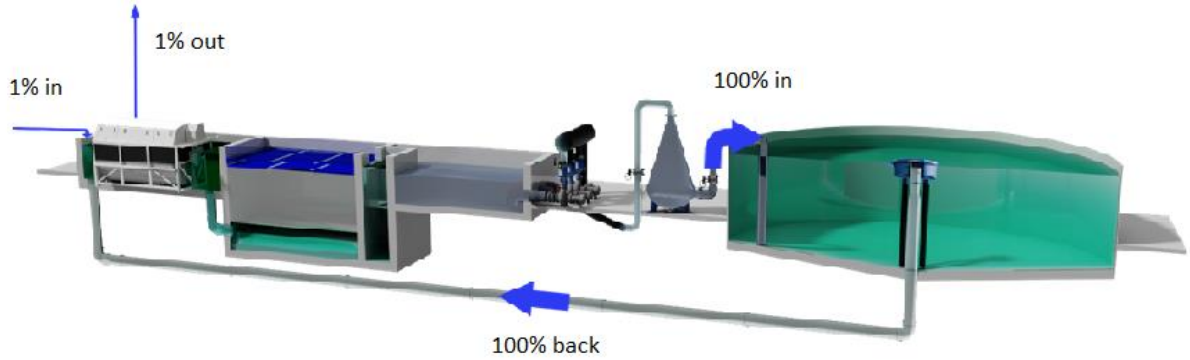

Figure 2.1: The basic principle of a RAS facility. This figure shows the inlet of new water being disinfected together with water coming from the tanks. A mechanical filter is used to remove particles from the water and some water is removed during this process as well. Buffer is added, and the water is sent through a biofilter, before it reaches the oxygen tank where oxygen is added, and the CO_2 concentration is reduced. From there the water is pumped into the fish tanks before returning back to the inlet (Ole Gabriel Kverneland, Akva Group).

To ensure an acceptable financial outcome the density of the fish needs to be kept high to produce as much fish as possible. This needs to be done without reducing the quality of the fish with regards to growth and fish health. With a high density of fish in the tanks, oxygen concentration decreases, and CO_2 concentration increases. This needs to be adjusted with the addition of oxygen pumped into the water to counteract the loss of oxygen and the accumulation of CO_2. A study on density in a RAS facility on sea bass (*Dicentrarchus labrax*) was conducted with densities of 10, 40, 70 and 100 kg m^{-3}. No significant differences were observed between densities up to 70 kg m^{-3}, however above this density the specific growth rate showed a reduction. This suggests an optimum density of 70 kg m^{-3} for farming sea bass in RAS (Sammouth et al., 2009). For Atlantic salmon densities of up to 62 kg m^{-3} did not show any significant differences in specific growth rate compared to lower densities (Kolarevic et al., 2014).

The water conditions in RAS tanks can be kept stable all year (temperature, oxygen, CO_2, NH_4, NO_2, and NO_3) which gives the producer control and predictability and the production is not affected by season to the degree of a flow-through system. As 95-98% of the water is reused and harmful pathogens entering the facility can represent a potentially huge problem, it's crucial with good internal control and protocols. The water inlet is disinfected (UV and

ozone) to remove all potential harmful organisms. To remove any solids and large particles a mechanical filter is used to filtrate the water. This removes particulate debris from the water, but not small organic matter and nitrogen compounds. The biofilter converts ammonia and nitrite into less toxic nitrate. The biofilter consist of many plastic elements filled with nitrifying bacteria with a huge surface area (Lin et al., 2003).

As part of the production period in the RAS facility salmon will undergo several stressful periods. These include, but are not limited to, transfer between tanks, sorting, vaccination and smoltification followed by adaption to salt water. When these periods are planned, the fish will also be fasted to reduce the metabolic stress. Consequently, these periods of stress and starvation should be kept as short as possible, to ensure good health and welfare to the fish. It is also important to understand how appetite is controlled and affected during periods of fasting, refeeding and stress.

1.3 Appetite control

Feed intake is controlled by centers in the brain. In vertebrates, the hypothalamus serves a key role in controlling appetite and ingestion of food and is also involved in control of allocation and growth (Rønnestad et al., 2017). Both endocrine and neuroendocrine signals reach the hypothalamus from other parts of the brain and also from peripheral organs such as the gastrointestinal tract (GI) and the liver. An area called the arcuate nucleus (ARC) in the hypothalamus is the primary part of the hypothalamus involved in regulating appetite. Two groups of peptides are produced in this area, 1; orexigenic neuropeptides (Neuropeptide Y (NPY) and Agouti-related peptide (AgRP)) and 2; anorexigenic neuropeptides (Cocaine and amphetamine regulated transcript (CART) and Pro-opiomelanocortin (POMC)) (Valassi et al., 2008). Orexigenic factors stimulate the appetite, while anorexigenic factors inhibits appetite. For some of these peptides different isoforms have been discovered in Atlantic salmon e.g. four isoforms of POMC (-a1, -a2, -a2s and -b), (Valen et al., 2011, Murashita et al., 2011).Other peptides involved in controlling food intake includes Orexins (OXs), Galanin (GAL), Melanin-concentrating hormone (MCH), corticotropin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH) and neurotensin (NT) (Rønnestad et al., 2017, Wan et al., 2011). In this thesis NPY, CART, Agouti-related peptide-1 (AgRP-1) and Pro-opiomelanocortin-a2s (POMCa2s) was selected for study because of their strong relationship with appetite regulation in mammalian species.

Not much is known of how other parts of the brain than the hypothalamus contributes to appetite control in Atlantic salmon. Most of the studies that have been done so far on gene expression is based on analysis of the whole brain (Valen et al., 2011, Rønnestad et al., 2017). Studies done for cod larvae (*Gadus morhua*) suggest involvement in appetite regulation in both forebrain and midbrain by NPY and CART (Le et al., 2016). By dissecting the brain into different parts, the mRNA concentration in each tissue can be assessed. This will further expand our understanding of the interaction between different parts of the brain in the appetite regulation in Atlantic salmon.

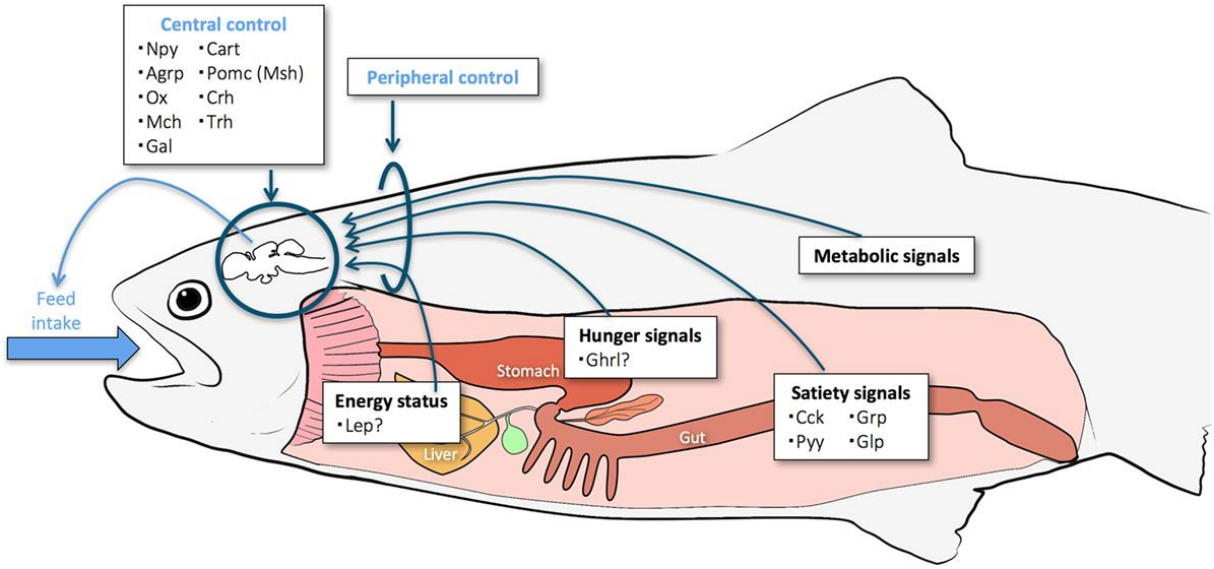

Figure 1.3: Key organs and signaling pathways believed to be involved in control of appetite in fish (Rønnestad et al., 2017).

1.4 NPY

NPY is apparently one of the strongest orexigenic neuropeptides in mammals (Valassi et al., 2008, Rønnestad et al., 2017). However, in fish NPY seem to have variable impact on appetite in different species. In some species like Zebrafish (*Danio rerio*) and Rainbow trout, injections (into the third ventricle) of NPY results in an increase of short-term appetite (Yokobori et al., 2012, Aldegunde and Mancebo et al., 2006). In other species, among them Atlantic cod, NPY levels are elevated around the time of feeding, suggesting that NPY plays a role in short-term appetite increase (Kehoe and Volkoff, 2007). Another study on cod larvae showed high levels of NPY in the forebrain and the midbrain, suggesting that other parts of the brain is involved in appetite regulation. It is also suggested other functions of NPY from this study (Le et al., 2016). In salmon however NPY show increased mRNA levels post-

feeding which might suggest a lesser role of feed regulation in Atlantic salmon (Valen et al., 2011).

1.5 CART

CART is a peptide with a strong anorexigenic effects in mammals (Valassi et al., 2008, Rønnestad et al., 2017). Multiple types of the CART gene have been discovered in different species e.g. rat and other mammals and birds in addition to fish species. Some fish species have multiple variants of CART, like goldfish (*Carassius auratus*) and zebrafish, but unexpectedly, only one version of the CART gene is found in salmon (Murashita et al., 2009, Valen et al., 2011). The mRNA expression of CART post-feeding has been shown to increase within 3h in salmon which is an indicator of an anorexigenic effect (Valen et al., 2011). Fasting for 6 days reduced the mRNA expression of CART in the brain further implicating an anorexigenic effect in salmon (Murashita et al., 2009). CART is also believed to have other functions than appetite regulation, such as in olfactory and visual effect pathways (Le et al., 2016).

1.6 AgRP

AgRP is also a strong orexigenic peptide together with NPY in mammals (Valassi et al., 2008, Rønnestad et al., 2017). This neuropeptide has been identified in several mammals, birds and a variety of different fish species. AgRP works as an antagonistic gene of the receptors MC3R and MC4R and participates in body weight regulation, appetite control and energy homeostasis. High mRNA expression of AgRP in both rats and zebrafish is correlated with obesity, which is a strong indicator of an orexigenic effect also in fish (Wan et al., 2012, Harrold et al., 1999). In common carp (*Cyprinus carpio*) AgRP-1 is strongly expressed in the brain, eye, testis, intestine, while AgRP-2 is only expressed strongly in the brain and the gills (Wan et al., 2012). In salmon two different isoforms of AgRP has been identified (AgRP-1 and AgRP-2). AgRP-1 is highly expressed in the pituitary and the skin for the Atlantic salmon, while AgRP-2 is highly expressed in gonads, mid-gut and red muscle. Salmon starved for 6 days showed unexpectedly a decrease in mRNA expression for AgRP-1. This suggest a species-specific regulation of AgRP-1 and an anorexigenic effect for salmon (Murashita et al., 2009).

1.7 POMC

POMC is an anorexigenic neuropeptide and a precursor peptide processed into different melanocortins which includes melanocyte-stimulating hormones (α-, β- and γ-MSH) and adrenocorticotropic hormone (ACTH) (Valassi et al., 2008, Rønnestad et al., 2017, Murashita et al., 2009). In mammals and birds only one gene of POMC is known, but in many fish multiple isoforms of POMC are present (Rønnestad et al., 2017). In salmon four isoforms of POMC (POMCa1, POMCa2, POMCa2s, POMCb) has been identified, mainly in the pituitary. POMCa1 and POMCb showed a short-term response after feed intake in Atlantic salmon. A study on salmon on the effect of fasting with regards to POMC showed reduced POMCa1 expression after 6 days, and no significant difference with the other isoforms of POMC (Valen et al., 2011).

1.8 Objective

Currently there is little known of the location of the different neuropeptides in the different parts of the brain. Similar to other vertebrates, the appetite regulation for salmon is believed to be located in the ARC of the hypothalamus. This study is divided into two parts. The first part is to describe the mRNA expression of the four selected neuropeptides (NPY, CART, AgRP-1 and POMCa2s) in different parts of the brain (forebrain, midbrain, cerebellum, hypothalamus, *Saccus vasculosus* and pituitary). The second part describes changes in the mRNA expression of the selected neuropeptides over two weeks with a period of fasting prior to vaccination and a recovery period until feeding is reestablished to normal levels.

The following hypothesis were formulated for this experiment:

$H0_{1a}$: NPY is only expressed in the hypothalamus.

$H0_{1b}$: CART is only expressed in the hypothalamus.

$H0_{1c}$: AgRP-1 is only expressed in the hypothalamus.

$H0_{1d}$: POMCa2s is only expressed in the hypothalamus.

$H0_2$: The mRNA expression of the different genes (NPY, CART, AgRP-1 and POMCa2s) in the different parts of the brain (forebrain, midbrain, cerebellum, hypothalamus, *Saccus vasculosus* and pituitary) is not affected by stress induced starvation.

2 Materials and methods

2.1 Hardingsmolt facilities

The Hardingsmolt RAS facility is located on land in Tørvikbygd in Kvam municipality. Hardingsmolt was built in 2006 and was one of the first recirculating facilities in Norway. At Hardingsmolt 95-98% of the water is recycled, which leads to a much lower water consumption compared to a regular flow-through system. The total intake of water is 43200 L h^{-1} from the Tørvikavatnet lake which lies in close proximity to the facility.

2.2 Fish and rearing conditions

2.2.1 Fish

The Atlantic salmon used in this experiment were reared at Hardingsmolt AS. The eggs came from Salmobreed (Nystølen and Erfjord Stamfisk) (March 16th, 2017) and were incubated and hatched at the Hardingsmolt facility at ~5°C for approximately 500 degrees day (mean temperature times amount of days) following standard protocols. After hatching the temperature was slowly raised with 0.5°C to ~13°C (0.5 °C every other day), until the time of first feeding in May 2017.

At first feeding (May 25th) the fish was transferred to 6m tanks (50m^3) and kept for 3.5 months (13°C) until an average body weight of ~15g. They were then transferred to 12m tanks (300m^3) on September 8th. During the following period (September 8th to October 17th) the fish had continuous light, LL (light light). From October 18th to November 27th the light changed from LL to LD (light dark) 12:12. From November 28th the light was set back to LL.

Temperature was kept at 13 ± 1 °C which is considered the optimum temperature for salmon in this size range (Handeland et al., 2008). The dissolved oxygen levels ranged from 85-95% with a set-point at 90% (outlet water). The density of fish in the tank ranged from 25 kg m^{-3} to approximately 70 kg m^{-3} before transfer to the 16m tank.

During the production cycle all water quality parameters (CO_2 <15.0 mg L^{-1}; NO_2 <0.5 mg L^{-1}; NH_4 <2 mg L^{-1}; NO_3 <250 mg L^{-1}) and temperature were kept stable. This was monitored closely by an automatic system and control tests were performed by the staff as an extra

security. Dead fish were removed each day (1-80 individuals each day out of 250.000 fish in the experimental tank) to avoid diseases. The first day (November 16th) and the last days (November 24-30th) the fish was fed with commercial dry feed (Biomar CPK40). For the rest of the rearing period, the fish was fed commercial feed correlating to the size and temperature based on industry standards (Biomar, Norway).

Figure 2.2: The large hall at Hardingsmolt facility with all the 12m tanks. The closest tank is empty at the time when the picture was taken. Bridges to walk on can be seen right over the tanks for the farmers to have an easy access to each of the tanks (Heima, 2016).

2.2.2 Vaccination

The fish were vaccinated following standard protocols for the plant. The fish was vaccinated with 1 PD (ALPHA JECT micro® 1 PD) and Micro 6 (ALPHA JECT micro® 6) at November 19th. The mortality was low (<0.1% day^{-1}) during the time of the vaccination. A specialized team was hired to do the vaccination, which took several days to perform on all the tanks. The fish from the 12m tank was pumped to a sedation tank to be anesthetized. When the fish was sedated it was sent to a table were four people arranged the salmon facing the right way for the vaccination. Each of the salmon then got the vaccine with a syringe through their abdominal cavity before being transported back to a different 12m tank.

Figure 2.3: The left image shows the tank were the fish was sedated. This is a small tank, and not too many fish can be in this tank at the same time. This tank is closely monitored to keep oxygen and CO_2 levels acceptable. The middle image shows the table were the fish was sorted for size and arranged correctly. The right image shows the salmon after vaccination being transported back to a different 12m tank.

2.3 Experimental design

The aim of the sampling design was to document responses in appetite controlling neuropeptides during a standard vaccination protocol. Sampling started with fed fish that was then fasted prior to vaccination, and then the group was followed until appetite and feed intake had resumed and reached normal levels for the size and temperature conditions used. There were all together seven samplings around the vaccination (November 16th – November 30th); 2 samples before vaccination and 5 after vaccination (Figure 2.4).

Pre-vaccination: Two samplings were taken before the vaccination, one control that was fed and with food in the GI tract (November 16th) and one group (N=8) sampled after three days of starvation (November 19th).

Post-vaccination: 5 samplings were taken after the vaccination, four of these were starved fish (November 19th, November 20th, November 22nd and November 26th), while the last sampling was fed fish (November 30th).

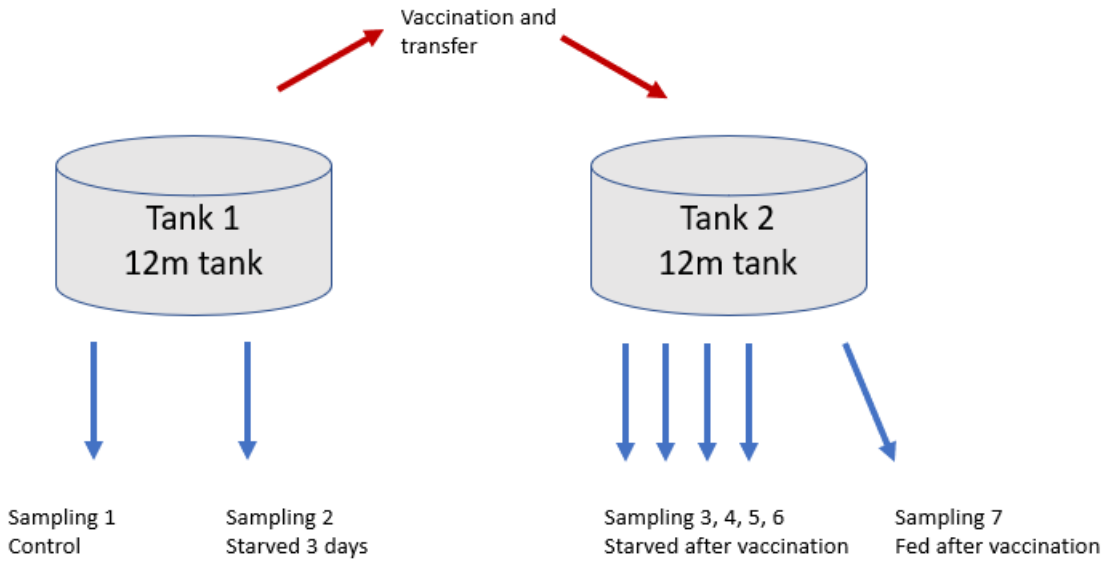

Figure 2.4: Schematic overview of the experiment with samples taken before and after the vaccination and after feeding had resumed. See text for further descriptions.

2.4 Sampling protocol

A total of 8 fish was collected at each sampling. A hand dip net was used to collect fish randomly from the experimental tank. Only one fish was collected each time and then placed into a bucket with high concentration of Finquel vet. (100% metacain) to reduce the time from euthanasia until tissue collection to preserve the tissues as best as possible (Figure 2.5). The fish was measured in length and weight (Ohaus Valor 2000 and a length scale) (Figure 2.6). The whole brain was dissected out and put in RNA-later (SIGMA) in a Sterile 5ml Centrifuge Tube (Eppendorf®). The pituitary was removed separately and put in RNA-later in Axygen® 1.5mL MaxyClear Microtubes. The samples were brought back to University of Bergen and stored in -80^{o}C until dissection.

Figure 2.5: Salmon in the bucket with high concentration of Finquel vet.

Figure 2.6: Weight and length scale.

2.5 Dissection of the brain

The brains of the salmons were thawed while still in RNA-later to protect the integrity of the RNA. Next, the brain was dissected into 5 different parts (Figure 2.7). The pituitary was already separated at sampling and proceeded to analysis without any further processing. The first cut isolated the forebrain which consists of the olfactory bulb and the telencephalon. The pineale gland (Epiphysis) were not present on all the samples (some were lost during the sampling). In order to standardize the forebrain, the pineale was therefore removed from all brains to avoid differences caused by the concentration of neuropeptides in the pineale. On a few test brains, the olfactory bulb was removed. However, this proved to be challenging since the cutting was not consistent on all fish. The olfactory bulb was then decided to be included, to get consistency between all the brain samples. The dissection cut was made from the dorsal side and downwards to the ventral side between the forebrain (Telencephalon) and the midbrain (Tectum opticum) on the same location on all brains.

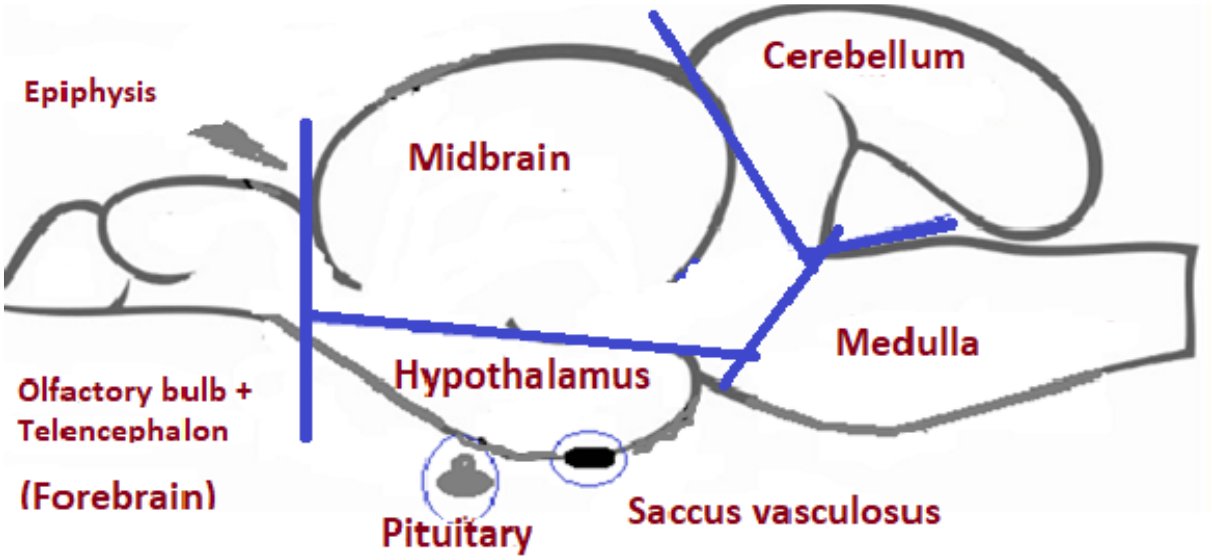

Figure 2.7: Schematic drawing of the salmon brain. The blue lines indicate location of the cuts that separated the brain into sections (Illustration by Tharmini Kalananthan, 2018).

The next cut was made between the midbrain and the hindbrain (Cerebellum) and was also made from the dorsal side and downwards to the ventral side. Next, the medulla oblongata was removed, carefully to avoid removing any part of the cerebellum and hypothalamus in the

process (Figure 2.7). After this cut the brain was then turned around and the hypothalamus was removed with a cut from the anterior to the posterior side. Following, the *Saccus vasculosus* was cut off from the hypothalamus. This structure was easy to recognize due to its dark red color and was removed with as little extra tissue as possible. The remaining part consisted of the midbrain. The different parts of the brain were frozen in separate Eppendorf tubes at -80ºC until RNA-extraction.

2.6 RNA extraction

RNA-extraction of the dissected brain samples was conducted using a protocol for RNA extraction with TRI reagent based on Chomczynski, (1993.). Centrifuge tubes (2 ml) were prepared with 0.6-0.7g Precellys® Zirconium Oxide Beads (1.4mm) using a balance (Sartorius BP 61S). Each tube was added 1 ml of TRIzol Reagent. The frozen tissue was added to the Eppendorf-tube after being squeezed on a paper to remove any leftover RNA-later and left on ice-block for 5 minutes. The samples were subsequently homogenized using a Precellys 24 homogenizer for 15 seconds at 5000 RPM (rotations per minute). The homogenized samples were left at room temperature for 5 minutes for the foam to disperse, before adding 200µl chloroform (≥99.5%) and vortexing for 1 minute. The tubes were then placed in a centrifuge (5415 R) for 15 minutes at 4ºC at 13.2 RCF (relative centrifugal force) while new Eppendorf tubes where prepared for the next phase. The tubes from the centrifuge had 3 layers, an aqueous phase on top, an interphase in the middle, and an organic phase at the bottom. The RNA is located in the aqueous phase, and this was carefully transferred with a pipette to the newly prepared Eppendorf tubes, while avoiding any white interphase. In the new tubes with the aqueous phase, 500µl of Isopropanol (≥99.5%) was added and left at room temperature for 10 minutes before the samples was put back in the centrifuge for 10 minutes at 4ºC at 13.2 RCF.

After this step, a little RNA-pellet could be observed as a white lump at the bottom of the Eppendorf tube in some of the samples, but some of the tissue had a too small starting concentration for this to be visible. This was the case for *Saccus vasculosus* and the pituitary for the most part, as these tissues were the smallest. With the RNA-pellet in the Eppendorf tube, the supernatant was decanted as best as possible, and 1ml 80% EtOH was added to the pellet as a washing step. Another round in the centrifuge at 5 minutes at 4ºC and 7500 RCF,

then the supernatant was removed carefully with a pipette and the RNA-pellet was left to dry for 5-10 minutes. The pellet was reconstituted in nuclease free water using 50µl for forebrain, midbrain, cerebellum and hypothalamus, and 15µl for *Saccus vasculosus* and the pituitary. This was based on the pellet size. The last step on this procedure was to precipitate RNA by adding 1/10 of the volume of the water with 3M NaAc, pH 5.2 and 2 time the volume of the water with -20ºC 100% EtOH. This represented 5µl NaAc and 100µl 100% EtOH in the forebrain, midbrain, cerebellum and hypothalamus. In the *Saccus vasculosus* and the pituitary this came out to be 1.5µl NaAc and 30µl 100% EtOH. Then all samples were stored in -80ºC freezer until cDNA synthesis.

2.7 cDNA synthesis

The RNA samples were transferred from -80ºC to the centrifuge and spun for 30 minutes at 4ºC at 13.2 RCF. This was followed by decanting of the supernatant, flash spin and removing the last drop with a pipette. The pellet was then air dried for 5-10 minutes and then resuspended in nuclease free water again. The volume ranged from 10µl for the smallest tissue to 40µl in the largest tissue. The RNA was then quantified using NanoDrop Spectrophotometer (Thermo Scientific™ NanoDrop™).

8µl of RNA at 125ng μl^{-1} concentration were used and added to 1µl of 10X ezDNase Buffer (SuperScript IV VILO™) and 1µl ezDNase enzyme (SuperScript™ IV VILO™) and then incubated (Applied Biosystems 2720 Thermal Cycler) at 37ºC for 2 minutes to make gDNA. Mastermix was made using 4µl SuperScript™ IV VILO™ Master Mix and 6µl Nuclease-free water. 10µl of master mix was added to the 10µl of gDNA for a total of 20µl. A Negative RT was created adding 4µl of SuperScript™ IV VILO™ No RT Control and 6µl of nuclease-free water to 10µl of gDNA from a sample with high volume. This was mixed and incubated (Applied Biosystems 2720 Thermal Cycler) for 25ºC for 10 minutes, 50ºC for 10 minutes, and 85ºC for 5 minutes. The result is cDNA with 50ng μl^{-1} concentration.

A dilution was prepared using 15µl cDNA (50ng μl^{-1}) and 22.5µl nuclease-free water resulting in a final concentration of 20ng μl^{-1} cDNA.

Another dilution was made by taking 2µl of the previous dilution of 20ng μl^{-1} and adding 78µl nuclease-free water, which gives 80µl of 0.5ng μl^{-1} cDNA. The two cDNA dilutions were stored at -20ºC until further use in the quantitative polymerase chain reaction (qPCR).

2.8 qPCR

The qPCR, also known as real-time polymerase chain reaction (Real-time PCR) was used to quantify the expression of the target genes (NPY, CART, AgRP-1 and POMCa2s). CFX96™ Real-Time System (BIO-RAD) was used, coupled with the software Bio-Rad CFX manager (version 3.1) to perform the qPCR. For the qPCR a 96-plate well (BIO-RAD) was used. Each well consisted of 10µl iTaq™ Universal SYBR® Green Supermix, 0.6µl primer F (table 2.1), 0.6µl primer R (table 2.1), and 6.8µl nuclease-free water. 2µl of cDNA with concentration 20ng μl^{-1} was added in addition for a total of 20µl in each well, resulting in 40ng RNA per well. The protocol used for the qPCR was 95ºC for 30 sec, then 40 cycles of 95ºC for 5 sec and 60ºC for 25 sec. All samples were run in duplicates to exclude pipetting errors. All plates also had a duplicate of NTC (non-template control) and NRT (No-reverse transcriptase control) to exclude genomic DNA contamination in the samples. The first plate on each gene had a standard dilution curve with 1:10 dilution (made from PCR products), and all plates had a cDNA pool used as BPC (between plate control) to correct for differences between the different plates. Ef1a was used as housekeeping gene to normalize the qPCR results on the gene expression analysis. Ef1a has previously demonstrated to be a stably expressed gene in salmon and used by the department laboratory as references for relative expression studies (Olsvik et al., 2005).

Table 2.1: Primer name and sequence used in this experiment

Gene	Primer name	Primer sequence 5' → 3'	Accession no.	Reference
NPY	NPY Fw4 NPY Rv6	ACTGGCCAAGTATTACTCCGCTCTCA CTGTGGGAGCGTGTCTGTGCTCTCCTTC	(AB455539)	Valen et al., 2011
CART	CART Fw4 CART Rv1	AGCAACTGCTTGGAGCACTACATGAC CAGTCGCACATTTTGCCGATTCTCGCGCCC	(AB455538)	Valen et al., 2011
AgRP-1	AgRP-1 Fw3 AgRP-1 Rv3	GCGTTCTCCCCGTCGCTGTA TGTTAGGGGCGCCTGTGAGC	(AB455536)	Valen et al., 2011
POMC-A2s	POMCA Fw7 POMCA Rv6	AGACGAGAGCTGGGGGGAGT CGTCCCAGCTCTTCATGAAC	(AB462420)	Valen et al., 2011
Ef1a	SsEF1 SYBR Fw SsEF1 SYBR Rv	GAGAACCATTGAGAAGTTCGAGAAG GCACCCAGGCATACTTGAAAG	(AF321836)	Valen et al., 2011

2.9 Statistical analysis

For the statistical analysis in this study the statistical program STATISTIKA 13.2 was used. All data sets fulfilled the requirements of normality, independence of individuals and homogeneity of variances to perform the ANOVA.

To determine the level of significance for the mRNA expression in the different tissues (forebrain, midbrain, cerebellum, hypothalamus, *Saccus vasculosus* and pituitary) for each of the selected neuropeptides (NPY, CART, AgRP-1 and POMCa2s) a one-way ANOVA was conducted. For NPY, CART and POMCa2s a significant difference was observed from the one-way ANOVA and a post-hoc test was performed (Student-Newman-Keuls test) to identify the difference between the brain tissues.

In addition, a one-way ANOVA was conducted for each part of the brain for each neuropeptide with regards to the different samplings. This was done to examine any differences between the samplings regarding each tissue and neuropeptide. A post-hoc test (Student-Newman-Keuls test) was performed when a significant difference was observed in the one-way ANOVA.

One fish was removed from the second sampling (morning November 19th) due to values outside of normal. All statistical results are given in Appendix, table 1-35. Differences were considered significant when $p<0.05$. All data in figures are given as a mean ± standard error of mean (SEM).

3 Results

3.1 Weight and length

The experiment lasted for two weeks and there were altogether seven sampling points. There were no significant differences in weight and length between the samplings (November 16th, 19th in the morning, 19th in the afternoon, 20th, 22nd, 26th and 30th). The average body weight and length of all the fish in this experiment was 58.45 ± 1.60 g and 16.38 ± 0.15cm (n=56) (Figure 3.1 and 3.2). The average body weight (measured as part of the routine production protocol at Hardingsmolt) in the tank was 51.4g (November 20th).

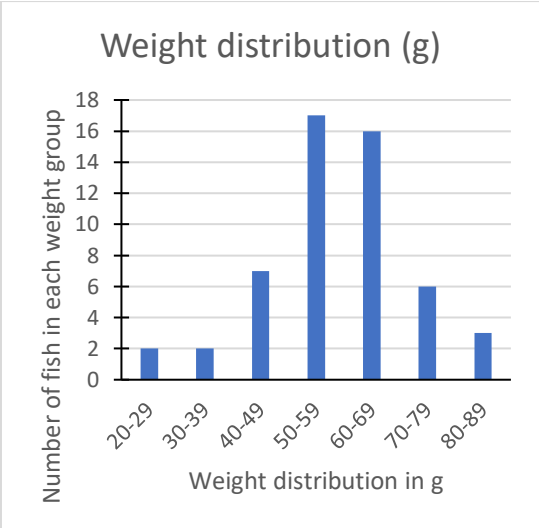

Figure 3.1: Weight (g) of the experimental fish, distributed in groups of 10g.

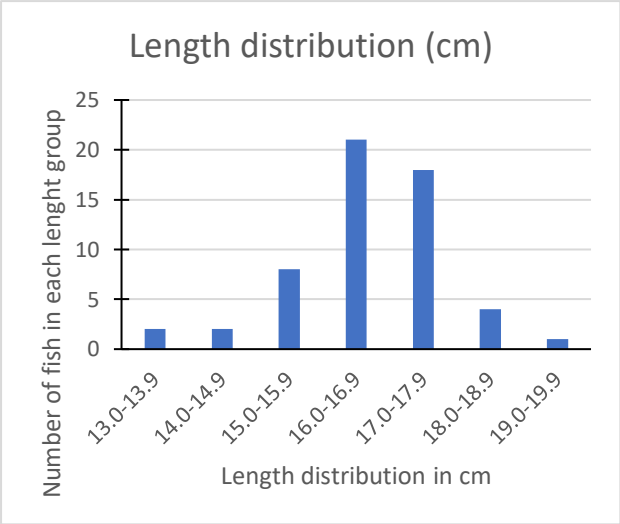

Figure 3.2: Length (cm) of the experimental fish distributed in groups of 1cm.

3.2 Gene expression

The brain was dissected into five sections + the pituitary. The expression of mRNA for each of the selected neuropeptides was quantified in each part.

3.2.1 NPY relative expression

There were significantly ($p<0.05$) higher mRNA expression of NPY in the forebrain than the other brain tissues (Figure 3.3). There were no significant differences between expressed mRNA levels of NPY in the other parts of the brain.

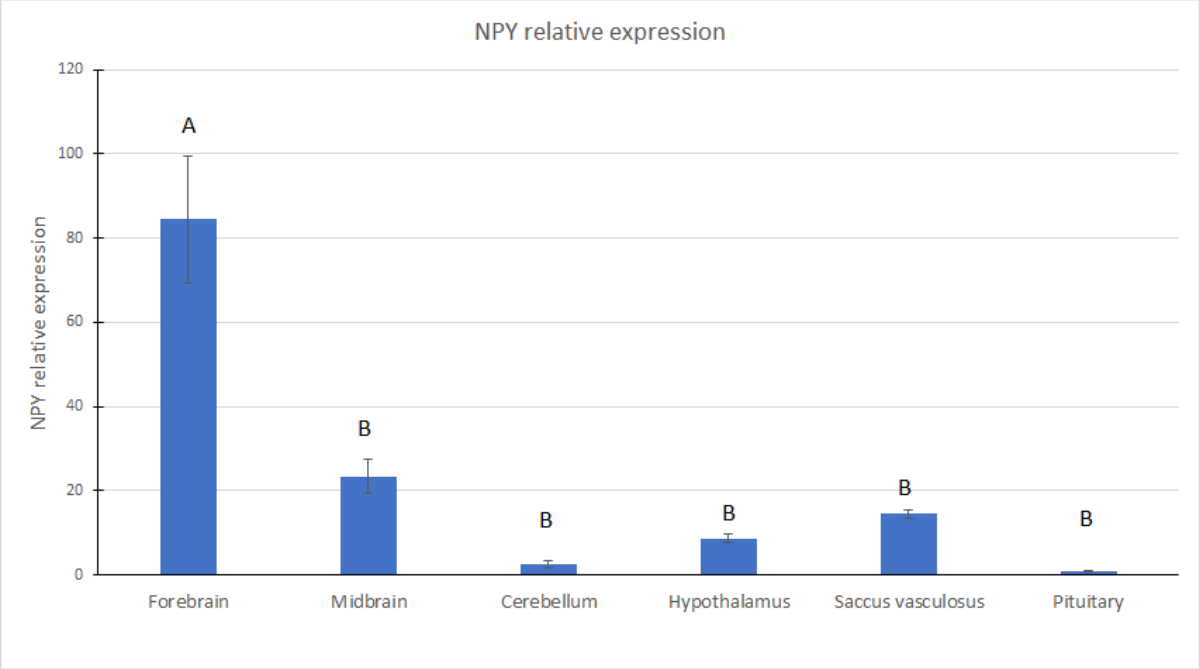

Figure 3.3: Expressed mRNA levels of NPY, relative to the housekeeping gene (Ef1a) in the different brain tissues from the first sampling (November 16^{th,} n=3). Different letters represent significant difference in mRNA expression between groups ($p<0.05$). Value presented as mean ± SEM.

3.2.2 CART relative expression

There was a significant difference ($p<0.05$) in mRNA expression of CART between forebrain and cerebellum and between forebrain and pituitary ($p<0.05$) (Figure 3.4). There was no significant difference between forebrain and midbrain, forebrain and hypothalamus and forebrain and *Saccus vasculosus*. There was no significant difference between the other parts of the brain.

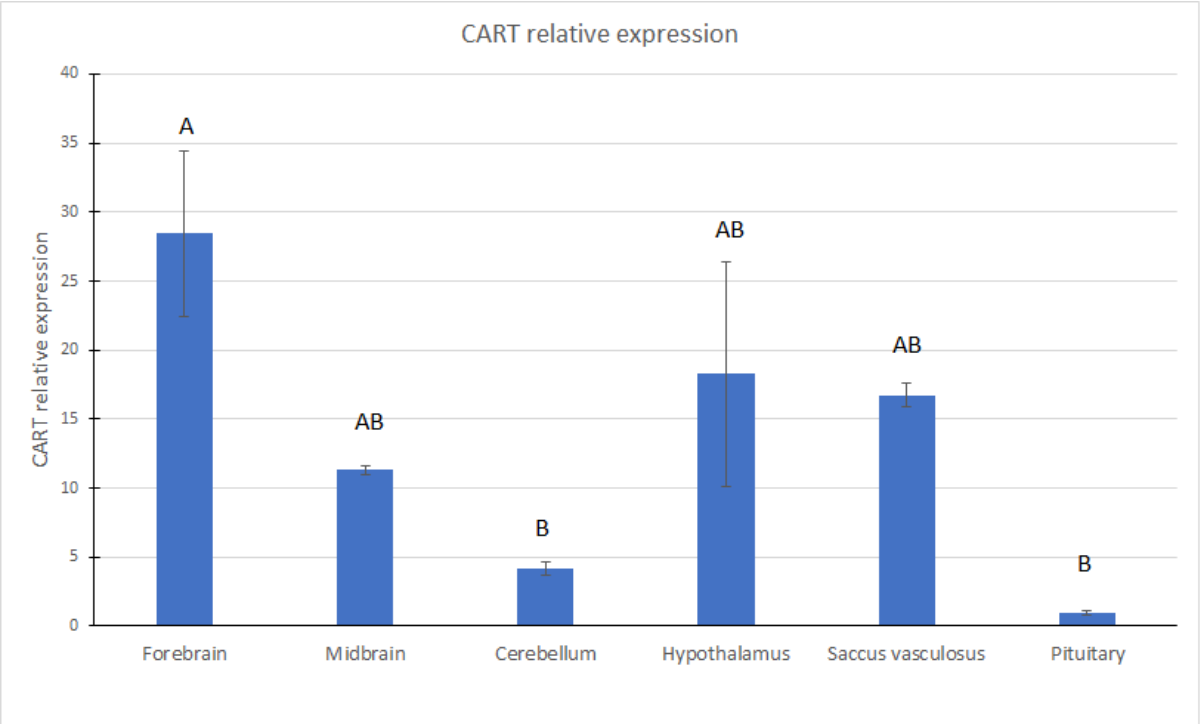

Figure 3.4: Expressed levels of CART, relative to the housekeeping gene (Ef1a) in different brain tissue from the first sampling (November 16th, n=3). See figure 3.3 for more information.

3.2.3 AgRP-1 relative expression

There was no significant difference between any of the different tissues regarding the expressed mRNA for AgRP-1. The mean values of hypothalamus, *Saccus vasculosus* and pituitary (72.11, 75.81 and 85.95 times higher mRNA concentration than midbrain respectively) were higher than the mean value of forebrain, midbrain and cerebellum, but these differences were not significant (Figure 3.5). For hypothalamus and *Saccus vasculosus* the difference in CT value from the qPCR differ substantially between each fish, which is illustrated at the error bars. Only 3 fish from this sampling is used for the analysis which gives a greater variation.

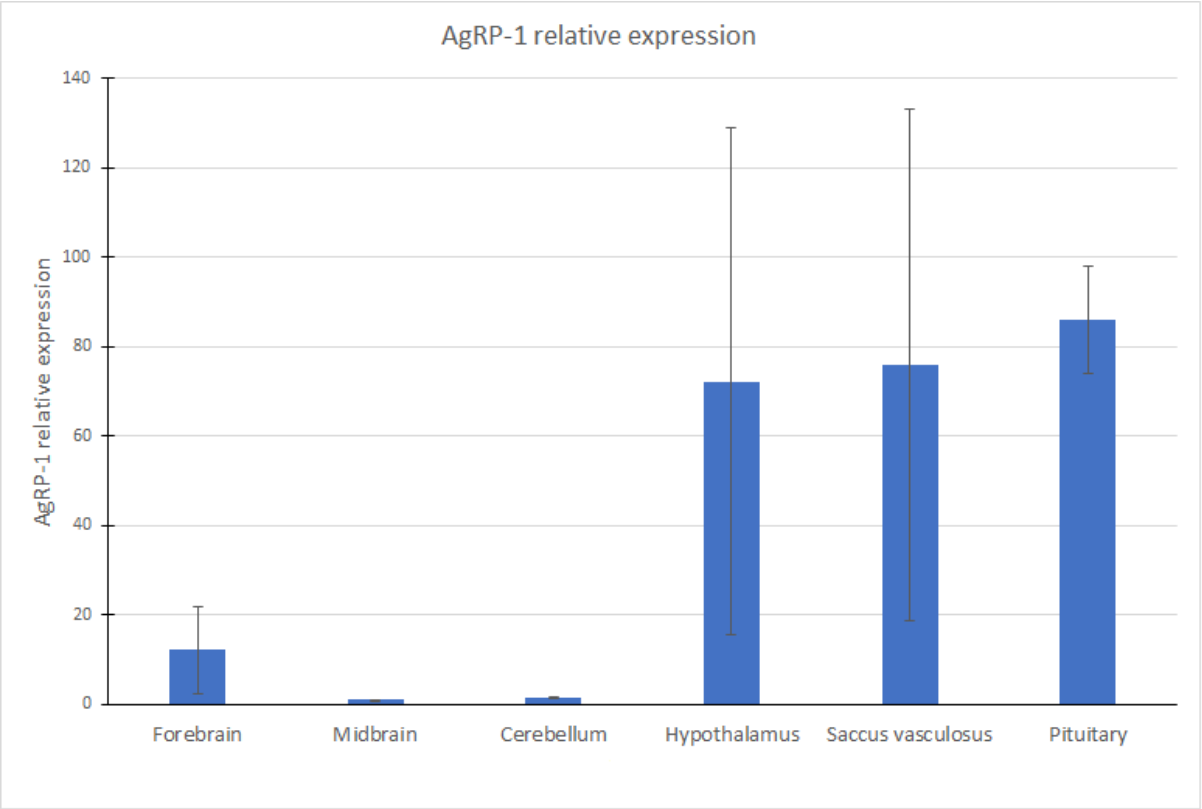

Figure 3.5: Expressed levels of AgRP-1, relative to the housekeeping gene (Ef1a) in different brain tissue from the first sampling (November 16th, n=3). See figure 3.3 for more info.

3.2.4 POMCa2s relative expression

There was a significantly higher ($p<0.05$) level of mRNA expression of POMCa2s in pituitary compared to the other parts. Hypothalamus and *Saccus vasculosus* showed a higher average (8.56 and 7.05 times higher mRNA expression than the cerebellum respectively) mRNA expression than the forebrain, midbrain and cerebellum, however the difference was not significant (Figure 3.6).

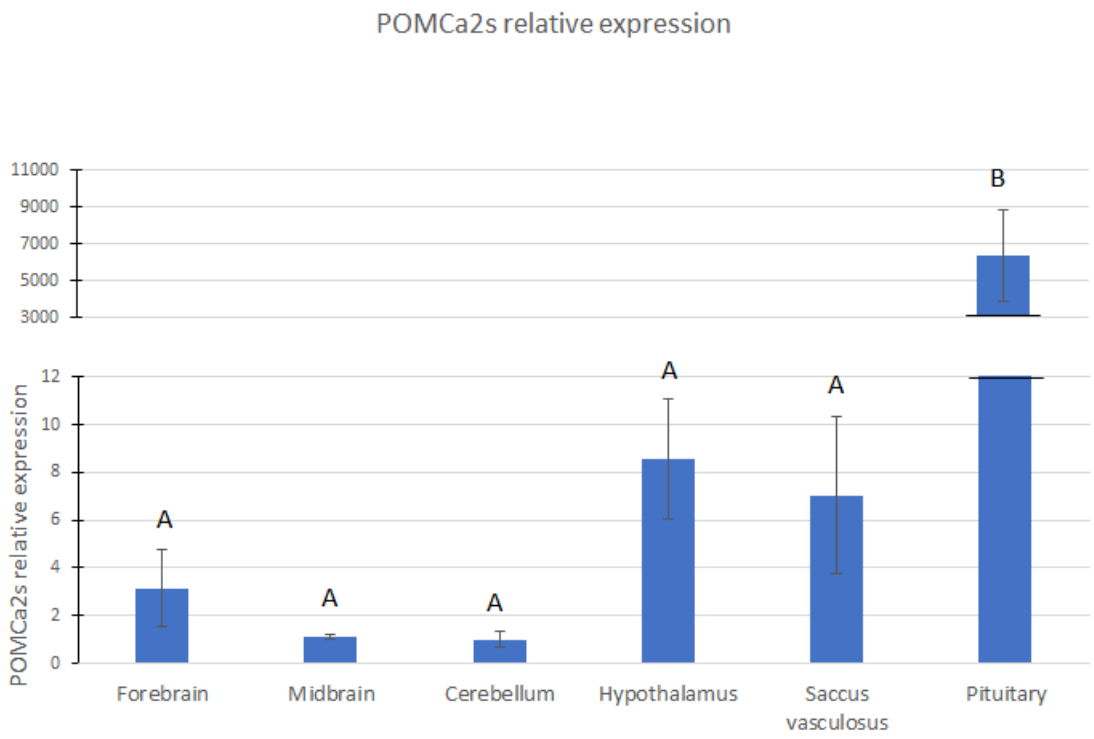

Figure 3.6: Expressed levels of POMCa2s, relative to the housekeeping gene (Ef1a) in different brain tissue of the first sampling (November 16th, n=3). Pituitary continues over the broken axis. See figure 3.3 for more info.

3.3 Changes in mRNA expression during the vaccination protocol

3.3.1 Development of mRNA expression in Forebrain

The mRNA expression of NPY, CART and AgRP-1 in the forebrain were not significant different between the samplings during the experiment. The expression of mRNA of POMCa2s showed significant higher ($p<0.05$) values in the first sampling (November 16th) than the rest of the samplings (Figure 3.7).

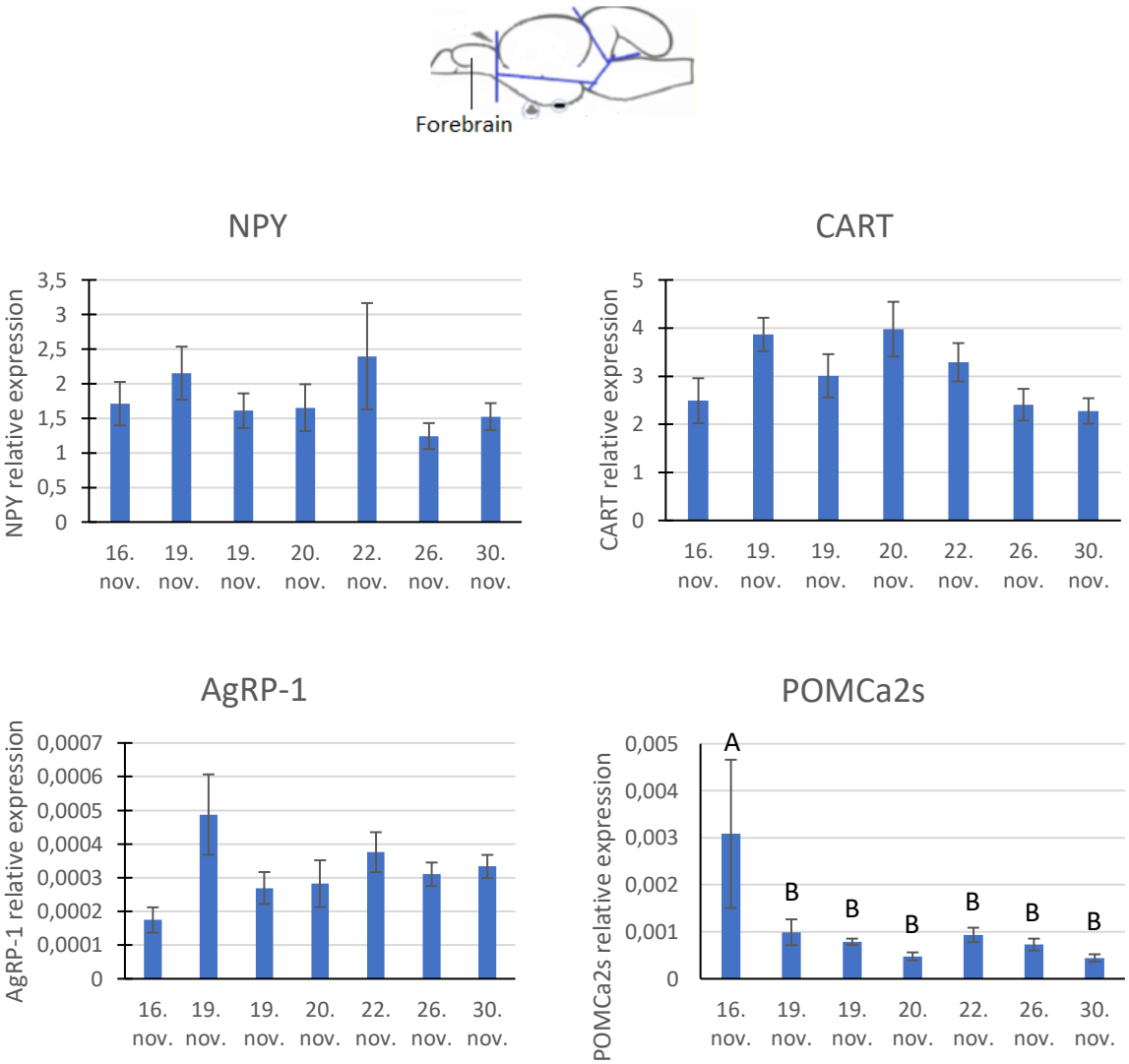

Figure 3.7: The mRNA expression of NPY, CART, AgRP-1 and POMCa2s during the time of the experiment in the forebrain in relation to the housekeeping gene; EF1a. Different letters represent a significant difference ($p<0.05$) in mRNA expression. N=3, 4, 5, 4, 6, 4 and 5 respectively for the different samplings. Mean value ± SEM is shown in the charts.

3.3.2 Development of mRNA expression in Midbrain

There was a significant difference ($p<0.05$) in mRNA expression for NPY in the forebrain between sampling 2 (November 19th) and 7 (November 30th). No significant differences were seen between the other samplings in regard to NPY. CART, AgRP-1 and POMCa2s showed no significant difference in mRNA expression in the midbrain (Figure 3.8).

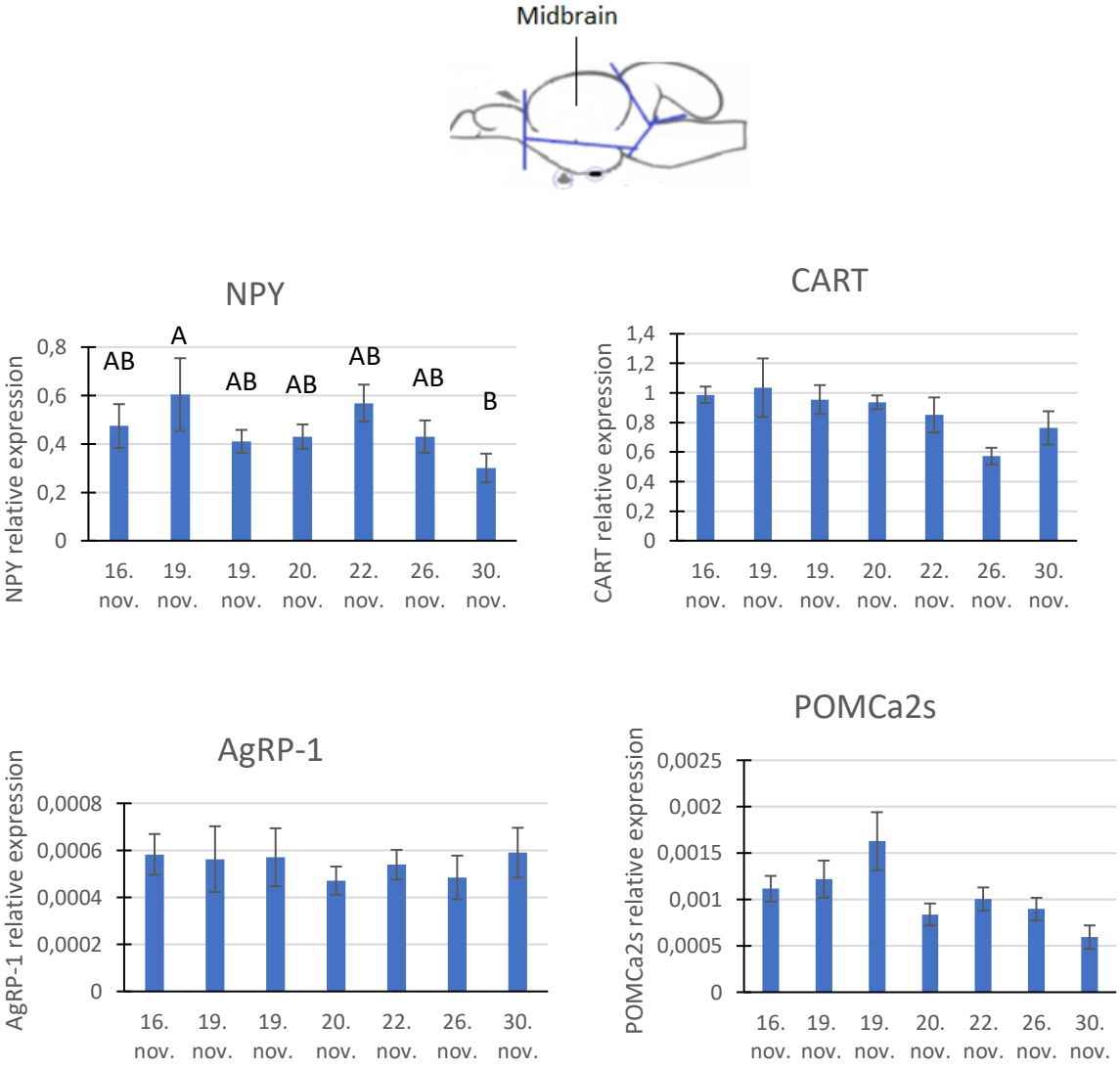

Figure 3.8: The development of mRNA expression NPY, CART, AgRP-1 and POMCa2s during the time of the experiment in the midbrain. See figure 3.7 for more information.

3.3.3 Development of mRNA expression in Cerebellum

There was no significant difference in mRNA expression of NPY, CART, AgRP-1 and POMCa2s between the different samplings in cerebellum (Figure 3.9).

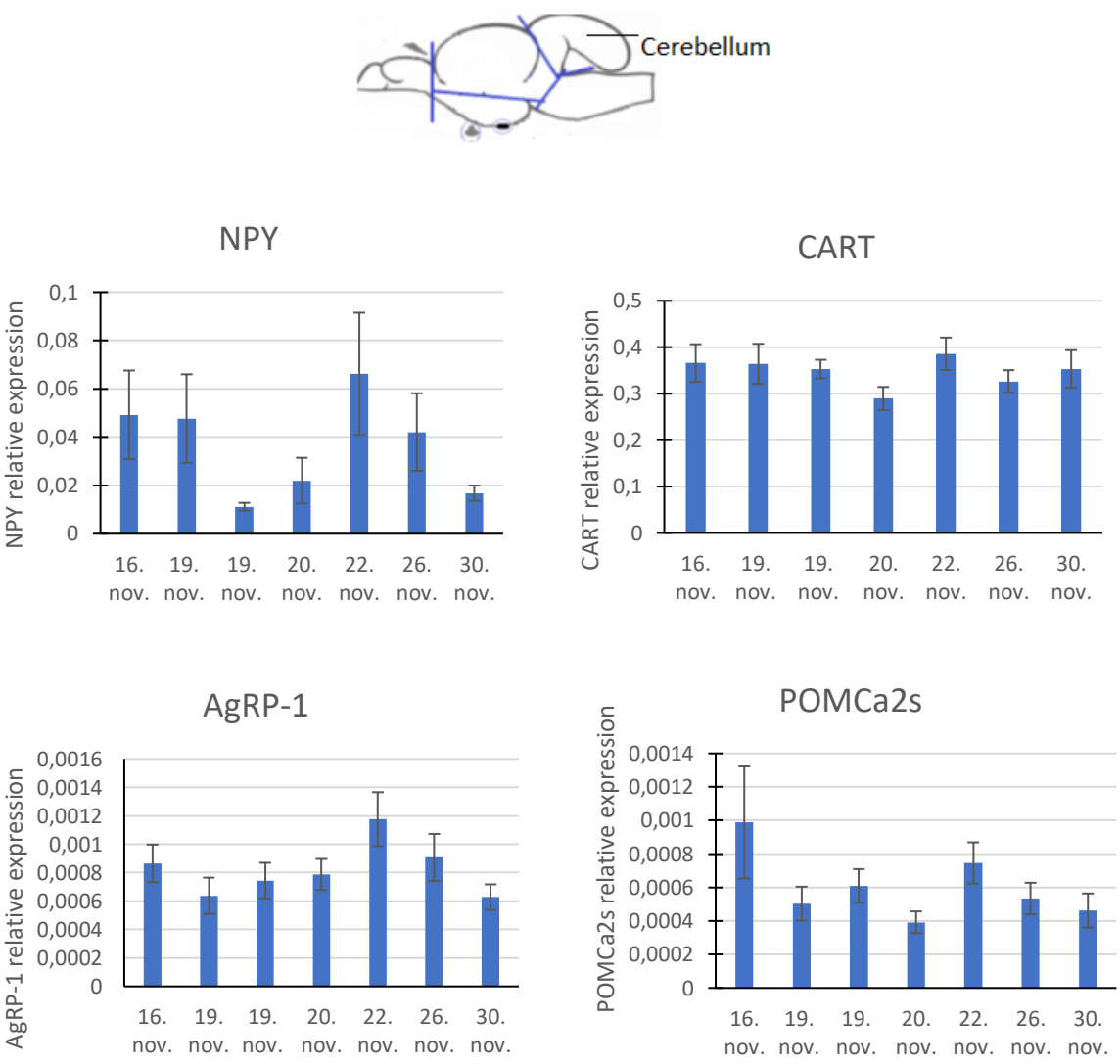

Figure 3.9: The development of mRNA expression of NPY, CART, AgRP-1 and POMCa2s during the time of the experiment in the cerebellum. See figure 3.7 for more information.

3.3.4 Development of mRNA expression in Hypothalamus

There was no significant difference ($p>0.05$) in mRNA expression of NPY, CART, AgRP-1 and POMCa2s between the different sampling in hypothalamus (Figure 3.10).

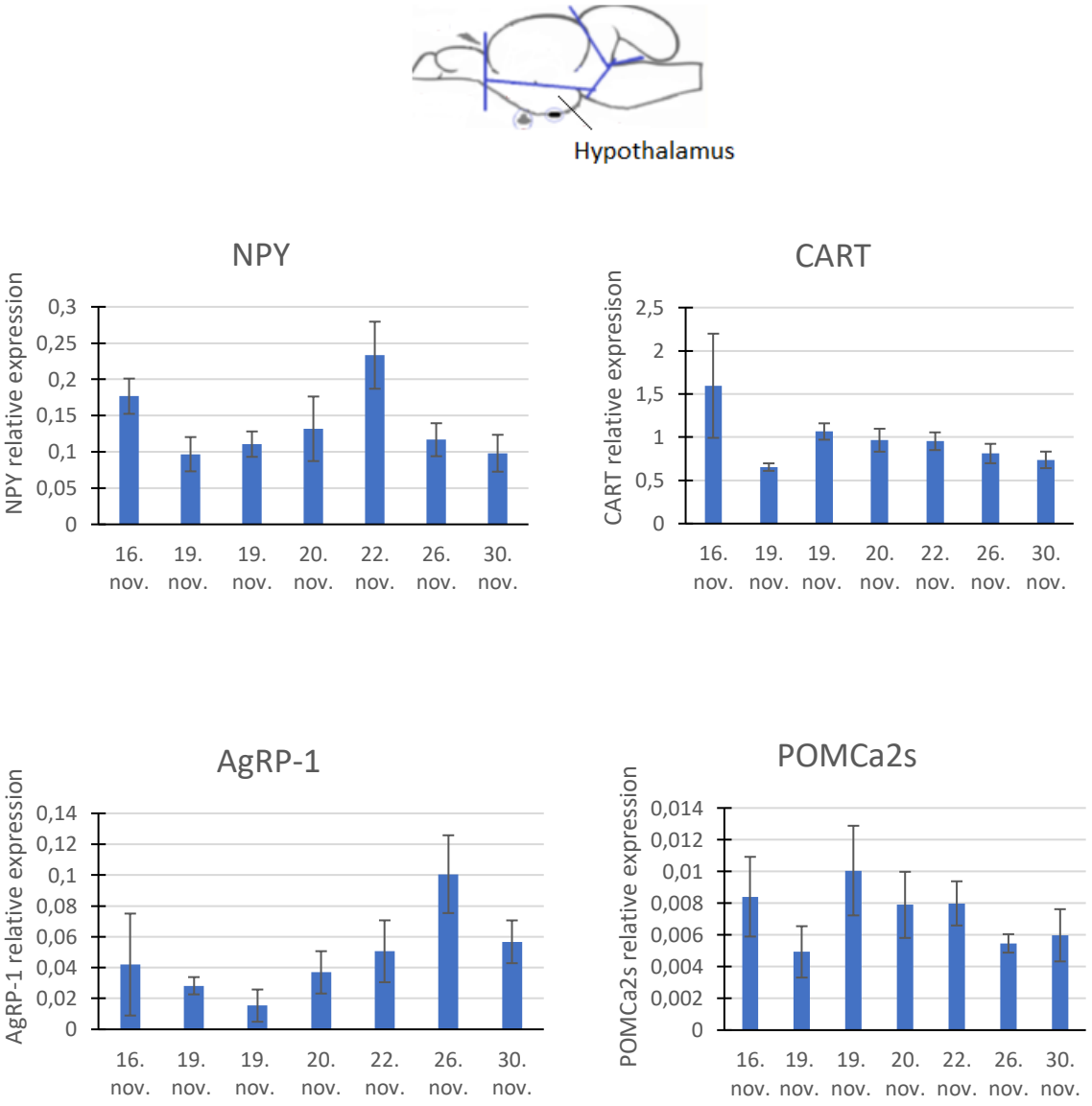

Figure 3.10: The development mRNA expression of NPY, CART, AgRP-1 and POMCa2s during the time of the experiment in the hypothalamus. See figure 3.7 for more information.

3.3.5 Development of mRNA expression in Saccus vasculosus

There was no significant difference in mRNA expression of NPY, CART, AgRP-1 and POMCa2s between the different samplings in *Saccus vasculosus* (Figure 3.11).

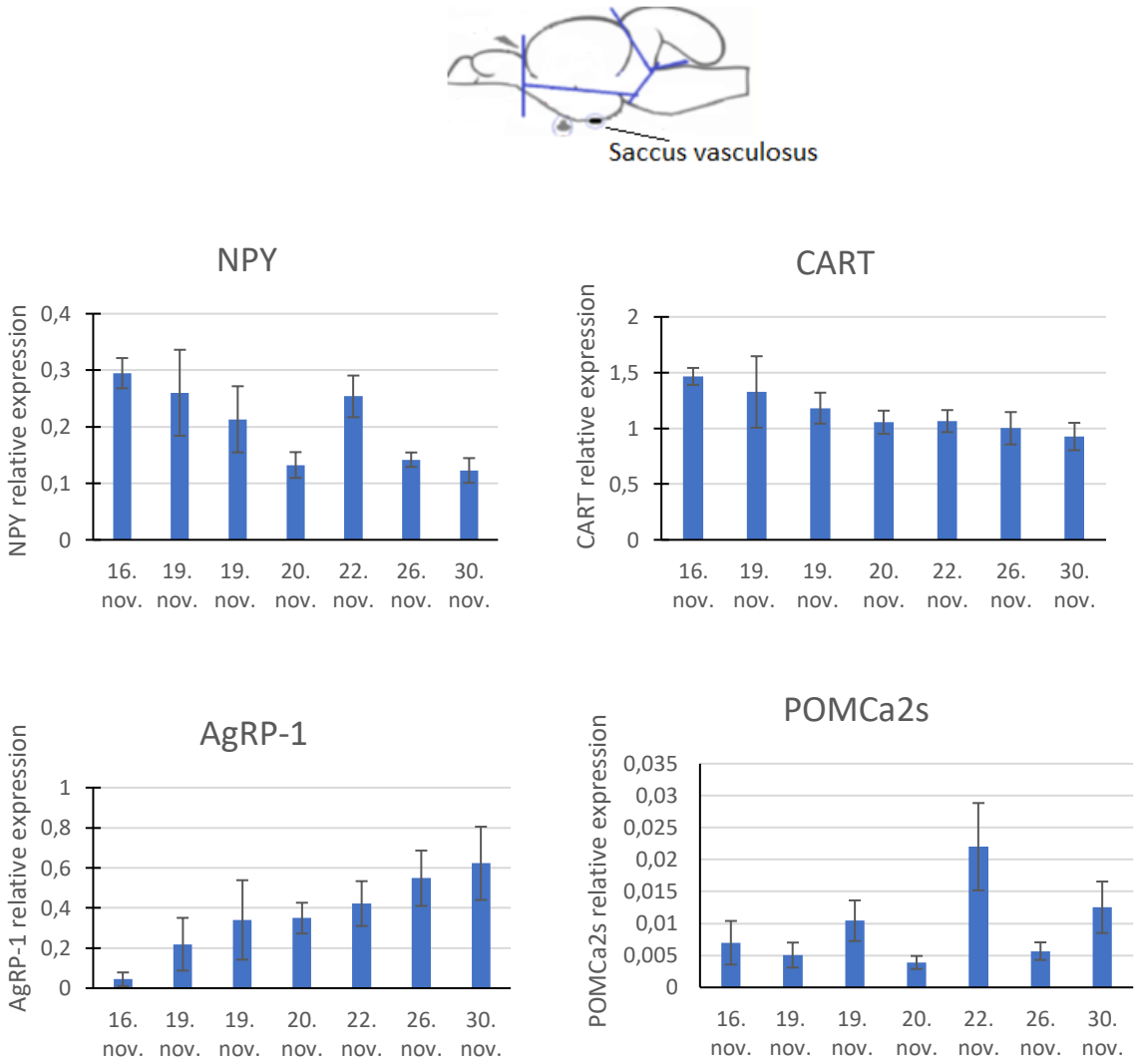

Figure 3.11: The development of mRNA expression of NPY, CART, AgRP-1 and POMCa2s during the time of the experiment in the Saccus vasculosus. See figure 3.7 for more information.

3.3.6 Development of mRNA expression in Pituitary

There was no significant difference in mRNA expression of NPY, CART, AgRP-1 and POMCa2s between the different sampling in pituitary (3.12).

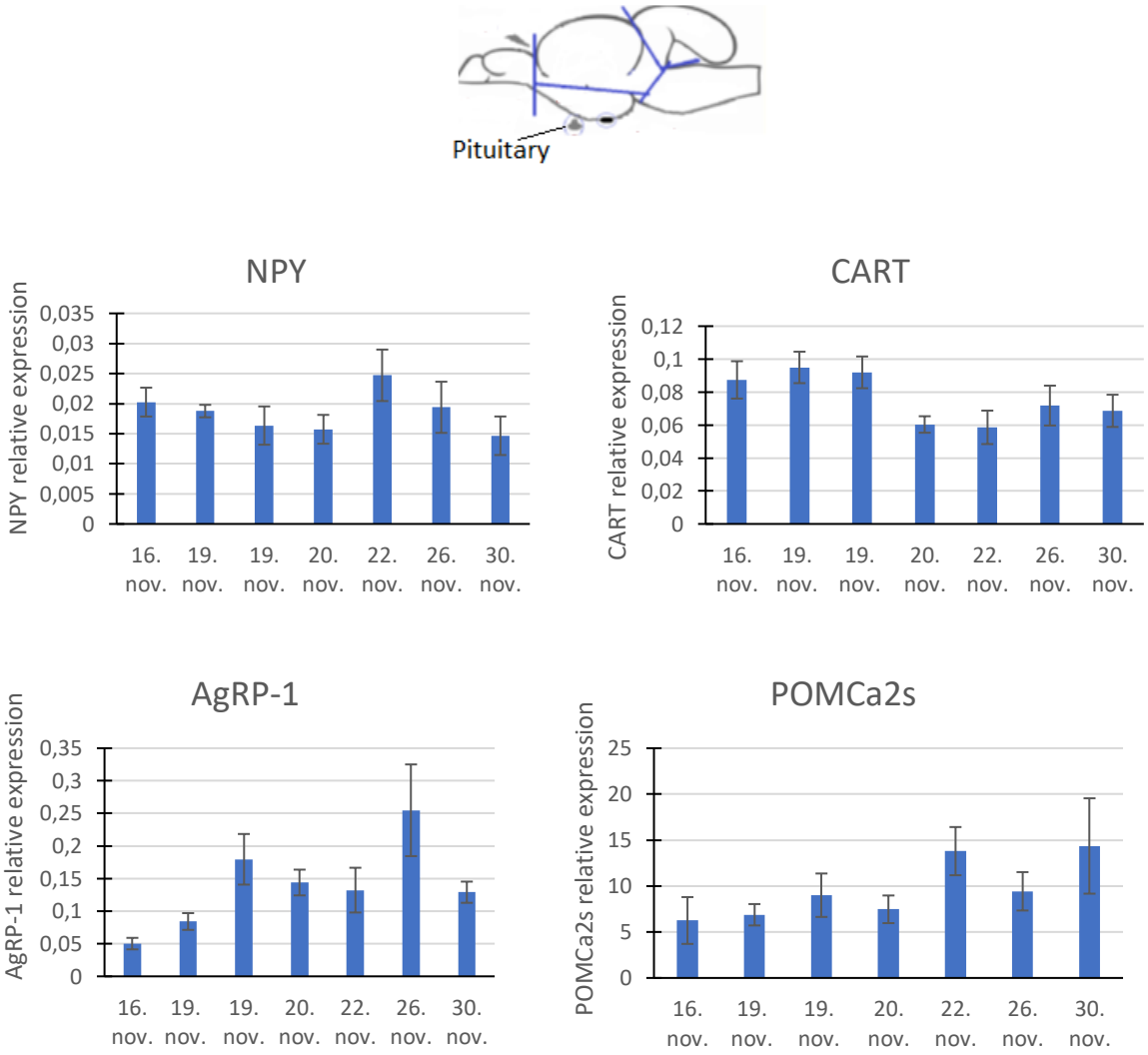

Figure 3.12: The development of NPY, CART, AgRP-1 and POMCa2s during the time of the experiment in the pituitary. See figure 3.7 for more information.

4 Discussion

4.1 Water parameters

This experiment was conducted at a RAS facility with a high level of control of the water parameters including temperature, oxygen, CO_2, NO_2, NO_3 and NH_4. The production data from the facility shows that the water parameters was kept stable and at satisfactory levels during the whole experimental period (Appendix, table 35). Therefore, these factors did not affect the results for this experiment.

4.2 Discussion of methods

The sampling was conducted over two weeks at approximately 10 a.m. each time, except for one day with two samplings; November 19th, one sampling in the morning in fish before the vaccination and one in the afternoon after the vaccination. 8 fish were collected at each sampling and the tissues were brought back on RNA-later to Høyteknologisenteret in Bergen and stored in freezer at -80^{o}C for further dissection and analysis in the laboratory. In the laboratory the brains were dissected, RNA was extracted from each part, cDNA was made, and qPCR were performed for each neuropeptide in addition to Ef1a as a reference gene. After the RNA extraction the quality of the RNA was quantified with the NanoDrop Spectrophotometer. From this quantification the problem with the low mRNA concentration in *Saccus vasculosus* and in the pituitary were encountered. Many of the samples had to be dismissed at this point. The reason for the low mRNA concentration was that the experimental fish was small (average weight 58.45 ± 1.60 g) which also corresponds to a small brain. This caused quite a few fish to have a very low mRNA concentration in the *Saccus vasculosus* and also in the pituitary. The mRNA concentration after RNA extraction was below 125ng μl^{-1} and could not be used to create cDNA, since 125ng μl^{-1} is the required minimum to be able to make the cDNA concentration needed for this analysis. This was an unforeseen issue and 8 fish from each sampling proved to be insufficient. Only 3 of 8 fish from the control group could be used for the analysis.

POMCa2s showed a melting curve of poor quality with no single peak with regards to forebrain, midbrain and the cerebellum (Figure 4.1). For hypothalamus, *Saccus vasculosus* and the pituitary, the melting curve showed a single peak, which is an indicator of high

specificity of the primers (Figure 4.2). In an earlier study (Valen et al., 2011) the same primers were used successfully for POMCa2s, but that analysis was conducted with the whole brain, and not dissected into different parts. The primers for POMCa2s didn't seem to be specific for the forebrain, midbrain and the cerebellum, but showed good results for the hypothalamus, *Saccus vasculosus* and the pituitary. Other qPCR tests run at this department with the same primers also confirmed the same problem with the forebrain, midbrain and cerebellum for POMCa1, POMCa2 and POMCb. New primers were designed to be more specific for the different isoforms of POMCa2s, but these could not be tested and validated in time for this thesis.

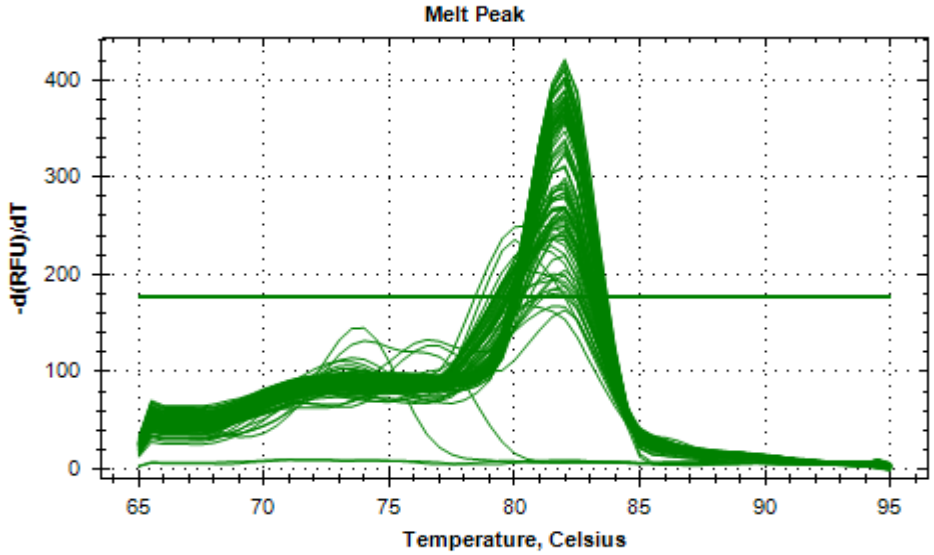

Figure 4.1: Melting curve for POMCa2s for all tissues of the brain. Green straight line is the threshold line for the CT value. 8 fish with all brain tissues and replicates are included for this graph.

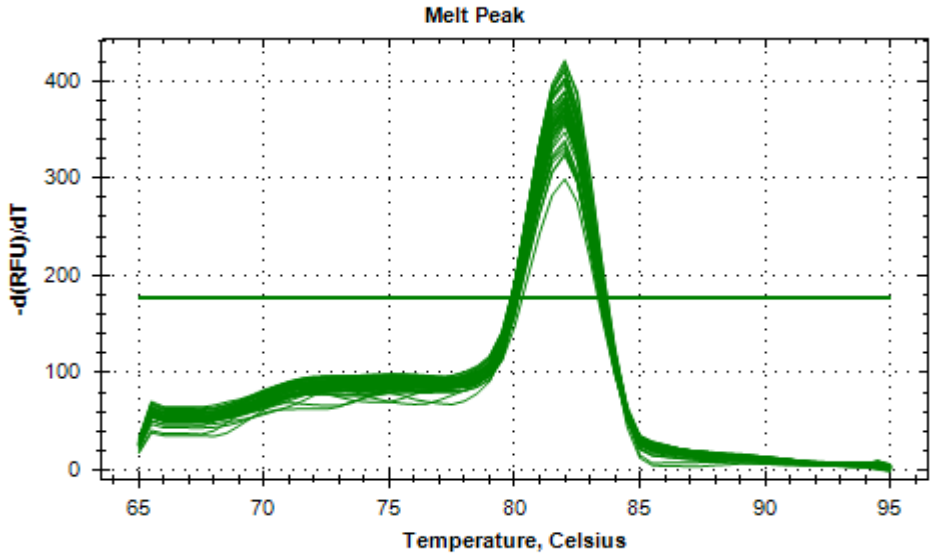

Figure 4.2: Melting curve for POMCa2s for hypothalamus, Saccus vasculosus and the pituitary. Green straight line is the threshold line for the CT value. The same 8 fish that is shown in figure 4.1 is present here, but forebrain, midbrain and cerebellum are not included. NRT and NTC is excluded as well.

Another challenge for this experiment was the experimental design. From the first to second sampling (November 16th – 19th) the only effect taking place was starvation for 3 days, so differences in mRNA expression here can be accounted for as differences caused by starvation. For sampling three to six (November 19th, 20th, 22nd and 26th) the fish was both starved and stressed from vaccination. Two different parameters were deviant from normal conditions during these samplings. These different parameters (starvation and stress) have an opposite effect on the appetite and will work against each other. The last sampling consisted of vaccinated fish, but on this sampling the fish was fed. For the rest of the samplings there will be an uncertainty to what effect that will cause differences. Either starvation or stress can be contributing factors to a difference in mRNA expression.

Low sample size increases the variance and standard error, and it will be harder to observe any significant differences. This is shown by large error bars in the results and many of the results are not significantly different. If this experiment is replicated, a larger sample size should be used, and larger fish collected to reduce the probability of too low mRNA concentration in the smaller parts of the brain (*Saccus vasculosus* and pituitary). The design of

the experiment should also be different, to only have starvation or stress as a factor. This will be a challenge to conduct during a vaccination protocol as the fish is starved as a standard protocol. To overcome this issue a study of the appetite can be conducted with a starvation period of two weeks to see the impact on the mRNA expression in each of the brain tissues. When the expression is known with the effect of starvation, the experiment can be duplicated with a vaccination protocol with the same starvation. This way the effect of the vaccination can be seen in relationship with the starvation effect.

4.3 Discussion of results

4.3.1 Spatial and temporal changes in NPY

NPY has been identified as one of the most potent orexigenic peptides in mammals as well as other species (Valassi et al., 2008, Rønnestad et al., 2017). NPY seems to have variable roles in appetite control in different fish species. In a study on Atlantic salmon NPY did not affect short-term feeding to the extent it does in some other species e.g. cod and rainbow trout (Aldegunde and Mancebo, 2006, Kehoe and Volkoff, 2007, Murashita et al., 2009). In Chinook and Coho salmon (*Oncorhynchus tshawytscha, Oncorhynchus kisutch*) mRNA expression of NPY was shown to increase in hypothalamus during fasting which indicates an orexigenic effect (Silverstein et al., 1998). In Atlantic salmon however 6 days of fasting showed no significant expression of NPY in the brain (Murashita et al., 2009).

NPY has different roles in addition to be a powerful enhancer of the appetite. These include physiological processes such as cardiovascular control, anxiety, sexual behavior and circadian rhythms (Dumont et al., 1992, Murashita et al., 2009). NPY has also been found in high concentration in the eye and also in the forebrain of the salmon. Since the primary center for the appetite regulation is the ARC in the hypothalamus, this suggest different roles for NPY in other regions of the brain (Murashita et al., 2009).

For this experiment all the brains were dissected into 6 different parts, and the concentration of NPY was studied for all the different parts. Hypothalamus showed a low concentration (10.2%) of expressed mRNA compared to the forebrain. There was a significant higher ($p<0.05$) mRNA concentration in the forebrain than all the other parts of the brain. No other significant difference was found between other parts of the brain (Figure 3.3). The expressed levels of mRNA in the midbrain apparently were higher than in the cerebellum and pituitary,

but these differences are not significant. With a higher mRNA concentration in the forebrain than in the hypothalamus, this may suggest that NPY has other roles in this part of the brain or that feeding is also affected by pathways in the forebrain. In a study with cod larvae, NPY also had the highest expression in the forebrain (telencephalon), as well as in the midbrain (Le et al., 2016). This indicate some participation in appetite control by the forebrain previously not described in the salmon. With a higher sample size, the results may reveal significant differences between the other parts as well, but it still needs to be verified that the expression of NPY in this section responds with feeding, hunger and satiety.

The mRNA concentration of NPY showed no significant differences between the different samplings in all parts of the brain except in the midbrain between the second sampling (November 19th) and the last sampling (November 30th) (Figure 3.8). The fish from the second sampling had been starved for 3 days and the fish from the last sampling was recently fed. Since NPY is assumed to be an orexigenic neuropeptide, this will naturally decrease as feeding occurs in fish. This could be a reason for the mRNA expression to be significantly lower in the last sampling than in the second sampling. The difference is however in the midbrain, not a tissue normally identified with feed regulation. This might suggest other properties of the NPY in the midbrain of the salmon, that is influenced by starvation and feeding. It could also indicate that midbrain of Atlantic salmon is involved in appetite regulation previously not described.

4.3.2 Spatial and temporal changes in CART

CART was first discovered in rats in 1995 and it is a potent anorexigenic neuropeptide in mammals as well as in fish. Multiple CART variants have been described for a variety of different animals and fish species, but only one CART has been described in Atlantic salmon. This CART isoform will be the focus for this thesis (Valassi et al., 2008, Murashita et al., 2009, Kehoe and Volkoff, 2007, Valen et al., 2011).

In Atlantic salmon fasting for 6 days decreases the mRNA expression of CART in the brain which suggest an anorexigenic effect in salmon. CART regulates both the short-term and long-term feeding and plays a role in central control of the body weight (Valen et al., 2011). The involvement of CART in food regulation is also believed to be in the ARC in the hypothalamus, but this still needs further investigation. CART is also highly expressed in the

eye, which suggests other properties for CART in addition to signaling an anorexigenic effect (Murashita et al., 2009). For cod larvae CART has been suggested to play a role in olfactory and visual processes as well as appetite regulation (Le et al., 2016).

In this experiment mRNA expression in the forebrain was significantly ($p<0.05$) higher than in the cerebellum and in the pituitary (Figure 3.4). There was no significant difference between the forebrain and the other parts of the brain. Between midbrain, cerebellum, hypothalamus, *Saccus vasculosus* and the pituitary there was no significant difference in mRNA expression of CART. Hypothalamus showed a higher (4.38 and 18.25 times respectively) mRNA concentration than in the cerebellum and the pituitary, however this was not a significant difference.

No significant difference in mRNA expression was observed between the different samplings in any of the brain tissues. From previously mentioned studies (Murashita et al., 2009), the levels of mRNA expression in the whole brain decreased with fasting for multiple days, however this was not observed in this experiment. There are a few possible reasons to why the mRNA expression of CART did not show a significant difference during this experiment. The first reason could be the low sampling size in the different samplings (n=3-6), which causes an uncertainty of the results, were one fish can affect the result in both positive and negative terms. Another explanation of the undifferentiated CART expression could be the experimental design. Fasting has shown a decrease in the expressed levels of CART, however stress has been shown to reduce the appetite of Atlantic salmon (as well as other species) (Madaro et al., 2015, Bernier, 2006). CART stimulates CRF (Corticotropin releasing factor) secretion which in turn is a hormone associated with a stress response in salmon as well as other species. Stress regulates the CART mRNA expression in the hypothalamus which works in contrast to the previous mentioned effect of a decrease in CART expression during fasting (Koylu et al., 2006). Therefore, it may be two opposite effects taking place during this experiment which makes these results hard to interpret.

4.3.3 Spatial and temporal changes in AgRP-1

AGRP coupled with NPY are strong orexigenic factors in the ARC are the main inducers of feeding in mammals. AgRP works as a competitive antagonist against POMC derivatives

Melanocortin-4-receptor (MC4R) and α-melanocyte-stimulating hormone (α-MSH) in rodents (Harrold et al., 1999, Rønnestad et al., 2017, Ollmann et al., 1997).

In a study done on rats including a control group, a dietary-obese group and a food-restricted group, the obese group showed a significant increase in of AgRP concentration and the restricted group showed a significant decrease in AgRP concentration (Harrold et al., 1999). This further confirms the role of AgRP as a potent orexigenic neuropeptide. In Atlantic salmon two different forms of AgRP has been discovered; AgRP-1 and AgRP-2. AgRP-1 is mainly expressed in the pituitary as well as the skin, but also in different tissues like the brain ovaries and the eyes. AgRP-1 is also present in the female gonads (not in males) which suggests a gender difference in other functions than the orexigenic factor. AgRP-2 was present and expressed in all tissues examined. This is an indicator of AgRP-1 and AgRP-2 has different roles in Atlantic salmon (Murashita et al., 2009).

In this experiment the mRNA expression of AgRP-1 showed no significant differences between the different tissues in the brain (Figure 3.5). The expressed levels of AgRP-1 in hypothalamus, *Saccus vasculosus* and the pituitary is around the same levels (72.1, 75.8 and 86.0 times the concentration of the midbrain respectively), but these differences are not significant, and no conclusion can be drawn from this experiment to which tissues contains the highest concentration of AgRP-1.

For the second part of this experiment AgRP-1 showed no significant differences between any of the different samplings in the different tissues. Murashita et al., 2009 showed a decrease in mRNA expression for AgRP-1 during 6 days of fasting for the whole brain. This experiment could not replicate these results, most likely due to differences in analysis of whole and dissected brains, the previously mentioned sample size and the experimental design with both fasting and stress from vaccination simultaneously.

4.3.4 Spatial and temporal changes in POMCa2s

POMC is a group of potent anorexigenic peptides and has been described in many different groups of animals, including mammals and fish. Cleavage products from POMC includes α- β- and -γ MSH and ACTH all of which are believed to play a role in appetite regulation.

(Valassi et al., 2008, Klovins et al., 2004). POMC are also involved in other physiological functions such as stress response, steroid synthesis and lipolysis (Murashita et al., 2011). In the Atlantic salmon four different isoforms of POMC has been identified (-a1, -a2, -a2s and -b). During an experiment with fasting for 6 days only POMCa1 showed a significant difference ($p<0.05$) in mRNA expression (Murashita et al., 2009). This could be an indication of a stronger anorexigenic effect in POMCa1 than the three other isoforms. This experiment will however be focusing on POMCa2s.

For the first part of this experiment the mRNA expression of POMCa2s in the different brain tissues was examined. The pituitary showed a significant higher mRNA expression of POMCa2s than the other tissues in the brain. Hypothalamus and *Saccus vasculosus* showed a higher concentration of POMCa2s than forebrain, midbrain and cerebellum, however there was no significant difference between any of these tissues (Figure 3.6). Even though the ARC located in the hypothalamus is the primary area involved in regulation of feed intake (Valassi et al., 2008, Rønnestad et al., 2017), the mRNA expression in this tissue is much lower than in the pituitary (0.13%). This either suggest a feed regulation for POMCa2s in the pituitary or another function of POMCa2s expressed in the pituitary. In Coho salmon POMC-related peptides is produced in pars intermedia in the pituitary (a region between the anterior- and the posterior pituitary) (Rand-Weaver et al., 1992). Because of the close relationship between Atlantic salmon and Coho salmon (both parts of the subfamily *Salmonidae*) an assumption can be made that Atlantic salmon also produces POMC in the pars intermedia in the pituitary.

The mRNA expression in midbrain, cerebellum, hypothalamus, *Saccus vasculosus* and pituitary showed no significant difference between the samplings. In the forebrain there was a significant difference from the first sampling to all the other samplings. From a previous study, no significant difference in mRNA expression of POMCa2s was observed from 3hpf to 24hpf in the brain (Valen et al., 2011). The study analyzed the whole brain and did not divide the brain into different parts. This experiment divided the brain and the expression of POMCa2s in the forebrain only contained 0.05% of the concentration in the pituitary. This could be a reason no significant difference was observed during the other experiment which used the whole brain, however a significant difference in the forebrain could be observed for this experiment with dissected parts.

5 Conclusion

In conclusion this experiment showed that all the four neuropeptides believed to be key players in appetite control of Atlantic salmon are expressed in the hypothalamus, but also to a very variable degree in other parts of the brain. There was a significant difference in mRNA expression between the different brain tissues for three of the four studied neuropeptides (NPY, CART and POMCa2s). However, for AgRP-1 there was no significant differences, even though there was a tendency for a higher concentration in hypothalamus, *Saccus vasculosus* and pituitary than the forebrain, midbrain and cerebellum.

Between the samplings during this experiment, the only significant difference observed was in the forebrain for POMCa2s (between the first sampling and all the others) and NPY in the midbrain (between the second and the last sampling). Whether the observed difference here is from the starvation or the stress cannot be stated with certainty. Based on these results the $H0_{1a, b, c, d}$ can be rejected, however the $H0_2$ cannot be rejected as there were little significant differences in mRNA concentration between the different parts of the brain during this experiment. If e.g. an increase in mRNA expression for NPY occurs in the hypothalamus it might not be a significant increase due to the high concentration of NPY in the forebrain if the brain is analyzed as a whole. For further studies on the appetite control in Atlantic salmon, dissection of the brain is therefore recommended to be able to distinguish in which tissues the appetite regulation take place. This could also further the knowledge of other roles of the different neuropeptides.

6 References

Aldegunde, M., Mancebo, M. 2006. Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (*Oncorhynchus mykiss*). *Peptides*, 27, 719-727.

Bernier, N. J. 2006. The corticotropin-releasing factor system as a mediator of the appetite-suppressing effects of stress in fish. *General and Comparative Endocrinology*, 146, 45-55.

Chomczynski, P. 1993. A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *BioTechniques*, 15, 532-534.

Costello, M. J. 2009. The global economic cost of sea lice to the salmonid farming industry. *Journal of fish diseases*, 32,115-118.

Dumont, Y., Martel, J. C., Fournier, A., St-Pierre, S., Quirion, R. 1992. Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. *Progress in Neurobiology*, 38, 125-167.

FAO, 2016. The state of world fisheries and aquaculture contributing to food security and nutrition for all. 2-10

Gutierrez-Wing, M. T., Malone R. F. 2006. Biological filters in aquaculture: Trends and research directions for freshwater and marine applications. *Aquacultural Engineering*, 34, 163-171.

Handeland, S. O., Imsland, A. K., Stefansson, S. O. 2008. The effect of temperature and fish size on growth, feed intake, food conversion efficiency and stomach evacuation rate of Atlantic salmon post-smolts. *Aquaculture*, 283, 36-42.

Harrold, J. A., Williams, G., Widdowson, P. S. 1999. Changes in Hypothalamic Agouti-Related Protein (AGRP), but not α-MSH or Pro-Opiomelanocortin Concentrations in Dietary-Obese and Food-Restricted Rats. *Biochemical and Biophysical Research Communications*, 258, 574-577.

Kehoe, A. S., Volkoff, H. 2007. Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 146, 451-461.

Klovins, J., Haitina, T., Fridmanis, D., Kilianova, Z., Kapa, I., Fredriksson, R., Gallo-Payet, N., Schiöth, H. B. 2004. The Melanocortin System in Fugu: Determination of POMC/AGRP/MCR Gene Repertoire and Synteny, As Well As Pharmacology and Anatomical Distribution of the MCRs. *Molecular Biology and Evolution*, 21, 563-579.

Kolarevic, J., Baeverfjord, G., Takle, H., Ytteborg, E., Reiten, B. K. M., Nergård, S., Terjesen, B. F. 2014. Performance and welfare of Atlantic salmon smolt reared in recirculating or flow through aquaculture systems. *Aquaculture*, 432, 15-25.

Koylu, E. O., Balkan, B., Kuhar, M. J., Pogun, S. 2006. Cocaine and amphetamine regulated transcript (CART) and the stress response. *Peptides*, 27, 1956-1969.

Le, H. T. M. D., Angotzi, A. R., Ebbesson, L. O. E., Karlsen, Ø., Rønnestad, I. 2016. The Ontogeny and Brain Distribution Dynamics of the Appetite Regulators NPY, CART and pOX in Larval Atlantic Cod (*Gadus morhua* L.). *PLoS ONE*, 11:4

Lin, Y. F., Jing, S. R., Lee, D. Y. 2003. The potential use of constructed wetlands in a recirculating aquaculture system for shrimp culture. *Environmental pollution*, 123, 107-113.

Liu, Y., Olaussen, J. O., Skonhoft, A., Wild and farmed salmon in Norway—A review. *Marine policy*, 35, 413-418.

Ljones, T. 2016. Stor settefisk treng kort produksjonstid i fjorden. *Heima*, 2, 26-28 (In Norwegian)

Madaro, A., Olsen, R. E., Kristiansen, T. S., Ebbesson, L. O. E., Nilsen, T. O., Flik, G., Gorissen, M. 2015. Stress in Atlantic salmon: response to unpredictable chronic stress. *Journal of Experimental Biology*, 218, 2538-2550.

Murashita, K., Kurakawa, T., Ebbesson, L. O. E., Stefansson, S. O., Rønnestad, I. 2009. Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (*Salmo salar*). *General and Comparative Endocrinology*, 162, 160-171.

Murashita, K., Jordal, A. E. O., Nilsen, T. O., Stefansson, S. O., Kurokawa, T., Björnsson, B. T., Moen, A. G. G., Rønnestad, I. 2011. Leptin reduces Atlantic salmon growth through the central pro-opiomelanocortin pathway. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 158, 79-86.

Ollmann, M. M., Wilson, B. D., Yang, Y. K., Kerns, J. A., Chen, Y., Gantz, I., Barsh, G. S. 1997. Antagonism of Central Melanocortin Receptors in Vitro and in Vivo by Agouti-Related Protein. *Science*, 278, 135-138.

Olsen, R. E., Sundell, K., Hansen, T., Hemre, G. I., Myklebust, R., Mayhew, T. M., Ringø, E. 2002. Acute stress alters the intestinal lining of Atlantic salmon, *Salmo salar* L.: An electron microscopical study. *Fish physiology and Biochemistry*, 26, 211-221.

Olsvik, P. A., Lie, K. K., Jordal, A. E. O., Nilsen, T. O., Hordvik, I. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. *BMC Molecular Biology*, 6, 21-29.

Rand-Weaver, M., Swanson, P., Kawuchi, H., Dickoff, W. W. 1992. Somatolactin, a novel pituitary protein: purification and plasma levels during reproductive maturation of coho salmon. *Journal of Endocrinology*, 133, 393-403.

Rønnestad, I., Gomes, A. S., Murashita, K., Angotzi, R., Jönsson, E., Volkoff, H. 2017. Appetite-Controlling Endocrine Systems in Teleosts. *Front. Endocrinol.*, 8:73.

Sammouth, S., d'Orbcastel, E. M., Gasset, E., Lemarié, G., Breuil, G., Marino, G., Coeurdacier, J. L., Fivelstad, S., Blancheton, J. P. 2009. The effect of density on sea bass (*Dicentrarchus labrax*) performance in a tank-based recirculating system. *Aquacultural engineering*, 40, 72-78.

Silverstein, J. T., Breininger, J., Baskin, D. G., Plisetskaya, E. M. 1998. Neuropeptide Y-like Gene Expression in the Salmon Brain Increases with Fasting. *General and Comparative Endocrinology*, 110, 157-165.

Valassi, E., Scacchi, M., Cavagnini, F., 2008. Neuroendocrine control of food intake. *Nutrition, Metabolism and Cardiovascular Diseases*, 18, 158-168.

Valen, R., Jordal, A. E. O., Murashita, K., Rønnestad, I. 2011. Postprandial effects on appetite-related neuropeptide expression in the brain of Atlantic salmon, *Salmo salar*. *General and Comparative Endocrinology*, 171, 359-366.

Wan, Y., Zhang, Y., Ji, P., Li, Y., Xu, P., Sun, X. 2012. Molecular characterization of CART, AgRP, and MC4R genes and their expression with fasting and re-feeding in common carp (*Cyprinus carpio*). *Molecular Biology Reports*, 39, 2215-2223.

Yokobori, E., Azuma, Nishiguchi, R., Kang, K. S., Kamijo, M., Uchiyama, M., Matsuda, K. 2012. Neuropeptide Y Stimulates Food Intake in the Zebrafish, *Danio rerio*. *Journal of Neuroendocrinology*, 24, 766-773.

7 Appendix

Table 1. Test results from a one-way ANOVA on mRNA expression for sampling 1 (November 16th) for NPY.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	3.715524	1	3.715524	49.30206	0.000014
Gr	6.112261	5	1.222452	16.22097	0.000056
Error	0.904349	12	0.075362		

Table 2. Test results from a one-way ANOVA on mRNA expression for sampling 1 (November 16th) for CART.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	24.39542	1	24.39542	57.58610	0.000006
Gr	11.56579	5	2.31316	5.46028	0.007544
Error	5.08360	12	0.42363		

Table 3. Test results from a one-way ANOVA on mRNA expression for sampling 1 (November 16th) for AgRP-1.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.010475	1	0.010475	6.118759	0.029299
Gr	0.008341	5	0.001668	0.974437	0.471416
Error	0.020544	12	0.001712		

Table 4. Test results from a one-way ANOVA on mRNA expression for sampling 1 (November 16th) for POMCa2s.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	19.62781	1	19.62781	4.285424	0.060674
Gr	97.36960	5	19.47392	4.251826	0.018599
Error	54.96157	12	4.58013		

Table 5. Test results from a one-way ANOVA on mRNA expression between samplings for NPY in forebrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	90.27248	1	90.27248	80.42889	0.000000
NPY	4.34537	6	0.72423	0.64526	0.693324
Error	26.93730	24	1.12239		

Table 6. Test results from a one-way ANOVA on mRNA expression between samplings for NPY in midbrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	6.468116	1	6.468116	284.7343	0.000000
NPY	0.372825	6	0.062137	2.7354	0.036067
Error	0.545192	24	0.022716		

Table 7. Test results from a one-way ANOVA on mRNA expression between samplings for NPY in cerebellum.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.083500	1	0.083500	10.21401	0.003879
NPY	0.070184	6	0.011697	1.43085	0.244054
Error	0.196200	24	0.008175		

Table 8. Test results from a one-way ANOVA on mRNA expression between samplings for NPY in hypothalamus.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.625607	1	0.625607	90.29484	0.000000
NPY	0.069297	6	0.011549	1.66695	0.172502
Error	0.166284	24	0.006928		

Table 9. Test results from a one-way ANOVA on mRNA expression between samplings for NPY in *Saccus vasculosus*.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	1.252887	1	1.252887	153.1362	0.000000
NPY	0.132619	6	0.022103	2.7016	0.037850
Error	0.196356	24	0.008182		

Table 10. Test results from a one-way ANOVA on mRNA expression for between samplings for NPY in pituitary.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.009872	1	0.009872	148.4911	0.000000
NPY	0.000385	6	0.000064	0.9642	0.469946
Error	0.001596	24	0.000066		

Table 11. Test results from a one-way ANOVA on mRNA expression between samplings for CART in forebrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	265.5782	1	265.5782	291.2552	0.000000
NPY	10.1370	6	1.6895	1.8528	0.130988
Error	21.8842	24	0.9118		

Table 12. Test results from a one-way ANOVA on mRNA expression between samplings for CART in midbrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	24.24030	1	24.24030	293.4843	0.000000
NPY	1.11022	6	0.18504	2.2403	0.073906
Error	1.98228	24	0.08259		

Table 13. Test results from a one-way ANOVA on mRNA expression between samplings for CART in cerebellum.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	4.600418	1	4.600418	81.98073	0.000000
NPY	0.415060	6	0.069177	1.23275	0.324667
Error	1.346780	24	0.056116		

Table 14. Test results from a one-way ANOVA on mRNA expression between samplings for CART in hypothalamus.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	28.84457	1	28.84457	147.5717	0.000000
NPY	1.74573	6	0.29096	1.4886	0.224320
Error	4.69107	24	0.19546		

Table 15. Test results from a one-way ANOVA on mRNA expression between samplings for CART in *Saccus vasculosus*.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	40.69751	1	40.69751	330.1366	0.000000
NPY	1.21917	6	0.20319	1.6483	0.177321
Error	2.95859	24	0.12327		

Table 16. Test results from a one-way ANOVA on mRNA expression between samplings for CART in pituitary.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.166170	1	0.166170	285.5864	0.000000
NPY	0.004961	6	0.000827	1.4211	0.247562
Error	0.013965	24	0.000582		

Table 17. Test results from a one-way ANOVA on mRNA expression between samplings for AgRP-1 in forebrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.000050	1	0.000050	4.195356	0.051625
NPY	0.000123	6	0.000020	1.722032	0.159002
Error	0.000285	24	0.000012		

Table 18. Test results from a one-way ANOVA on mRNA expression between samplings for AgRP-1 in midbrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.000017	1	0.000017	15.42632	0.000633
NPY	0.000007	6	0.000001	1.15258	0.363500
Error	0.000026	24	0.000001		

Table 19. Test results from a one-way ANOVA on mRNA expression between samplings for AgRP-1 in cerebellum.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.000023	1	0.000023	115.9783	0.000000
NPY	0.000001	6	0.000000	0.9373	0.486863
Error	0.000005	24	0.000000		

Table 20. Test results from a one-way ANOVA on mRNA expression between samplings for AgRP-1 in hypothalamus.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.090893	1	0.090893	26.28885	0.000030
NPY	0.011239	6	0.001873	0.54179	0.771211
Error	0.082980	24	0.003457		

Table 21. Test results from a one-way ANOVA on mRNA expression between samplings for AgRP-1 in *Saccus vasculosus*.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	4.613143	1	4.613143	19.10248	0.000206
NPY	1.674008	6	0.279001	1.15531	0.362113
Error	5.795866	24	0.241494		

Table 22. Test results from a one-way ANOVA on mRNA expression between samplings for AgRP-1 in pituitary.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.559537	1	0.559537	81.38450	0.000000
NPY	0.103167	6	0.017195	2.50094	0.050528
Error	0.165006	24	0.006875		

Table 23. Test results from a one-way ANOVA on mRNA expression between samplings for POMCa2s in forebrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.000033	1	0.000033	32.16641	0.000008
NPY	0.000016	6	0.000003	2.66907	0.039655
Error	0.000024	24	0.000001		

Table 24. Test results from a one-way ANOVA on mRNA expression between samplings for POMCa2s in midbrain.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.000481	1	0.000481	2.204521	0.150624
NPY	0.001550	6	0.000258	1.185349	0.347170
Error	0.005232	24	0.000218		

Table 25. Test results from a one-way ANOVA on mRNA expression between samplings for POMCa2s in cerebellum.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.000013	1	0.000013	80.67433	0.000000
NPY	0.000001	6	0.000000	1.17516	0.352181
Error	0.000004	24	0.000000		

Table 26. Test results from a one-way ANOVA on mRNA expression between samplings for POMCa2s in hypothalamus.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.002463	1	0.002463	23.54679	0.000060
NPY	0.000440	6	0.000073	0.70078	0.651700
Error	0.002511	24	0.000105		

Table 27. Test results from a one-way ANOVA on mRNA expression between samplings for POMCa2s in *Saccus vasculosus*.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	0.002684	1	0.002684	26.16421	0.000031
NPY	0.001227	6	0.000204	1.99270	0.106477
Error	0.002462	24	0.000103		

Table 28. Test results from a one-way ANOVA on mRNA expression between samplings for POMCa2s in pituitary.

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	2938.768	1	2938.768	61.17245	0.000000
NPY	251.509	6	41.918	0.87256	0.529301
Error	1152.977	24	48.041		

Table 29. Test results from a post-hoc test (Newman-Keuls test) on mRNA expression between different tissues for NPY during sampling 1 (November 16th).

Cell no.	Gr	{1} 1,7119	{2} 0,47413	{3} 0.04902	{4} 0.17604	{5} 0.29467	{6} 0.02026
1	Forebrain		0.000284	0.000203	0.000273	0.000278	0.000218
2	Midbrain	0.000284		0.279908	0.406527	0.439086	0.311605
3	Cerebellum	0.000203	0.279908		0.581513	0.534510	0.900167
4	Hypothalamus	0.000273	0.406527	0.581513		0.606455	0.770938
5	Saccus vasculosus	0.000278	0.439086	0.534510	0.606455		0.624283
6	Pituitary	0.000218	0.311605	0.900167	0.770938	0.624283	

Table 30. Test results from a post-hoc test (Newman-Keuls test) on mRNA expression between different tissues for CART during sampling 1 (November 16th).

Cell no.	Gr	{1} 2.4867	{2} 0.98718	{3} 0.36449	{4} 1.5955	{5} 1.4637	{6} 0.08739
1	Forebrain		0.064459	0.012665	0.119512	0.174155	0.007236
2	Midbrain	0.064459		0.264185	0.506566	0.387698	0.247654
3	Cerebellum	0.012665	0.264185		0.148729	0.138554	0.611710
4	Hypothalamus	0.119512	0.506566	0.148729		0.808490	0.089949
5	Saccus vasculosus	0.174155	0.387698	0.138554	0.808490		0.095311
6	Pituitary	0.007236	0.247654	0.611710	0.089949	0.095311	

Table 31. Test results from a post-hoc test (Newman-Keuls test) on mRNA expression between different tissues for POMCa2s during sampling 1 (November 16th).

Cell no.	Gr	{1} 0.00308	{2} 0.00111	{3} 0.00098	{4} 0.00841	{5} 0.00692	{6} 6.2449
1	Forebrain		0.999225	0.999999	0.999995	0.998375	0.017548
2	Midbrain	0.999225		0.999950	1.000000	0.999995	0.025946
3	Cerebellum	0.999999	0.999950		1.000000	1.000000	0.034945
4	Hypothalamus	0.999995	1.000000	1.000000		0.999444	0.004013
5	Saccus vasculosus	0.998375	0.999995	1.000000	0.999444		0.010098
6	Pituitary	0.017548	0.025946	0.034945	0.004013	0.010098	

Table 32. Test results from a post-hoc test (Newman-Keuls test) on mRNA expression between different samplings for NPY in midbrain.

Error: Between MS = 0.22272, df = 24.000

Cell no.	NPY	{1} 0.47413	{2} 0.66754	{3} 0.41061	{4} 0.43028	{5} 0.56634	{6} 0.42902	{7} 0.29894
1	1		0.200273	0.926741	0.675641	0.381916	0.901031	0.456849
2	2	0.200273		0.200731	0.153090	0.387091	0.210830	0.028645
3	3	0.926741	0.200731		0.980378	0.569378	0.860403	0.291432
4	4	0.675641	0.153090	0.980378		0.401008	0.990472	0.590676
5	5	0.381916	0.387091	0.569378	0.401008		0.555470	0.140149
6	6	0.901031	0.210830	0.860403	0.990472	0.555470		0.432564
7	7	0.456849	0.028645	0.291432	0.590676	0.140149	0.432564	

Table 33. Test results from a post-hoc test (Newman-Keuls test) on mRNA expression between different samplings for NPY in *Saccus vasculosus*.

Error: Between MS = 0.00818, df = 24.000

Cell no.	NPY	{1} 0.29467	{2} 0.28158	{3} 0.21296	{4} 0.13193	{5} 0.25242	{6} 0.14153	{7} 0.12270
1	1		0.834956	0.562400	0.130814	0.777188	0.132442	0.123948
2	2	0.834956		0.520509	0.147139	0.643094	0.137353	0.147064
3	3	0.562400	0.520509		0.406392	0.531357	0.261536	0.480027
4	4	0.130814	0.147139	0.406392		0.238615	0.878523	0.883160
5	5	0.777188	0.643094	0.531357	0.238615		0.195853	0.257314
6	6	0.132442	0.137353	0.261536	0.878523	0.195853		0.950751
7	7	0.123948	0.147064	0.480027	0.883160	0.257314	0.950751	

Table 34. Test results from a post-hoc test (Newman-Keuls test) on mRNA expression between different samplings for POMCa2s in forebrain.

Error: Between MS = 0.00000, df = 24.000

Cell no.	NPY	{1} 0.00308	{2} 0.00088	{3} 0.00079	{4} 0.00048	{5} 0.00093	{6} 0.00073	{7} 0.00044
1	1		0.010624	0.014028	0.010660	0.004839	0.017812	0.012571
2	2	0.010624		0.893175	0.936111	0.941842	0.974191	0.968312
3	3	0.014028	0.893175		0.895904	0.976155	0.935141	0.959156
4	4	0.010660	0.936111	0.895904		0.963571	0.718121	0.961656
5	5	0.004829	0.941842	0.976155	0.963571		0.991174	0.979408
6	6	0.017812	0.974191	0.935141	0.718121	0.991174		0.910220
7	7	0.012571	0.968312	0.959156	0.961656	0.979408	0.910220	

Table 35. Temperature (°C), CO_2 (mg L^{-1}), NO_2 (mg L^{-1}), NH_4 (mg L^{-1}) and NO_3 (mg L^{-1}) during the experiment. Data provided by Hardingsmolt.

	16/11	17/11	18/11	19/11	20/11	21/11
Temperature °C	13.1	12.9	12.0	12.8	13.2	13.3
CO_2 mg L^{-1}	NA	7	NA	NA	0.5	NA
NO_2 mg L^{-1}	NA	0.096	NA	NA	0.12	NA
NH_4 mg L^{-1}	NA	0.05	NA	NA	0.11	NA
NO_3 mg L^{-1}	NA	250	NA	NA	250-	NA
	22/11	23/11	24/11	25/11	26/11	27/11
Temperature °C	13.3	13.5	13.1	12.8	13.2	13.5
CO_2 mg L^{-1}	3	NA	8	NA	NA	10
NO_2 mg L^{-1}	0.115	NA	0.175	NA	NA	0.180
NH_4 mg L^{-1}	0.02	NA	0.015	NA	NA	0.13
NO_3 mg L^{-1}	250-	NA	100+	NA	NA	250
	28/11	29/11	30/11			
Temperature °C	13.7	12.9	13.4			
CO_2 mg L^{-1}	NA	11	NA			
NO_2 mg L^{-1}	NA	0.470	NA			
NH_4 mg L^{-1}	NA	0.1	NA			
NO_3 mg L^{-1}	NA	250	NA			