Master Thesis

Effects of rapid temperature changes on Atlantic salmon, *Salmo salar*, post-smolts

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Abstract

Year round production of Atlantic salmon post-smolts in closed or semi-closed systems opens up for use of seawater from different depths, which will permit stocking of fish from closed containment system to the open sea cage at different seasons. This may be accompanied by greater temperature gradients, which in turn may result in growth depression, increased stress and mortality. In current study, post-smolts of 250 grams were directly transferred from 10°C to 13°C, 16°C and 18°C seawater. After 45 days weight and length increased significantly in all temperature groups, with no differences between rearing temperature. Post-smolt growth, measured as Thermal Growth Coefficient (TGC) in individually pit-tagged fish were lower in the 13, 16 and 18°C compared to the control (10°C) group. Markers for stress responsiveness (plasma cortisol, telencephalic CRF, CRFbp) and neural plasticity (telencephalic NeuroD and BDNF) were assessed following one and seven days' temperature transfer and after acute confinement stress test (ACT). A transient 50-60-fold increase in plasma cortisol were observed in the 13 and 16°C groups following one and seven days' transfer, while cortisol low in the 10 and the 18°C groups. After 45 days all temperature groups displayed low cortisol levels and responded to ACT by more than a 110-fold increase in plasma cortisol. Surprisingly only minor significant changes in CRF and CRFbp mRNA expression were found in response to abrupt changes in temperature, or in response to an acute challenges test in current study. There was up-regulation of telencephalic BDNF following acute challenge stress test in the 18^0C group. Taken together, our findings indicate that post-molts increase in size following direct transfer to higher temperatures, yet growth rate measured as TGC decrease with increasing temperature. Post-smolts responded well physiologically (cortisol) but displayed only limited (telencephalic BDNF) cognitively to temperatures, which raises concerns from an animal welfare perspective. It should also be emphasized that additional stressors such as handling should be taken into account and it is therefore recommended that direct transfer should not exceed a temperature gradient more 5°C when post-smolts are kept in the temperature range between 10 and 16°C.

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1. Introduction

Norway has the largest Atlantic Salmon production in the world. According to Statistisk Sentralbyrå, Norway produced 1.31 million tonnes of salmon in 2015, which was a 6 % increase from 2014. The Norwegian government and industry has an offensive future scenario with a five-fold increase of salmon production by 2050 (Hersoug, 2015). The predominant production of salmon occurs in open sea cages, taking advantage of the beneficial conditions along the near coast and fjords of Norway. This strategy has allowed for an unprecedented growth over the last decades. However, the annual increment in production volume in today's industry has decreased notably during the last years due to challenges with salmon lice, organic pollution and escapees, sustainable growth is not reasonable. The industry is often forced to deal with several of these challenges, and the treats from sea lice in particular, by increasing the operational handlings, which in turn may effect fish welfare, growth and survival. It has been documented that approximately 16% of the fish transferred to seawater do not survive and a high percentage of this mortality occurs during the early seawater phase (Bleie and Skrudland, 2014, Gullestad, 2011). This results in large economic losses for the industry and is clearly a welfare issue that resonates negatively from a consumer perspective. In this situation development and implementation of alternative productive strategies may play a vital role to secure future sustainable growth of the salmon industry. One suggestion has been to produce larger and thus more robust smolts prior to transfer to open sea cages, a trend that already has been implemented by the industry (Bergheim et al 2009). The legislation has also been adopted so the industry now can produce salmon on land up to 1-kilogram (kg) before transfer to open sea cages. Currently the infrastructure of today's smolt facilities does not allow for such an increase in biomass on land, nor is it an economical viable option. A probable solution of this situation is increasing numbers of Recirculatory Aquaculture Systems (RAS) for production of large smolts up to 250-300 grams for subsequent transfer to either open sea cages, or to closed containment systems (CCS) in the sea where production of post-smolt occur in protective enclosures that allows for better control of production parameters and minimize the footprint on the surrounding environment before they are stocked into open sea cages as 1 kg salmon (Rosten et al 2013).

CCS production strategies opens up the use of seawater from different depths, which will permit a more optimal use of temperature, allowing production at relatively higher and/or optimal temperatures all year-around. Thus, subsequent stocking of fish from CCS to the open sea cage at different seasons may be accompanied by greater temperature gradients and this may result in growth depression, increased stress and mortality depending on size and temperature tolerance of post-smolts. This may result in compromised fish welfare and economical gain if not handled

with care. Knowledge about temperature optima during different developmental stages for particular fish species is very important for successful fish culture. Somatic growth of salmonids may be affected by many factors but is predominantly influenced by temperature and nutrition, and elevated temperature may affect salmonids due to thermal restrictions (Elliott and Ellott, 2010). One important factor for optimal temperature determination is age and size of the fish. As a general rule, with increasing body size and age fish may cope better with extreme water temperatures (Pedersen and Jobling, 1989). The industry has a substantial knowledge and experience with temperature limits in the earlier phases of salmon culture (Forseth et al., 2001; Handeland et al., 2000; 2008). More recent studies have also found that adult Atlantic salmon display reduced appetite and growth when exposed long-term to 17°C and 19°C seawater compared with fish reared at 13°C and 15°C (Hevrøy et al., 2013). It has also been demonstrated that 13°C is the optimal temperature for somatic growth in adult 1.6 (kg) Atlantic salmon, while exposures between 15 - 19°C had negative effects on growth, stress and cardiac functions (Hevrøy et al., 2015; Olsvik et al., 2013; Jørgensen et al., 2014). Optimal growth temperature in seawater for 70-150 grams' Atlantic salmon post-smolts has been suggested to be 12.8°C, while post-smolts between 170-300 grams post-smolts was suggested to be 14°C (Handeland et al., 2000; 2008). Common for most of the studies on temperature effects on salmon post-smolts and adults is based on a gradual acclimation to changing temperatures, often by one degree per day. Although differences in temperature tolerances probably will be higher in post-smolt and adults than those observed in smolts, new production strategies in CCS systems will open for direct year-around transfer from CCS and into sea cages for salmon during different grow-out phases. Given that different sized post-smolts exhibit different temperature optima for growth (Handeland et al., 2008) one may argue that they also may display different temperature preference and tolerance when directly transferred from CCS to open sea cages. The general rule of thumb is to not transfer fish when temperature differences is above 4-5°C and this may vary depending on the temperature gradient during which the fish is transferred and the amount of additional handling stress. Hence a better understanding of tolerance to abrupt changes in temperature among post-smolts and adult salmon would be beneficial.

Fish farming is associated with different operational and environmental factors that may affect fish performance and welfare, e.g. handling, sorting, vaccination, grading, transport, treatment and changes in environmental parameters (Barton and Iwama, 1991). Successful and profitable fish farmers needs to operate within boundaries where fish can overcome, cope with, and recover from challenges they meet during production. How fish respond to the above mentioned challenges is often referred to as stress, a term that in its simplest term may be construed as "a physiological response of the organism" (Schreck, 2000). Several definitions of stress in fishes

exist in the literature (See review by Schreck, 2016). In this thesis I use the term stress as defined by C. Schreck (2000) – "the physiological cascade of events that occurs when the organism is attempting to resist death or reestablish homeostatic norms in the face of insult", with the insult/stressor here being abrupt changes in water temperature and crowding of fish. Depending on duration of the stressor one may divided stress in acute (short-term) and chronic (long-term) phases. The impact of a specific stressor normally induces an integrated response from the cell to the whole fish organism. The terms acute and chronic are often dependent on the context during which they are used and therefore not always easily defined. It has been argued that the physiological response and consequences of the fish rather than the duration of a stressor should be the basis of weather the stressor is considered acute or chronic (Boonstra 2013). The stress response is dependent of the magnitude and duration of the stressor and will determine if the fish is able to completely overcome and adjust back to pre-stress level or, partially recover and compensate sufficiently. If the stressor is too severe the fish will not recover and ultimately lead to decreased growth and increased losses for the farmer. This study is focused on acute stress and adaptive responses during direct exposure to different water temperatures and crowding of fish, which can be compared with operational procedures in the industry.

The perception of a stressor results in a responsive cascade that is essential for the fish to adapt and cope with the stressor. This stress response is often viewed as a primary, secondary and tertiary response (Wendelaar Bonga, 1997, Schreck, 2010). The primary response of environmental stressors activates the hypothalamus – pituitary – interrenal axis (HPI) (Mommsen et al. 1999). This cascade involves the release of catecholamines (adrenaline and noradrenaline) from the head kidney and the hypothalamus releases corticotropin-releasing factor (CRF) to the pituitary, stimulating the synthesis of pro-opiomelanocortin (POMC), which in turn is processed into either adrenocorticotropic hormone (ACTH) or alpha-melanophore-stimulating hormone (α-MSH). Once released into the bloodstream both ACTH and α-MSH activates the synthesis and release of cortisol by the interrenal tissue (Fryer and Lederis, 1986). Cortisol is the primary corticosteroid released in response to stress, secreted by the interrenal tissue (steroidogenic cells) located in the head-kidney of teleost fish. This principal glucocorticoid has a various effect on secondary physical functions, including metabolism, immune ability, and osmoregulation (Iwama *et al.* 1999). If the fish experiences multiple stressors or the stressor persists this may lead to tertiary stress responses, which would include long-term suppression of growth and impaired behavior that ultimately lead to compromised physiological states and increased mortality (Shreck, 2010). Cortisol is responsible for redistribution and priority of energy from 'secondary' biological systems (e.g. immune or

reproductive) toward immediate biological requirements important during the stress response, e.g. cardiovascular system or energy mobilization (Gorissen and Flik, 2016). Once the stressor is no longer present, cortisol level may return to pre-stress concentrations within hours of activation, while under chronic stress conditions cortisol concentration can remain elevated for days and weeks (Madaro et al., 2016). However, cortisol could be depleted or down regulated through an effective negative feedback system (Gorissen and Flik, 2016). One regulator may be the crf binding protein (CRFbp), which binds crf with high affinity. According to Housing et al. (2008) CRFbp may control the HPI axis through inhibiting the biological activity of cCRF by binding CRF and CRF-related peptides and thus decrease their biological activity, which in turn may lead to reduced release of ACTH (Housing et al 2008; Gorissen and Flik, 2016) (Fig.1).

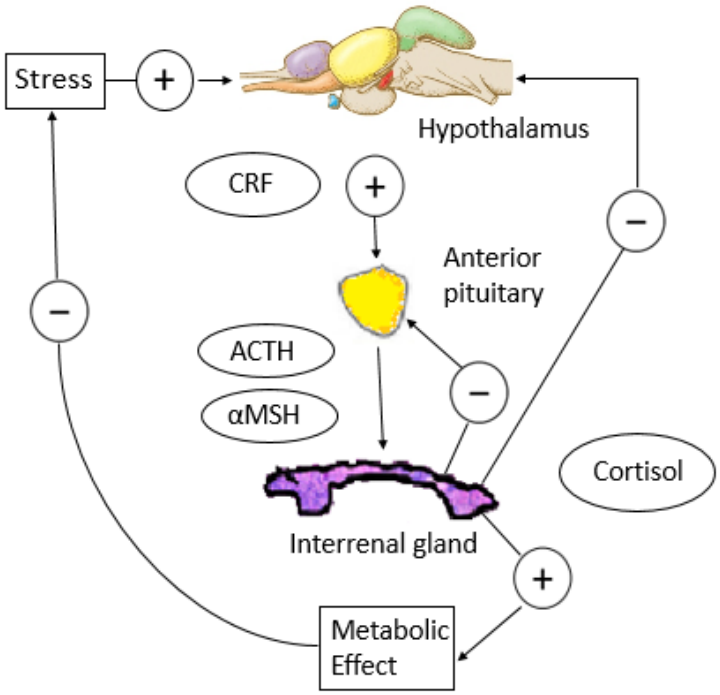

Figure 1: Schematic presentation of key aspects of the hypothalamus – pituitary – interrenal axis (HPI) in teleosts. CRF - corticotropin-releasing factor, ACTH - adrenocorticotropic hormone, αMSH-alpha melanocyte stimulating hormone. Modulated after Canavello et al. (2010).

An appropriate stress response is characterized by the ability to functionally respond both physiologically and cognitively (Ebbesson and Braithwaite, 2012). Cognition is the interaction between perception, learning and memory and recent studies have shown that stress condition, exercises and learning processes can modulate hippocampal neurogenesis which affect neurogenesis, synaptic plasticity in the brain (Rossi et al., 2006; Salvanes et al., 2013). Neurogenesis includes proliferation, migration, differentiation and survival of neurons, and is an integral step in learning and memory that is modulated by stress (Banasr and Duman, 2007).

Neurogenic differentiation factor (NeuroD) is a member of the family of proneural genes that regulates and controls neural differentiation, also in fish (Kiefer et al., 2005; Mueller and Wullimann, 2003). Recent studies suggest neuroD1 may be a reliable marker of neurogenesis in fish and may be a useful indicator of neural plastic changes associated with memory and learning (Grassie et al., 2013; Salvanes et al., 2013). Thus, stress-induced changes in learning could be linked to modifications in neurogenesis through differential expression of neuroD (Grassie et al., 2013). Depending on the species and brain structure, many different neurotrophic factors may affect on neural plasticity. Brain-derived neurotrophic factor (BDNF) is one of the most abundant members of the nerve growth factor family and is important for sculpting and refinement of synapses, promote neurogenesis and cell survival that identify high level of regulation of neural plasticity in multiple brain regions (Suri and Vaidya, 2013; Mattson, Maudsley and Martin, 2004). Recent studies have shown that environmental challenges affect bdnf mRNA expression in the telencephalon of Atlantic salmon (Vindas et al., 2017). Hence neurod1 and bdnf may be promising makers for adaptive changes robustness of fish in culture.

Common for most studies investigating temperature effects on salmon is based on a gradual acclimation to changing temperatures, often by one degree per day. Here we abruptly changed the temperature within 30 minutes. The main objective of this study was to evaluate effects of direct transfer of 250 grams post-smolts from 10°C to 13°C, 16°C and 18°C seawater. The following aspects were focused upon:

1) Long-term effects on growth physiology following abrupt transfer from 10°C to 13°C, 16°C and 18°C seawater.

2) Short and long-term responses in plasma cortisol and mRNA expression of telencephalic crf, crfbp, neurod1 and bdnf in post-smolts following abrupt transfer from 10°C and to 13°C, 16°C and 18°C seawater.

3) Responses in plasma cortisol and mRNA expression of telencephalic crf, crfbp, neurod1 and bdnf in post-smolts following acute challenge test of post-smolts acclimated for 45 days to 10°C, 13°C, 16°C and 18°C seawater.

2. Material and methods

2.1 Fish material

Atlantic salmon (*Salmo salar*) smolts from Lerøy Seafood Group ASA, type Salmobreed QTL duo were used for this experiment. Fish were fed a commercial dry diet (EWOS Microboost 30, pellet size 2.8 mm) and received standard's vaccines at the smolt facility prior experiment. The smolts arrived the Industrial and Aquatic Laboratory (ILAB) at the Bergen High Technology Center on May 11. A total of 1147 smolts (mean weight 99 g ± 0,61 SEM) were randomly distributed into eight (N=143 in each tank) experimental $1m^3$ indoor tanks (rearing volume; 400 L) reaching a biomass of 13,87 kg ± 0,09 SEM in each tank. The tanks were supplied with flow-through (20 L/min) fresh water (FW) at a constant control temperature of 10 ^{0}C ± 0.017 SEM. The oxygen levels were kept above 84% in the outlet or the duration of the whole study. All fish were exposed to constant light (LL) from fluorescent light tubes installed in the tank cover. The fish in each tank were fed by equal portions of commercial dry diet (EWOS, Norway) by an automatic feeder throughout the whole experiment, except for starvation before sampling and handling. Water temperature and oxygen were monitored in the outlet of each tank as a daily routine to keep environment as similar as possible for all experimental groups.

2.2 Experimental design

The experiment was conducted in Akvahall 2 at ILABs facility, starting on the 11th of May, and ending on 2nd October (see Fig. 1). On May 18th a gradual acclimation to higher salinity was initiated, with salinity being adjusted to 12 ‰ for 5 days, then raised to 22 ‰ for another 5 days before full strength seawater (34,5 ‰ ± 0.008 SEM) was given on 29th of May. After two weeks on full strength seawater the water flow rate was increased to 45 L/min and was maintained at this level throughout the whole experiment. When needed oxygen was added into header tanks to maintain O2 levels above 80% in the outlet.

On May 21 twelve fish from each tank were anesthetized with a nonlethal dose (50 mg L^{-1}) of $NaCO_3$-buffered tricaine methanesulphonate (MS222, Sigma-Aldrich, St Louis, MO, USA) and 1.2 mm pit-tags (Nonatec TM, Luxemburg) was implanted with ergonomic syringe at the dorsal-lateral area of the skin under the dorsal fin, resulting in 24 pit-tagged fish per group. Length and weight of pit tagged fish were also recorded on 11th August prior to temperature transfer and at the end of the experiment on 2nd October. Pit tagged fish that died was substitute by tagging new fish on August 11th so we could have a total of 24 fish in each group (N= 12 for each tank) (Fig.2).

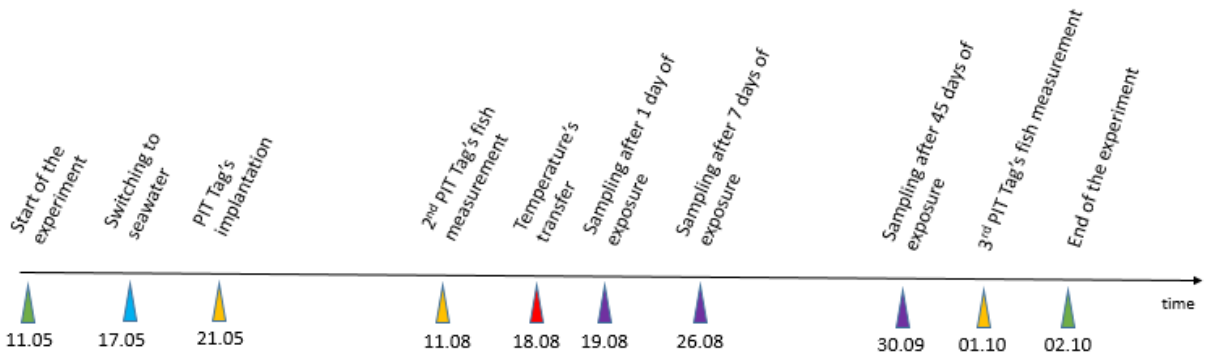

Figure 2: Schematic representation of experimental timeline and different sampling time points.

The water temperature was maintained at 10 ^{0}C in all tanks until the 18th of August when the post-smolts were challenged with a direct abrupt change in water temperature within 30 minutes, resulting in a total of 4 temperature groups, a 13 ^{0}C, 16^{0}C, 18 ^{0}C and 10^{0}C control group (Fig. 3). Fish in each temperature group were kept in duplicate tanks. Temperatures were maintained at these four levels until the end of the experiment (45 days).

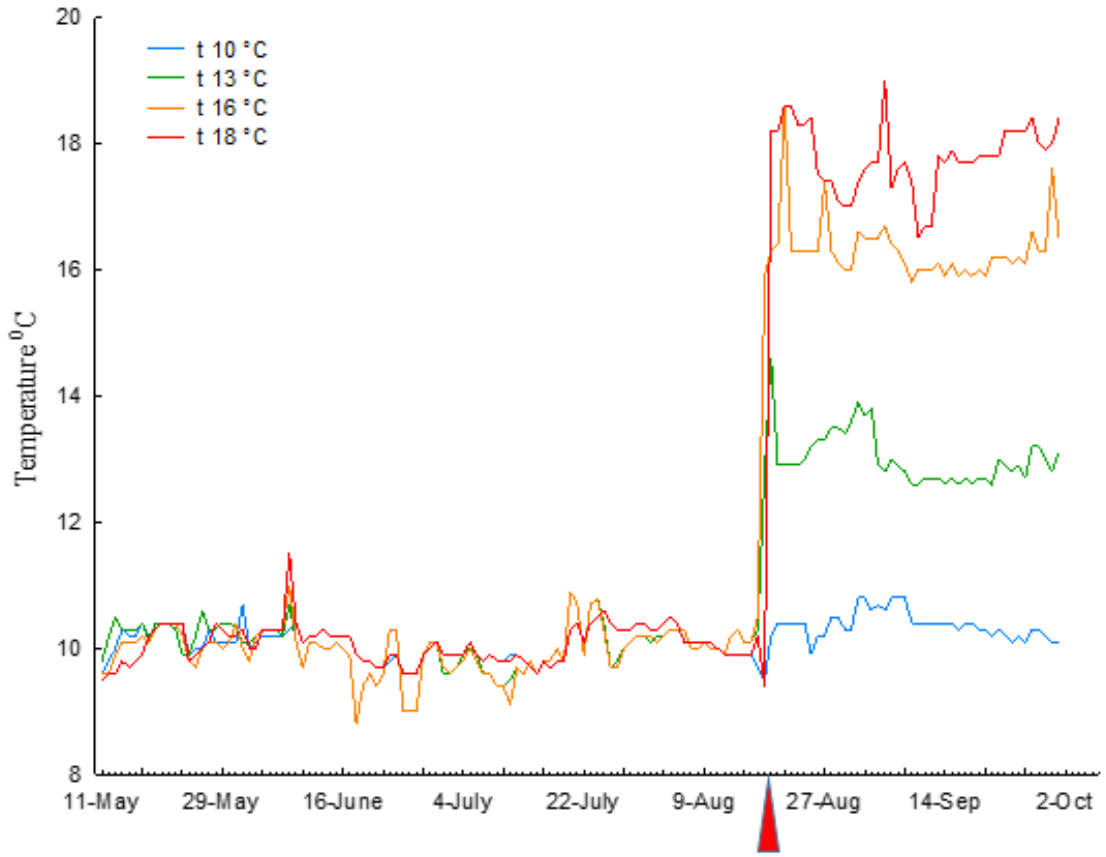

Figure 3: Shows the water temperature profile measured daily throughout the experimental period from May 11th and until October 2nd. Water temperature was adjusted to the target temperature within 30 min on 18th of August (red triangle on the graph).

Due to increasing biomass in each tank during the experiment, two biomass adjustments were done during the early post-smolt growth phase. On the 18th June fish in all tanks were sedated by Aqui S (Scan Aqua A.S, Norway) (25 mg/L) and 20 fish were removed from each tank. Length and weigh of that fish were measured. The second adjustment were done on Augusts 11th and 12th, resulting in a total of 90 post-smolts (weight 233 g ± 4,11 SEM and length 27,5 cm ± 0,16 SEM) with a biomass of 21 kg ± 0,37 SEM in each tank. Fish with fin erosions, other external skin defects and fish clearly deviating in size (looser fish) were eliminated from the tanks during biomass adjustments. This was done so there would be a so homogenous population of post-smolts as possible in all tanks prior to temperature changes was initiated.

To further understand how different temperatures, affect stress responsiveness and neural plasticity in post-smolts, an acute stress challenge test (ACT) was performed 45 days' post transfer to different temperatures. Subsets of six fish per tank (N = 12 per treatment) were sacrificed prior to ACT procedures and served as baseline measurements of hormone levels and mRNA transcript abundance. The ACT was performed by dip netting six fish per tank and then constraining them in a white bucket (15-L) with water from the original treatment for 30 minutes. To maintain sufficient oxygenated water (≥70 %), every 5 min 10 L water from the same experimental tank as the fish originated from were use to change water in the 15 L bucket. Afterwards, stressed fish were placed for 30 minutes to a blue tank (75*65*70 cm) in 125 L water, taken from the same tank as the fish originated from. Every 10 minutes 40 L of water was changed. After confinement stress manipulations, fish were sampled as described below.

2.3 Sampling

The fish were starved one day before six Atlantic salmon post-smolts were quickly dip netted out of the tanks (N = 12 per treatment) and put in a lethal dose (200 mg L^{-1}) of MS222 before weight and length were recorded to the nearest 0.1 gram and 0.1 cm, respectively. Samples were collected 1, 7 and 45 days after water temperature was changed. Blood was collected with heparinized syringes from the caudal veins. Then, as fast as possible tissues from the second gill arch of the left side of the fish, the liver, head kidney, brain, skin and white muscle (from the same place as skin's samples) were collected. Plasma was collected after 5-minutes' centrifugation (4^0 C, 5 min, 3000 rcf) and placed in two tubes, 100 mL in each. Plasma was stored at -20^0C for hormone analyses. All samples were kept on dry ice during sampling and stored at -80^0 C until further analysis.

2.4 Calculation of condition factor, specific growth rate and thermal growth coefficient

Condition factor (CF), specific growth rate (SGR) and thermal growth coefficient (TGC) were calculated according to the following equation:

$$CF = \frac{Total\ body\ weight\ (g) * 100}{Fork\ lenght\ (cm)^3}$$

$$SGR = \left[\frac{(ln\,W2 - ln\,W1)}{t}\right] * 100$$

$$TGC = \frac{\left(W2^{1/3} - W1^{1/3}\right) * 1000}{Day\ degree\ sum}$$

W2 – final weight

W1 – initial weight

t – days number between measurement

Day degree sum – sum of average day temperature between measurements

2.5 Plasma samples

Plasma cortisol was measured using a custom ELISA in a 96-well plate. All wells except the 'non-specifics' received 100 µl cortisol antibody (Cortisol Antibody [East Coast Bio; P01-92-94M-P], Protein A purified monoclonal antibody); 1: 3,000 in 50 mM $NaHCO_3$, 50 mM NaH_2CO_3, pH 9.6) and were incubated overnight at 4°C. The following day, the plates were washed three times with 200 µl/well wash buffer + Tween (100 mM Tris, 0.9% NaCl, 0.1% Tween20). Subsequently, non-specific sites were blocked by the addition of 200 µl blocking buffer (100 mM Tris, 0.9% NaCl, 0.1% Tween20, 2% Normal Calf Serum) to each well. Plates were covered and incubated for one h at RT on a plate shaker (300 rpm). Wells were emptied by decanting, after which 10 µl of standard (4–2048 pg cortisol/10 µl assay buffer containing 100 mM Tris, 0.9% NaCl, 0.1% 8-anilino-1-naphthalene-sulfonic acid, 0.1% Tween20) in triplicate, or 10 µl of undiluted plasma in duplicate was added to designated wells. Non-specifics and B_0 received 10 µl assay buffer (both in triplicate). After the addition of standards and samples, 90 µl

cortisol-HRP conjugate (1: 3,000 East Coast Bio; P91-92-91H, Cortisol-HRP Antigen) solution was added to all wells. Plates were incubated overnight at 4°C or 4 h at RT. The plates were then washed once with wash buffer + Tween, and twice with wash buffer without Tween. 100 µl 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate at room temperature (Sigma Aldrich) was added to each well. After 30 to 60 min (depending on the time required to develop a blue color) incubation in the dark on a plate shaker (300 rpm), 100 µl of stop solution (1M sulfuric acid) was added to all wells. Absorbance was measured within half an hour at 450 nm.

2.6 RNA isolation and quality measurements

The total RNA was isolated from telencephalon using the QIAsymphony SP purification robot and QIAsymphony RNA kit (Qiagen, Hilden, Germany) as described in the manufacturer's protocol. The telencephalon was dissected from the rest of the frozen brain on cooled Petri dishes. Then, telencephalon was weighted (20-25 mg, depending on brain size) and immediately placed into Eppendorf tubes containing 1ml RTL plus lysis buffer (Qiagen, Hilden, Germany) and 6-7 mg zirconium oxide beads (1,4 µm, Bertin technologies, Versailles, France) while tissue was still frozen, followed by tissue homogenization for 15 seconds at 5000 rpm in a Precellys 24 (Bertin technologies, Versailles, France). Homogenized samples were then left at least 5 minutes at room temperature for foam to subsidence and placed in the refrigerator until sufficient number of samples was prepared for RNA purification. Samples, reagents and consumables are loaded into the appropriate QIAsymphony SP drawer before each run. Each reagent cartridge contains all particular reagents, such as magnetic particles, lysis buffer, wash buffer, or elution buffer. RNA is purified through a silica-based RNA purification method with use of magnetic particles. RNA is purified from lysates in one step through its binding to the silica surface of the particles in the presence of a chaotropic salt. The particles are separated from the lysates using a magnet, and DNA is removed by treatment with RNase-free DNase. The purification procedure comprises four steps: lyse, bind, wash, and elute. The magnetic particles are efficiently washed, and RNA is eluted in RNase-free water. The QIAsymphony SP uses a magnetic head containing an array of 24 magnetic rods, that allow to process up to 24 samples simultaneously.

The total RNA concentration and purity was measured by the ND-1000 spectrophotometer (NanoDrop One Technologies, NC, USA) using 1.5 µl total RNA. The RNA to protein ratios (260/280nm) ≥1.8 and the 260/230 nm ratio ≥2.0 indicated that RNA was sufficiently pure of contaminants for further downstream analysis (Bustin et al 2013). The RNA integrity in a select number (24) of samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) with the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto,

CA, USA) following the Agilent RNA Nano protocol according to the manufacturer's recommendations. The Agilent 2100 software classify the RNA Integrity Number (RIN) of eukaryotic total RNA on a number system ranging from 1, being the most degraded, to 10 being the most intact (Mueller, 2004). All tested samples had RIN values above 9 and the assumption was made that this was representative for all samples. Samples were then stored at – 80 ^{0}C.

Four of the samples displayed low RNA concentration (≤ 90,9 ng/µl) and were precipitated by adding Sodium Acetate (NaAC) and 100% ethanol (EtOH) in order to achieve sufficient RNA concentration to proceed with cDNA synthesis. Briefly, RNA precipitation was performed by adding 1/10 of the sample's volume of 3M NaAC and 2.5 times the sample's volume of ice cold 100% EtOH and stored at temperature at – 80 ^{0}C. After samples' centrifugation (30 min, 12000 rcf 4 ^{0}C) the supernatant was decanted. The resulting pellet was then washed with 200 µl 80% EtOH and centrifuged again (5min, 1200 x g, 4 ^{0}C). The supernatant was decanted, tube flash spun and the residual EtOH were removed by pipetting. 10 µl of RNase free water were added to the pellet for dissolution. Samples were flash spun and kept at – 80 ^{0}C until used for cDNA synthesis.

2.7 cDNA synthesis

cDNA was synthesized using the SuperScript^{TM} III Reverse Transcriptase (RT) First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) based on the manufacturer's instructions. 1 µl of oligo (dT) (50µM) and 1µl of 10 mM dNTP Mix (10mM each dATP, dGTP, dCTP and dTTP at neutral pH) were added to 11 µl of total RNA for each sample before incubation at 65 ^{0}C for 5 min in the Thermal Cycler C 1000 Touch (Bio- Rad Laboratories, Inc. USA) to limit the formation of secondary structures. Then, samples were incubated on ice at least 1 minute. After a brief flash spin, the total RNA was added to a tube containing 7 µl of cDNA Synthesis mix containing 4 µl 5x First-Strand buffer, 1 µl 0.1M DDT, 1 µl RNaseOUT Recombinant RNase Inhibitor (40 units / µl) and 1 µl of SuperScript III RT (200 units / µl). All samples were run using 1 µg of total RNA in a total volume of 20 µl. The cDNA was synthesized at 50 ^{0}C for 60 minutes and incubated at 70 ^{0}C for 15 minutes to terminate the reaction. The cDNA samples were stored at – 20 ^{0}C until Real-time quantification PCR.

2.8 Real-time quantification PCR: quantitation of gene expression

Quantitative PCR (q-PCR) was performed to quantify the mRNA abundance of crf, crfbp, neuroD1 and bdnf in telencephalon, using the C1000 Touch Thermal Cycler, CFX96 Real-Time

System (Bio-Rad Laboratories, CA, USA) in conjunction with the software CFX Manager (version 3.1, Bio-Rad). The q-PCR reactions were performed in a total volume of 10µl containing 4,38 µl Gene Expression Master Mix (Bio-Rad Laboratories, CA, USA), 0,3 µl of forward and reverse primer (200 nm final concentration), 0,02 µl of nuclease free water and 3 µl of cDNA diluted 1:10. The q-PCR reactions were performed in 96-well plates (Bio-Rad) and the following thermal cycling protocol was used: 3 min at 95 ^{0}C, 37 repetitions of 15 sec at 95^{0}C and 1 min at 60^{0}C and in the end 10 sec at 95^{0}C, 5 sec at 65^{0}C and 5 sec at 95^{0}C. Information about primer sequences for each qPCR assay are given in the table 1. All samples were run in duplicates and to confirm absence of DNA contamination and residues, "non template control (NTC)" (Bio-Rad Laboratories, CA, USA) was included to the all plates. Furthermore, each plate had duplicate sample of pooled cDNA that was included on each plate and used for correction of differences between plates. Targets genes and reference gene for each individual sample were run in duplicates on separate plates.

Table 1. Primer sequences used in Real-time quantification PCR in present study.

Gene		Primer sequences (5′→3 ′)	Accession no.	Reference
S20	F	GCAGACCTTATCCGTGGAGCTA	BG936672	(Olsvik et al., 2005)
	R	TGGTGATGCGCAGAGTCTTG		
crf	F	AACCAGCTCGACGACTCGATGG	GBRB01035702	(Madaro et al., 2015)
	R	GCTATGGGCTTGTTGCTGTAACTG		
crfbp	F	TGAGCCCAACCAGGTCATCAATG	BT059529	(Madaro et al., 2015)
	R	TCCCTTCATCACCCAGCCATCAA		
bdnf	F	GAGGGCTGCCGTGGAATA	GU108576.1	(Vindas et al., 2014)
	R	CTGGGTTGTCCTGCATTGG		
neurod1	F	CAATGGACAGCTCCCACATCT	BT058820.1	(Salvanes et al., 2013)
	R	CCAGCGCACTTCCGTATGA		

Prior to analyze mRNA expression on all samples all pries set were validated by running two-fold dilution series made from a representative pool of cDNA taken from 54 samples covering all

time points and different groups. Based on the dilution series all qPCR assays were run with cDNA dilution 1 to 10.

The amplification efficiency (E) was determined by the slope of a regression line (threshold cycle (ct) values versus log cDNA dilution) from the dilution curve. The efficiency was calculated based on the following formula (Pfaffl, 2004):

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

Normalization of the genes expression based on target versus reference gene was calculated as follow (Pfaffl, 2004)

$$Ratio = E\,(Ct_{mean})ref \quad / \quad E(Ct_{mean})target$$

Ct - threshold cycle for each individual sample

E ref – qPCR efficiency of the reference gene (SsS20)

E target – qPCR efficiency of the target gene (CRF, CRFbp, NeuroD1, BDNF)

Ct mean – average Ct value of duplicate reaction of individual sample

2.9 Statistics

All data sets were tested for normality using the Kolmogorov-Smirnov test. The Harley F Max test was used to test for homogeneity of variances. Nonparametric analysis was used base on Kruskal-Wallis test. All statistical analyses were performed in Statistica version 13 (StatSoft, Inc., USA). Possible difference between replicates were tested with nested design ANOVA and replicates combined in case of non-significant ANOVAs. A one-way ANOVA was performed for body weight, length, condition factor, specific growth rate, thermal growth coefficient and crfbp, neurod1 and bdnf mRNA expression. Kruskal-Wallis tests were performed for plasma cortisol and crf mRNA expression. Samples with values that were more than 2x standard deviation (2SD) of the mean were considered statistical outliers (Zar, 1996) and excluded from dataset for crf, crfbp, neurod1 and bdnf. Tukey HSD post-hoc tests were used if the one-way ANOVA revealed significant effect between or within groups. Data are presented as means ± standard error of mean (S.E.M.), and p-level of < 0.05 were considered as statistically significant (Table 2-20, appendix B).

3. Results

3.1 Growth parameters

Body weight in pit tagged fish increased significantly from May 21 through August 21 and October 1 (one-way ANOVA, $p<0.001$), while no significant differences in body weight between temperature groups observed (table 2, appendix B), (Fig.4).

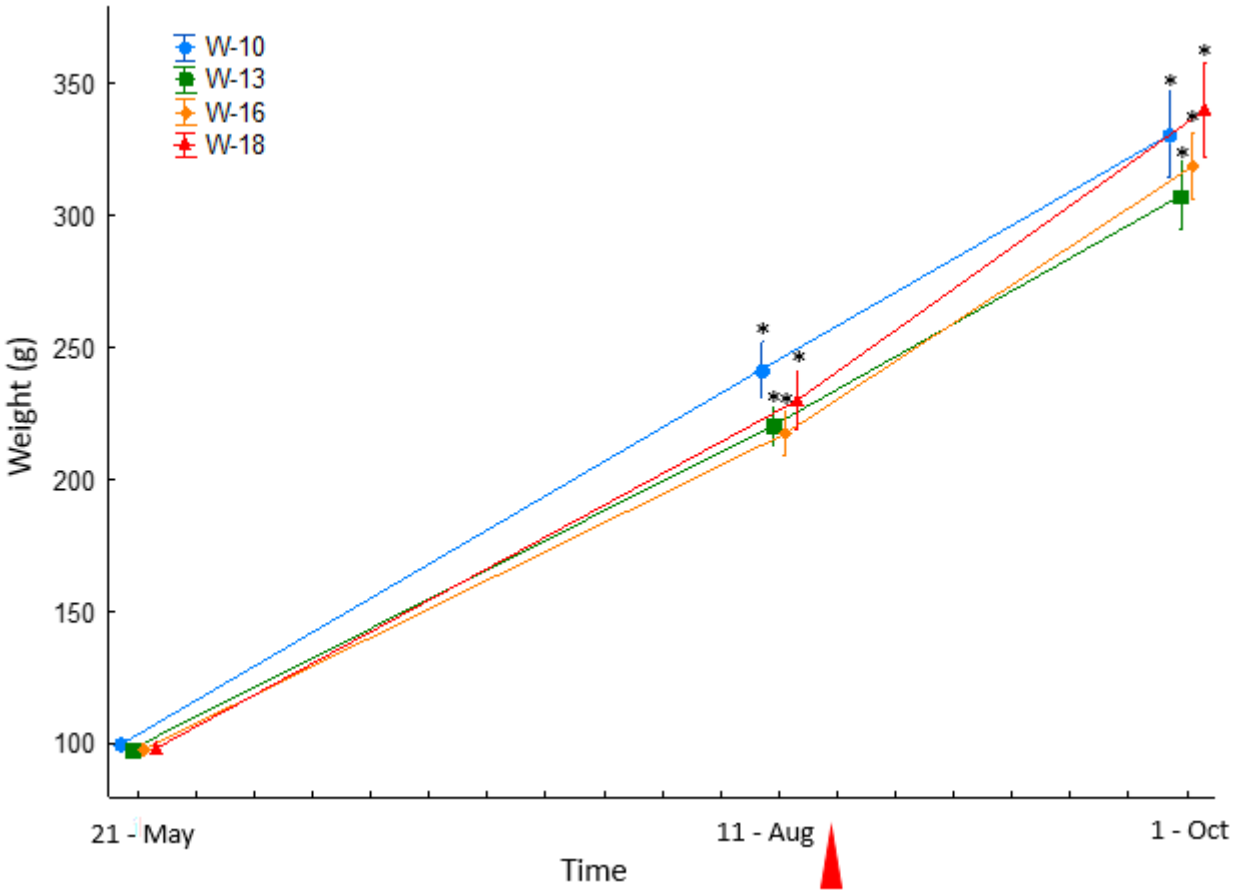

Figure 4. Changes in body weight (gram) of pit-tagged Atlantic salmon post-smolts in 10^0C seawater on May 21 and August 11th. On August 18th four different temperatures regimes (10, 13,16 and 18^0C) was established and weight of all pit tagged fish was measured 45 days' post-transfer (October 1). A total of 24 fish were measured in each group. Data are presented as mean ± and standard error of the mean (SEM). Red triangle indicates date of temperature transfer. Asterisks indicate significant difference from initial weight in 21st May.

Body length of pit tagged fish increased significantly from May 21 through August 21 and October 1 (one-way ANOVA, $p<0.001$), while no significant differences in body length between temperature groups observed (table 3, appendix B), (Fig.5)

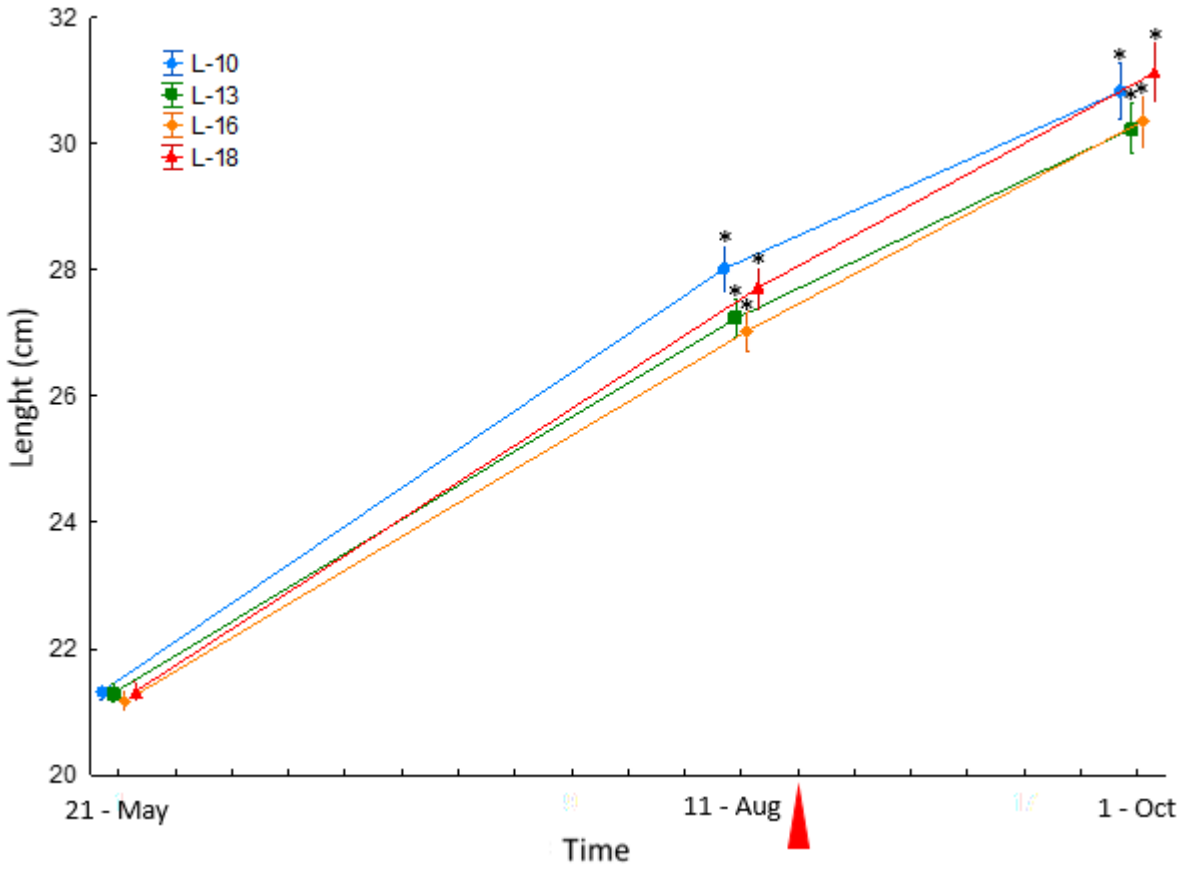

Figure 5. Changes in body length (cm) of pit-tagged Atlantic salmon post-smolts in 10^0C seawater on May 21 and August 11th. On August 18th four different temperatures regimes (10, 13,16 and 18^0C) was established and length of all pit tagged fish was measured 45 days' post-transfer (October 1). A total of 24 fish were measured in each group. Data are presented as mean ± and standard error of the mean (SEM). Red triangle indicates date of temperature transfer. Asterisks indicate significant difference from initial length in 21st May.

Condition factor in pit tagged fish increased significantly between May 21 and August 21 (one-way ANOVA, $p<0.001$). Condition factor in post-smolts increased significantly 45 days after transfer to 16 and 18^0C on October 1 (one-way ANOVA, $p<0.001$), while no significant changes were observed in the 10 and 13^0C groups (table 4, appendix B). No significant differences in condition factor were observed between post-smolts exposed to different temperatures on October 1 (table 5, appendix B) (Fig. 6).

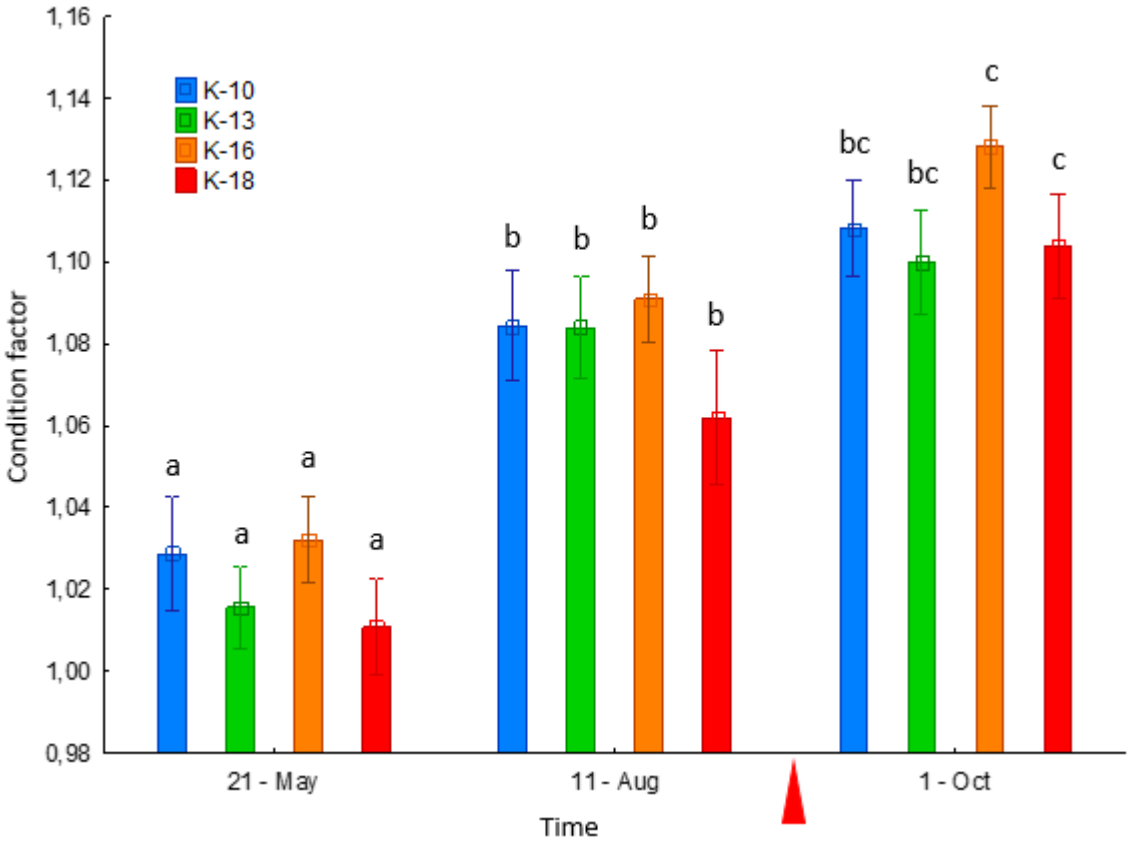

Figure 6. Changes in condition factor of pit-tagged Atlantic salmon post-smolts in 10^0C seawater on May 21 and August 11th. On August 18th four different temperatures regimes (10, 13,16 and 18^0C) was established and length of all pit tagged fish was measured 45 days' post-transfer (October 1). A total of 24 fish were measured in each group. Data are presented as mean ± and standard error of the mean (SEM). Red triangle indicates date of temperature transfer. Different letters indicate significant differences between groups independent from the time points

Specific growth rate was calculated for PIT tagged fish for two periods from 21 May to 11 August and 11 August to the 1 October. One-way ANOVA revealed significant difference between these two periods ($p<0.01$). Furthermore, there was found no significant difference between group in the May-August period ($p>0.05$). One-way ANOVA revealed significant difference between group ($p<0.01$) in August-October period (Fig.7). Tukey test shows that there is significant difference in specific growth rate between groups with treatment 10 ^{0}C and 16^0C, and 10^0C and 18^0C in the August-October period (table 6, Appendix B).

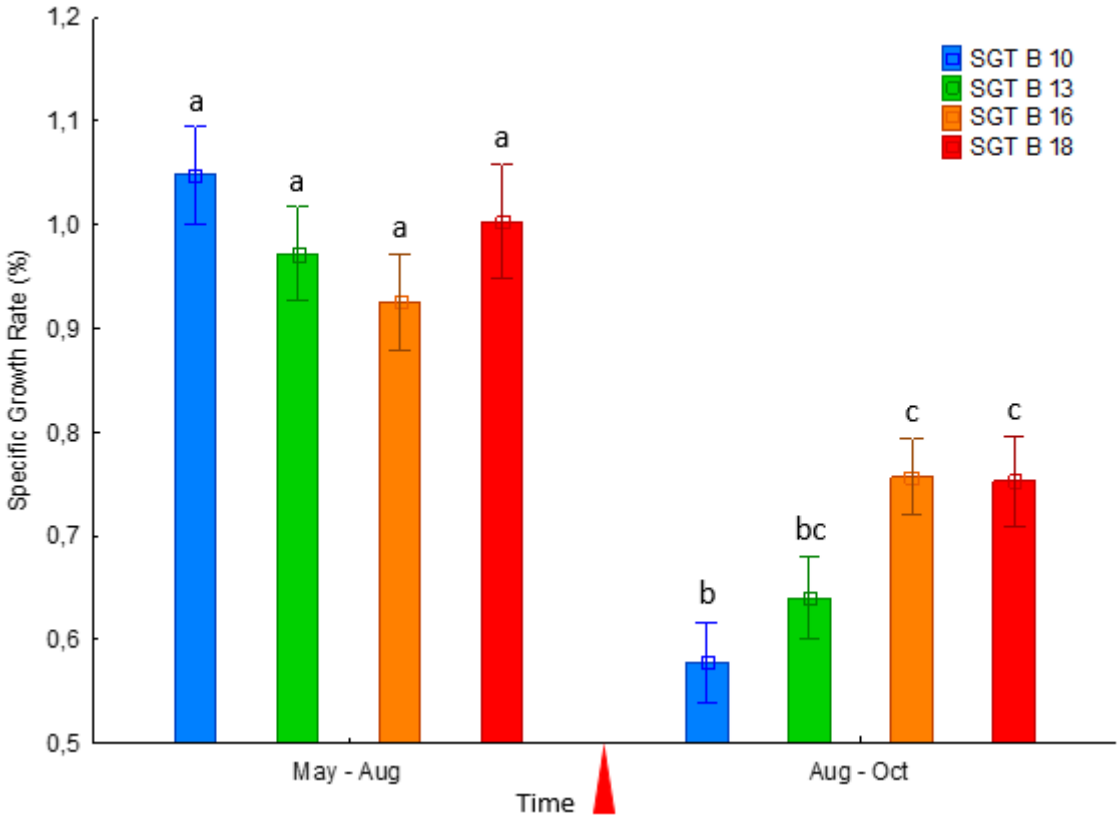

Figure 7. Box plot showing specific growth rate (SGR) of pit-tagged Atlantic salmon post-smolts from May 21st to August 11th and from August 11th and until October 1st. On August 18th four different temperatures regimes (10, 13,16 and 18^0C) was established. A total of 24 fish were measured in each group. Data are presented as mean ± and standard error of the mean (SEM). Red triangle indicates date of temperature transfer. Different letters indicate significant differences between groups independent from the time points.

Thermal growth coefficient was calculated for PIT tagged fish for a period from 21 May to 11 August and 11 August to the 1 October. One-way ANOVA revealed significant difference within treatment's group between these two periods ($p<0.001$) (table 7, Appendix B). Furthermore, there was found no significant difference between groups in the May-August period ($p>0.05$). One-way ANOVA revealed significant difference ($p=0,02$) between groups in August-October period (Fig.8). Tukey test revealed significant difference in thermal growth coefficient between groups with 10 and 18^0C treatment in the August-October period (table 8, Appendix B).

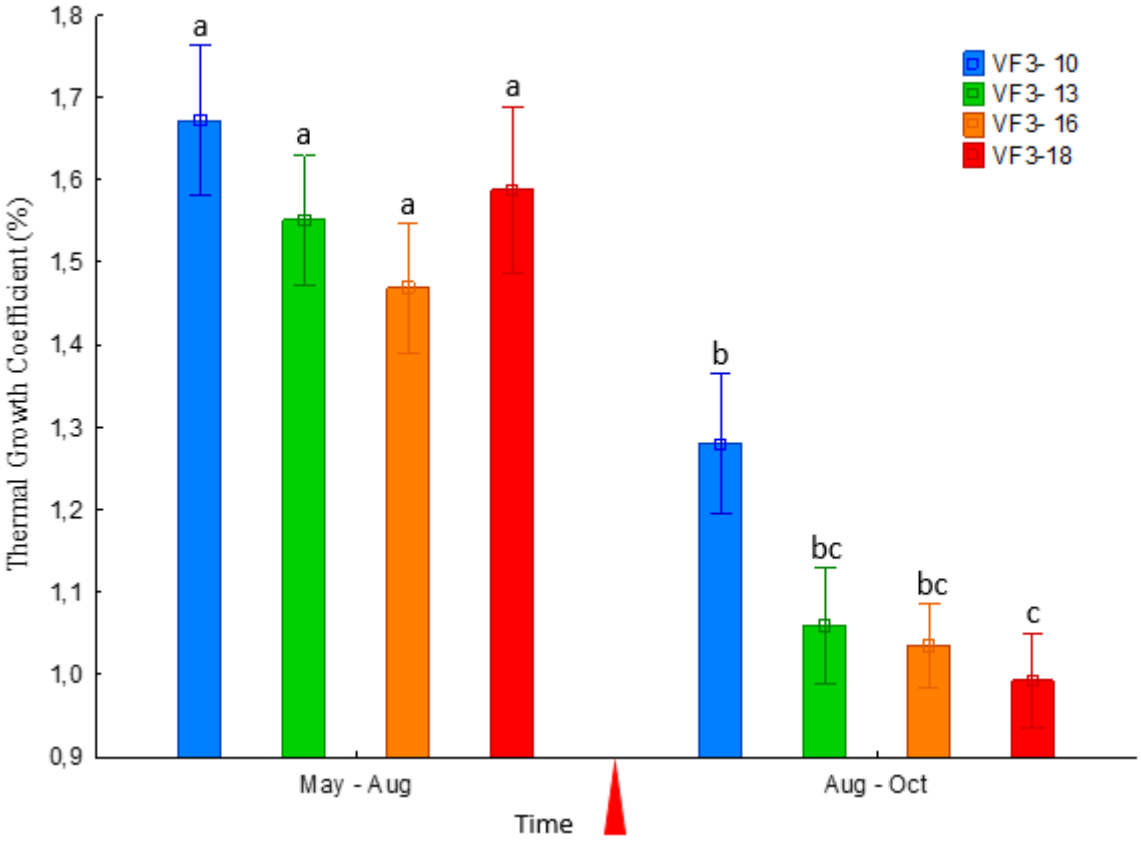

Figure 8. Box plot showing thermal growth coefficient (TGC) of pit-tagged Atlantic salmon post-smolts from August 11th and until October 1st. On August 18th four different temperatures regimes (10, 13,16 and 18^0C) was established. A total of 24 fish were measured in each group. Data are presented as mean ± and standard error of the mean (SEM). Red triangle indicates date of temperature transfer. Different letters indicate significant differences between groups independent from the time points.

3.2 Plasma Cortisol levels

Circulating plasma cortisol levels of 42.7 ± 11.5 ng ml^{-1} and 58.1 ± 13.0 ng ml^{-1} in post-smolts one day after transfer to 16 and 13^0C were significantly higher than cortisol levels observed in fish exposed to 10 and 18 (Kruskal-Wallis tests, p-value ≤ 0.001) (table 11, Appendix B). Cortisol levels remained low in all temperature groups following 7 and 45 days' post transfer, with no differences between groups. Circulating cortisol increased significantly 1 hour after an acute challenge test (ACT), with cortisol levels reaching 121.9 ± 10.2 ng ml^{-1} in the 10^0C group, 111.3 ± 9.7 ng ml^{-1} in the 13^0C group, 138.8 ± 8.4 ng ml^{-1} in the 16^0C group and 116.9 ± 20.5 ng ml^{-1} in the 18^0C group. No differences in plasma cortisol were noted between temperatures groups 1 hours post ACT (table 9-10, Appendix B) (Fig.9).

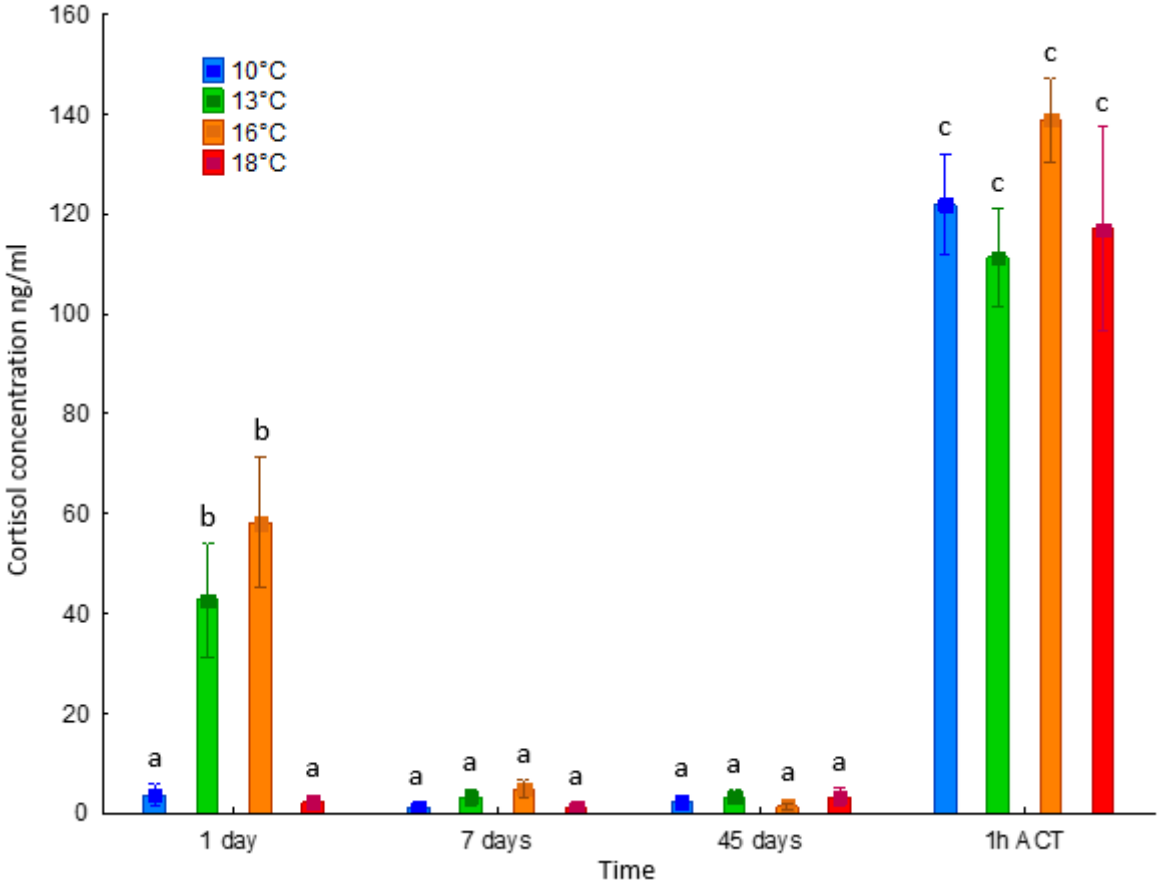

Figure. 9. Changes in plasma cortisol levels Atlantic salmon post-smolts following 1, 7 and 45 days direct transfer to from 10^0C and to 10, 13,16 and 18^0C. Cortisol levels one hour after an acute challenge stress test (ACT) after 45 days at different temperatures is also shown. A total of 12 fish were measured in each group. Data are presented as mean ± and standard error of the mean (SEM). Different letters indicate significant differences between experimental group throughout the whole experiment.

3.3 CRF, CRFbp, NeoroD1 and BDNF mRNA expression

Telencephalon crf mRNA expression was not different among post-smolts following one-day exposure to 10, 13, 16 and 18^{0}C, with crf mRNA levels remaining low after 7 and 45 days in the 10 and 18^{0}C groups. Relative crf mRNA levels in post-smolt exposed to 13^{0}C for 45 days were significantly higher than those observed among fish in the 10^{0}C and 18^{0}C groups (Kruskal-Wallis test, p-value < 0.05). No changes in relative crf mRNA levels were observed within or between groups of ACT challenged fish (table 12 and 14, Appendix B). Furthermore, no significant changes were found between initial level of relative crf mRNA expression and subsequent sampling points within the same treatment group (table 13, Appendix B) (Fig 10).

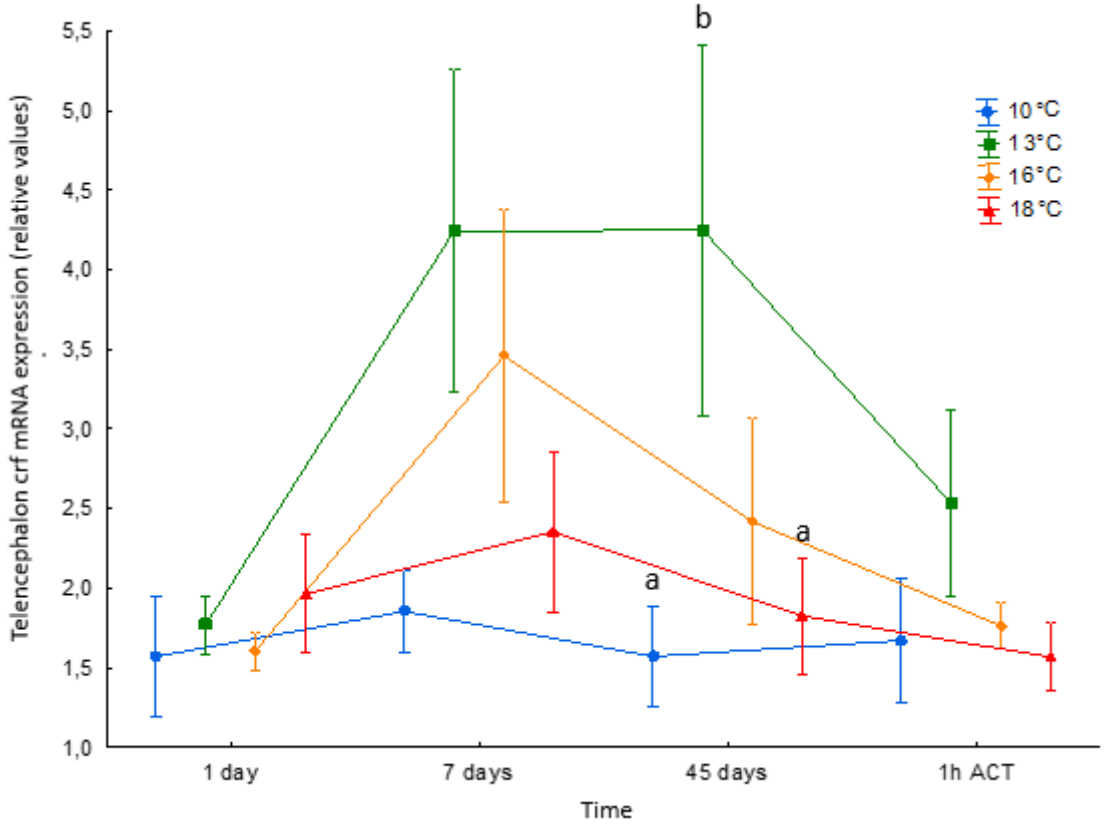

Figure. 10. Relative CRF mRNA expression in telencephalon of Atlantic salmon post-smolts following 1, 7 and 45 days direct transfer to from 10^{0}C and to 10, 13,16 and 18^{0}C. Relative CRF mRNA expression one hour after an acute challenge stress test (ACT) after 45 days at different temperatures is also shown. Significant differences between treatments within the same sampling point are represented by different letters (Kruskal-Wallis test). Data are presented as mean ± and standard error of the mean (SEM).

Telencephalon crfbp mRNA expression was significantly higher among post-smolts at 13 and 16^{0}C than those observed in the 18^{0}C following one-day post-transfer (One-way ANOVA, Tukey HSD test) (table 16, Appendix B). No significant difference was found between groups following one-week, 45 days' post transfer and under acute stress challenge (p-value>0.05) (table 12,

Appendix B). Relative crfbp mRNA levels in post-smolt exposed to 13 and 16^{0}C for 45 days' post-transfer and ACT respectively, were significantly lower than those observed after one-day exposure (p-value < 0.05 and 0.01) (table 15, Appendix B) (Fig 11).

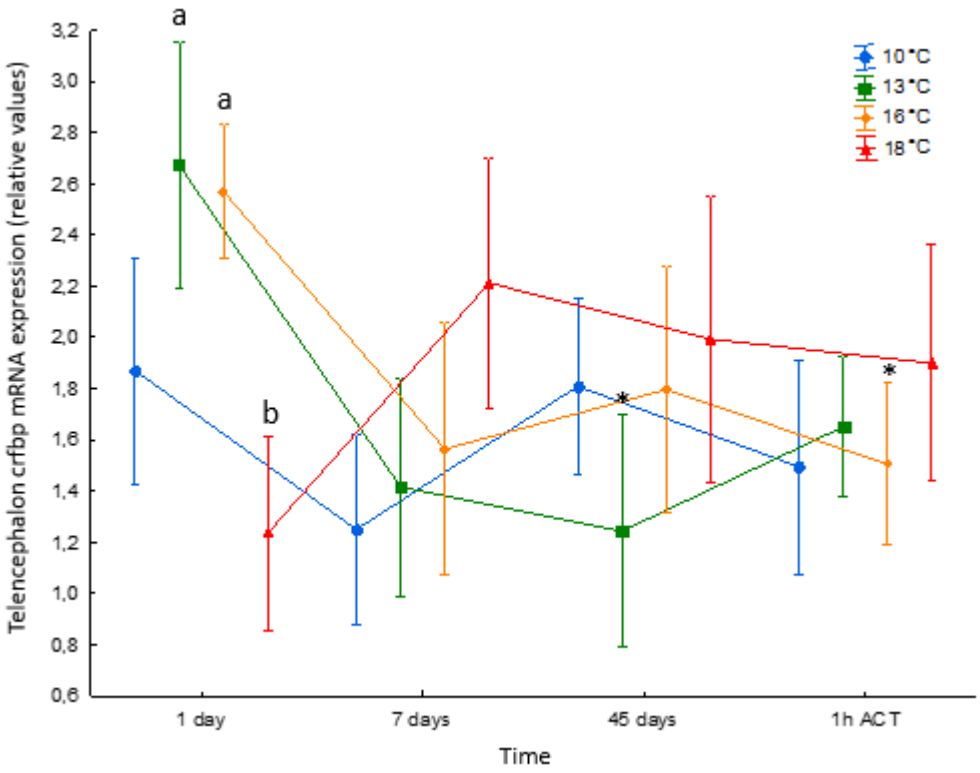

Figure 11. Relative CRFbp mRNA expression in telencephalon of Atlantic salmon post-smolts following 1, 7 and 45 days' direct transfer to from 10^{0}C and to 10, 13,16 and 18^{0}C. Relative CRFbp mRNA expression one hour after an acute challenge stress test (ACT) after 45 days at different temperatures is also shown. Significant differences between treatments within the same sampling point are represented by different letters (One-way ANOVA, Tukey HSD test). Asterisks indicate significant difference from initial level on 1-day post transfer (19 August) (One-way ANOVA). Data are presented as mean ± and standard error of the mean (SEM).

Telencephalon neuroD1 mRNA expression had no significant difference among post-smolts at each group following one-day, one-week, 45 days' post-transfer and under ACT challenge (One-way ANOVA, Tukey HSD test) (table 12, Appendix B). Relative neuroD1 mRNA levels in post-smolt exposed to 13 and 16^{0}C for ACT challenge and 45 days' post-transfer respectively, were significantly lower than those observed after one-day exposure (p-value = 0.05 and 0.01) (table 17, Appendix B) (Fig 12).

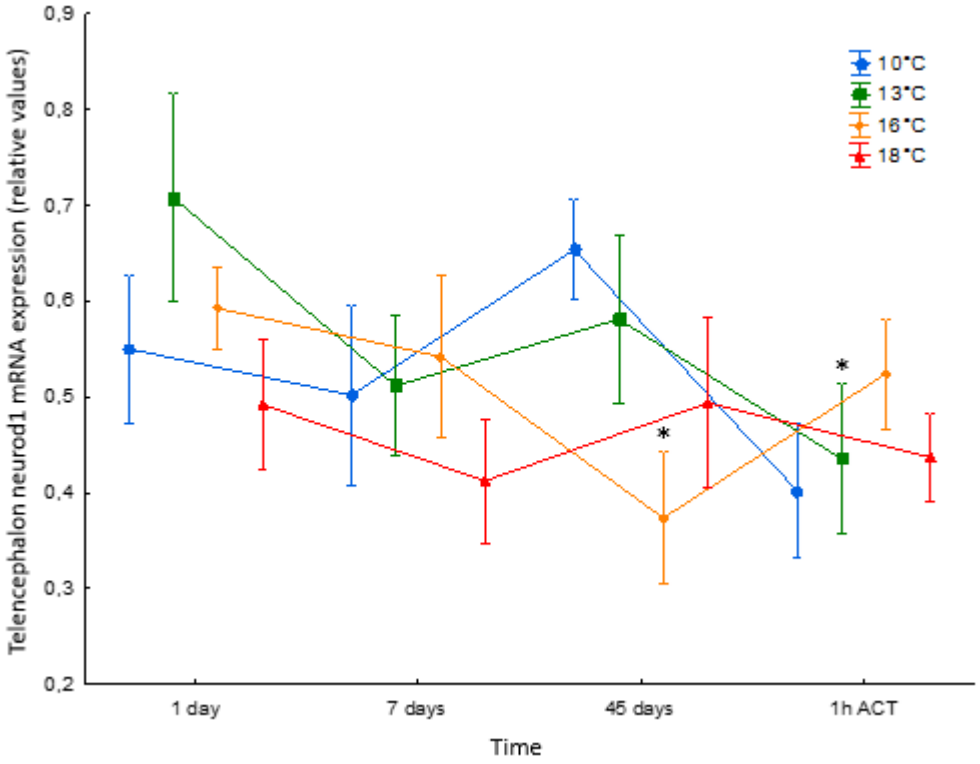

Figure 12. Relative NeuroD1 mRNA expression in telencephalon of Atlantic salmon post-smolts following 1, 7 and 45 days' direct transfer to from 10^0C and to 10, 13,16 and 18^0C. Relative NeuroD1 mRNA expression one hour after an acute challenge stress test (ACT) after 45 days at different temperatures is also shown. Asterisks indicate significant difference from initial level on 1-day post transfer (19 August) (One-way ANOVA). Data are presented as mean ± and standard error of the mean (SEM).

Telencephalon bdnf mRNA expression was not different among post-smolts following one-day and one-week exposure to 10, 13, 16 and 18^0C. Relative bdnf mRNA levels in post-smolt exposed to 13^0C for 45 days were significantly higher than those observed among fish in the 16^0C group (One-way ANOVA, p-value = 0.03) (table 12 and 19, Appendix B). Relative bdnf mRNA levels in post-smolt exposed to 18^0C for ACT challenged fish was significantly higher than those observed among 10 and 13^0C groups (p-value $\leq$ 0.01) (table 20, Appendix B). Relative bdnf mRNA level in post-smolt exposed to 18^0C for ACT challenge was significantly higher than those observed after one-day exposure (p-value < 0.01) (table 18, Appendix B) (Fig 13).

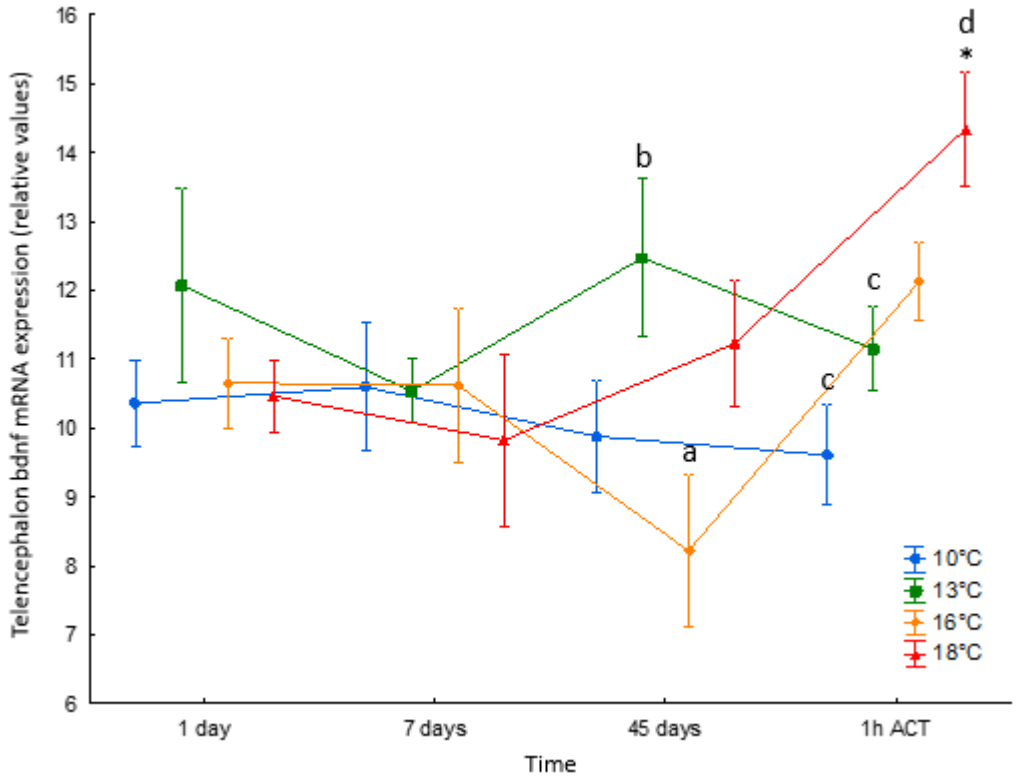

Figure. 13. Relative BDNF mRNA expression in telencephalon of Atlantic salmon post-smolts following 1, 7 and 45 days' direct transfer to from 10^0C and to 10, 13,16 and 18^0C. Relative BDNF mRNA expression one hour after an acute challenge stress test (ACT) after 45 days at different temperatures is also shown. Significant differences between treatments within the same sampling point are represented by different letters (One-way ANOVA, Tukey HSD test). Asterisks indicate significant difference from initial level on 1-day post transfer (19 August) (One-way ANOVA). Data are presented as mean ± and standard error of the mean (SEM).

4. Discussion

Temperature is considered a major environmental factor effecting growth of Atlantic salmon and has clear effects on metabolism and increases the efficiency of food energy transformation to net biomass development (Brett, 1979). Studies have suggested that salmon in seawater favour the highest offered temperature up to approximately 14°C (Oppedal et al., 2001), and avoid temperatures above 18°C (Johansson et al., 2009, Johansson et al., 2006), which was the high temperature group in present study. Negligible mortality and fin corrosions damage was observed in all temperature groups in present study, which suggests that post-smolts around 250 grams have an inherent robustness when it comes to tolerate abrupt changes from 10 and up to 18°C. The present study found that post-smolts in all temperature groups displayed an increased growth measured as body weight and length, yet no significant differences were revealed after 45 days of thermal treatment. This is in agreement with findings by Kullgren and co-authors (2017) who reported no significant difference between salmon during the first month of exposure to 8, 12 and 18°C. However, these authors found lower weight growth and condition factor after three months at 18°C. Different optimal temperatures for growth in post-smolts might also be size dependent (Handeland et al., 2008). Furthermore, post-smolts following transfer to elevated temperature (16 and 18°C) displayed significantly increase in condition factor, whereas condition factor of fish with low temperature treatment remained the same, probably due to difference in skeletal length growth versus muscle weight (Nicieza and Metcalfe, 1997). It has been found that the temperature optimum for growth of post-smolts between 70-150 g was 12.8°C, and increased with about 1.2 + 0.3°C in the size range 70-300 g (Handeland et al., 2008). It is likely that longer exposure to higher temperatures would have resulted in growth differences in present study as well, at least the 13 and 16°C groups.

The most common growth indexes used in today's industry are specific growth rate (SGR) (Jobling, 2003, Jobling, 1995, Austreng et al., 1987, Brett, 1979) and the thermal growth coefficient (TGC) (Alanärä et al., 2001, Iwama and Tautz, 1981). Since specific growth rate are limited for calculation of Atlantic salmon's growth range of 50-3000 g and a temperature range between 4 and 14°C (Thorarensen and Farrell, 2011), we also applied thermal growth coefficient, which is a temperature corrected growth measure in fish. Both SGR and TGC provide solid measures of growth in production, but they also comprise some inherent limitations since they overlook fundamental drivers of fish growth. For instance, SGR is based on the incorrect assumption that fish growth is continually exponential, and does not consider that the growth rate of fish is highly dependent on body weight and water temperature (Dumas et al., 2010). In the present study SGR and TGC displayed divergent growth patterns in all temperature groups during the last 45 days, despite weight and length increased in all groups as discussed

above. The overall lower SGR in all groups during the last 45 days compared to the first 82 days growth rate was somewhat surprising as SGR predictions are usually appropriate for short intervals (Dumas et al., 2010). Moreover, the average SGR values obtained in current study is lower than those observed in other studies (Hevrøy et al., 2013; 2015; Olsvik et al 2013). One explanation could be that growth studies in small-scale tanks generally yield lower growth rates. Another could be due to different temperature optimum for growth and/or that post-smolts may display different growth rates within different size ranges (Handeland et al., 2008). TGC is a more flexible tool for estimating growth in commercial production when temperature differ (Thorarensen and Farrell, 2011). However, the assumptions of TGC can, as with SGR, be violated in different production scenarios and should be used with caution. It was surprising that TGC were lower during the last 45 days compared with the first 82 days in the present study. It should be noted, however, that errors could be made when calculating TGC in situations where the temperature is above the optimum for growth, which was the case for the 18°C groups in present study. Overall, our general findings on TGC in different temperature groups are in line with other studies (Olsvik et al., 2013), which reports significant reduction in TGC among adult (1.6 kg) Atlantic salmon transferred from 13 to 19 ^{0}C after 45 days of exposure (treatment groups are 13, 15, 17, 19 ^{0}C). In contrast, Hevrøy and co-authors (2013) found decreasing SGR following transfer of adult salmon to increasing temperature. Our findings suggest that post-smolts handle direct and abrupt transfer from 10 to 13, 16 and 18^{0}C surprisingly well according to SGR and the increase in weight and length.

After an extensive literature review it became apparent that very few studies has addressed temperature effects on stress axis in farmed post-smolt Atlantic salmon. The closest study to present research is Olsvik et al. (2013) where he looked at transcriptional responses to temperature and low oxygen stress. A few studies have touched upon this topic, but they were focused on behavioral aspect of wild salmonids during elevated water temperature or developmental temperature stress of Sockeye salmon fry (Breau et al., 2011; Burt et al., 2011). Furthermore, some articles have addressed similar topics in non-salmonid fish, e.g. common carp (Jaxion-Harm and Ladich, 2014), seabass (Maulvault et al., 2017) and catfish (Dalvi et al., 2017).. Therefore, the present thesis report relatively novel experimental design and data regarding stress and cognitive responses in Atlantic salmon under chronic and confinement stress.

Plasma cortisol was measured as a primary parameter for stress response to temperature among post-smolts in the present study. Cortisol is a widely used accepted indicator of stress in fish (Schreck, 2016). The thermal shift in current experiment was immediate during the first 30

minutes, which significant diverge from the general approach of stepwise alteration of temperature in other studies (1 °C per day) (Hevrøy et al., 2013, 2015; Olsvik et al 2013). The industry also operates with a max temperature difference of 4-5^{0}C when transferring fish. The transient increase in plasma cortisol in fish after one day, with levels being back down to control level (10^{0}C group) seven days' post-transfer to 13 and 18 ^{0}C water suggest a functional stress response to temperature exposure. Hence one may argue that post-smolts directly transferred from 10 to 13 and 16^{0}C are without a prolonged (more than one week) disturbance of cortisol levels (metabolism) and thus became habituated to the new temperature regimes. This was not the case for the 18^{0}C group, were one may assume that during the first day of exposure to elevated temperatures these fish probably have fully metabolized all cortisol reserve in order to cope and adapt to 18^{0}C. However, these post-smolts did not elicit lethargic or apathetic behavior upon visual inspection. Depending on severity and type of stress, cortisol level of stressed salmonids returns to the initial condition after one hour to one day, or longer if the stressor persists (Pankhurst and King, 2010). The fact that cortisol level in all groups was reduced back down to the control group's level after one week suggests that habituation or desensitization took place (Madaro, 2016). We could not claim that group with highest temperature regime became completely adapted after one week, but zero mortality, similar weight/length growth and plasma cortisol as the other groups suggest that they at least became habituated after 45 days. Pankhurst and King (2010) found an inverse relationship between temperature and the duration of fish stress responses. After 45 days of 'acclimation' to all groups displayed similar plasma levels of cortisol, suggesting that the post-smolts had adapted well to their new temperature regimes. Following an acute confinement stress test of 1 hour it was clear that all groups elicited a rise in plasma cortisol, confirming that the fish from all temperature regimes were able to respond to an additional stressor. This variation likely reflects the dynamic actions of cortisol as a mediator of maintaining stability through change (allostasis), thereby initiating physiological adjustments to maintain internal stability (Korte, 2007). The increase plasma cortisol in the 18^{0}C contrasts the lack of response following one-day exposure to temperature. Hence depending on the sampling time point and the varying response to stressors, one should be critical when using plasma cortisol as a stress indicator in longer-term studies as this can be misleading, since it is not always a good predictor of functional output, such as learning and adaptation. On the other hand, most authors agree that cortisol is a good indicator of an acute stress condition and that handling and short-term confinement is an effective acute stressor in Atlantic salmon (Schreck, 2016; Madaro 2016a; Madaro 2016b). Hence, according to the concept of allostasis, fish in a state of good welfare will have the ability to increase cortisol levels to react to an acute challenge. It has recently been shown, in Atlantic salmon, that cortisol response to an acute stressor is reduced in

fish that have earlier been subjected to a chronic stress situation (Madaro et al., 2016a; Madaro et al., 2016b). Nevertheless, based on the clear responses in cortisol to an acute challenge it is concluded that the fish were performing well after 45 days' exposure to different temperatures.

CRF and CRFbp in teleost best known as a main hypothalamic factor of the HPI-axis, stimulating ACTH production in the pituitary glands, regulates the endocrine and behavioral stress response (Housing, 2004). According to previous physiological studies of fish stress, was found that CRFbp best explain and anticipate endocrine stress axis activity, thus suppression of CRFbp expression identify presence of stressful conditions, whereas up-regulation occurs under basal non-stress circumstance (Schreck, 2000; Geven et al., 2006). However, the CRF-system not only regulates the endocrine stress response but is also involved in extra-hypothalamic autonomic and behavioural responses to stress (Lowry and Moore, 2006). In mammals, telencephalic CRF is involved in the regulation of stress-related behavioural responses and affects memory and learning (Radulovic et al 1999). CRF mRNA production in the dorsal telencephalon has also been identified in zebrafish (Alderman and Bernier, 2007) and in Atlantic salmon (Vindas et al 2007), which suggests that the telencephalon is a conserved structure and that telencephalic CRF may have an important role in stress-related behaviours and learning in fish. Since the role of CRF as endocrine (HPI-axis) was beyond the scope of the present study, and because changes in telencephalic CRF might be easily masked as a result of large(r) changes in preoptic CRF we excluded the preoptic area from our samples.

Surprisingly very few significant changes in CRF and CRFbp mRNA expression were found in response to abrupt changes in temperature, or in response to an acute challenges test in current study. One should therefore exercise caution when interpreting telencephalic CRF and CRFbp expression following one and 7 and 45 days' exposure to different temperatures. Nevertheless, assuming that transcript abundance reflects changes at a protein level our findings suggest an inverse expression trend between CRF and CRFbp supporting the suggested role of CRF beyond the HPI-axis in teleosts (Schreck, 2016). However, the lack of major changes in CRF mRNA levels following an acute stress test contrasts previous findings of increased CRF mRNA in the telencephalon of Atlantic salmon in response to stress (Alderman and Bernier, 2007; Vindas et al., 2017). CRF binds with high affinity to CRFbp, which inhibits the biological activity of CRF, however, the exact functions of CRFbp under basal and stress conditions are not well known, especially in fish. It has been shown that CRFbp mRNA production occurs in the *area ventralis* of the telencephalon in both zebrafish and rainbow trout (Alderman et al., 2008; Alderman and Bernier, 2007). Co-localization of CRFbp with CRF and the related urocortins in the pallial areas of the dorsal telencephalon in fish (Alderman and Bernier, 2007) suggests a highly localized regulation of the CRF system. In the present study, we found that very few changes in CRFbp in

relation temperature and acute handling stress, which contrasts an up-regulation of telencephalic CRFbp after a stress challenge was observed in Atlantic salmon classified as having a reactive coping style (Vindas et al., 2017). Furthermore, hypoxia and social stress (subordinate fish) lead to an increase in CRFbp gene expression in the telencephalon of rainbow trout after 24 h (Alderman et al., 2008). Thus different timelines on expression pattern may have led to us missing peak changes in present study. Other studies also showed that whole-forebrain CRFbp decreases in response to a challenge (Gorissen et al., 2015), which further strengthens that CRFbp exerts a local fine-tuning of the CRF system in the telencephalon, independently of CRFbp actions in the HPI axis. Still, more studies are required to unravel the role under different high temperatures in Atlantic salmon post-smolts and other players of the HPI-axis should be further studied to get a more complete understanding.

Neural plasticity is a key aspect in adapting to environmental changes since it enables experiences to modulate the brain`s physical structure and functional output (Knudsen, 2004). Environmental enrichment increased neuroD1 in the forebrain of Atlantic salmon parr and improved learning ability in spatial task (Salvanes et al., 2013). Furthermore, anthropogenic factors such as aluminium exposure in acidified waters caused a down regulation in neuroD1 and impaired learning performance in a maze task (Grassie et al., 2013). Hence, an increased neuroD1 response to an acute challenge would indicate a greater capacity to emit neural and behavioral changes. However, we found no significant responses in neurod1 mRNA expression in the present study. Literature review of a similar research found that chronic stress has detrimental effects on learning and memory, where reduced neuroD1 expression of neuroD1 in the telencephalon have direct relationship with increased level of stress (elevated level of glucose and cortisol in the blood) (Grassie et al., 2013). Taken together, our findings indicate that post-molts in the resilience and robustness of fish in our study to respond to challenges, which may in turn be challenging from an animal welfare point of view.

Brain-derived neurotropic factor (BDNF) is important for synaptic plasticity and neurogenesis and it has recently been shown that environmental challenges alter BDNF expression in the telencephalon of Atlantic salmon (Vindas et al., 2017) In the present study we found an up-regulation of telencephalic BDNF following acute challenge stress test in the 18^0C group. Hence as for neuroD1 our result seems to contrast previous studies that report increased expression of BDNF mRNA levels in telencephalon, suggesting that with the exception of the 18^0C group there was a negative impact on the cognitive ability of post-smolts to respond to environmental challenges in the present study.

5. Conclusion

The main objective of this study was to evaluate effects of direct transfer of 250 grams post-smolts from 10°C to 13°C, 16°C and 18°C seawater. After 45 days' exposure to different temperature we found increased weight and length growth in all temperature groups but no significant differences between post-smolt rearing in different temperatures. Our findings are in agreement with previous research (Kullgren et. al., 2017; Olsvik et al., 2013; Hevrøy et al., 2013; 2015). Moreover, when correcting for temperature post-smolts exhibited a decreasing TGC with increasing temperature suggesting that growth is affected by temperatures as reported in previous research (Olsvik et al., 2013), where fish growth decrease with temperature increasing. The transient increase in plasma cortisol after one day, with levels being back down to control levels (10^0C group) after seven days in the 13 and 16 ^{0}C groups suggest a functional stress response in all groups except the 18 ^{0}C degree group. This was also evident after the ACT test (after 45 days' treatment). Taken together, our findings indicate that post-smolts display impaired resilience and robustness (stress and cognitive functions) when challenged, which may in turn have negative consequences for animal welfare. Furthermore, rearing temperature at 18°C or above is not optimal from both with respect to growth, stress and animal welfare perspective. It should also be emphasized that additional stressors such as handling should be taken into account and one may therefore recommend that direct transfer should not exceed more 5-6°C.

6. Reference

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Appendix A: Discussion of materials and methods

Fish Material and experimental design

The smolt facility had reared the smolt in 11^0C freshwater. Upon arrival and random distribution into all experimental tanks the smolts were kept several days in 10^0C freshwater so they could recover from the transport with minimum additional temperature differences. In addition to reduce potential stress effects by minimizing temperature differences, a stepwise increase in salinity was performed in order to provide a gradual acclimation with minimum osmotic stress during transfer to seawater (Handeland et al., 2014). The low mortality rate of only 10 fish after the stocking of smolts and during early seawater phase suggests appropriate and gentle transfer protocol in this experiment. Water temperature and oxygen saturation were all kept well within the recommended Atlantic salmon post-smolts production values (Thorarensen and Farrell, 2011). The water temperature were monitored daily and kept as stable as possible with a maximum of 0.5^0C deviation from target temperatures throughout the whole experiment. The biomass adjustments ensured that the density did not exceed 25 kg per tank (12.5 kg/m3). Once the direct changes in water temperatures were initiated the biomass density was 10.5 kg/m3, which is well within recent studies showing that post-smolt densities can be up to 75 kg/m3 if water quality and feeding is optimal (Calabrese et al., 2017a, 2017b). The daily inspections, good condition factor and no aggressive behavior of fish in our experiment indicates that the fish were healthy and were not affected by problems due to rearing conditions that would interfere with the results. For present study, duplicate tanks were set up for as to control for any tank effects. Usually, triplicate tanks are used for experiment with fish growth, but in our case restrictions due to capacity and resources only allowed duplicate treatment tanks. For the physiological and transcriptional parameters of the stress axis measured has been found to robust using duplicate tanks at the ILAB facility (Tom Ole Nilsen, Uni, 2017, personal comment). Automatic feeders fed the fish in duplicate tanks during the light hour of the natural light regime. Distribution of feed pellets secured that all fish got relatively equal amount of excess feed in order to avoid aggressive behavior and minimize development of hierarchies. No mortalities after direct transfer to different temperatures suggest that rearing conditions in all experimental groups was good.

Sampling procedure and tissues preservation

The sampled fish got a lethal overdose of MS222 and were immediately delivered to the dissection lab for further sampling. Great care was taken as to not disturb fish in tanks prior to and during samplings. Within 5 minutes after being dip netted out of the tanks and anesthetized

blood was collected also ensured minimal effect of sampling, as indicated by the low plasma cortisol values observed in present study. Sample acquisition and purification of RNA mark the initial step of every RT-qPCR, and the quality of the template is without doubt the most important determinant of the reproducibility for a successful expression result (Bustin and Nolan, 2004). It is well known that RNA is sensitive to degradation by post-mortem processes and inadequate sampling handling and/or storage conditions. Therefore, at least four people were involved in sampling process, thus six fish were processed by relatively short time (< 20 min). This is sufficient for avoiding any degradation of RNA in tissues used for subsequent gene expression analysis of the target genes analyzed in this thesis (Tom Ole Nilsen, Uni, 2017, personal comment). All surgical supplies were washed between each fish, furthermore all work surfaces and supplies were disinfected between fish parties. For avoiding RNA degradation all samples after taking were frozen directly on dry ice and stored in biofreezers at - 80^0C until processed. The use of liquid nitrogen for preserving tissues was chosen because other options such as RNAlater limits the sampled tissue to be used in other assays, such as protein quantification and measuring enzyme activity in addition to increased cost (Tom Ole Nilsen, Uni, 2017, personal comment). Moreover, studies have further suggested that tissue preserved in liquid nitrogen is preferred for subsequent RNA extraction compared to RNAlater from a quality point of view (Olsvik et al., 2007). The RNA integrity was good in our experiment as indicated by the high RNA Integrity Number (RIN) of our samples.

RNA isolation

Extraction of total RNA is one of several critical steps toward the gene expression results (Bustin, 2002). In this work, RNA isolation robot was used in conjunction with the Fast Prep Homogenization System, which allowed an effective isolation of the RNA for a large number of samples. The use of RNA purification robot allowed not only a high capacity and efficient extraction of high quality RNA but also permitted us to not use hazardous compounds associated with more manual protocols such as the TRI Reagent method by Chomczynski, 1993. Further discussion of the principles behind different methods of RNA extraction will not be discussed here as that is beyond the scope of this thesis work. Assessment of RNA purity and integrity is important for downstream analysis such as cDNA synthesis and Real time q-PCR. The A260/A280 estimates the RNA to protein ratio and low A260/A280 value indicates an impure sample. An over estimation of this ratio can occur if genomic DNA (gDNA) is present, caused by the absorption of gDNA at 260 nm wavelength (Imbeaud et al., 2005). In the present study all RNA samples were within recommended range (1.8-2.1) and was therefore considered to display

an acceptable purity for subsequent cDNA synthesis. The NanoDrop also gives a secondary qualitative measure, A260/A230, which can give indications of contaminates such as carbohydrates and phenol which are absorbed at 230 nm (Tom Ole Nilsen, Uni, 2017, personal comment). This ratio was within recommended values (1.8-2.2) for all tested samples in the present study.

The RNA integrity was assessed by electrophoresis (Agilent 2100 Bioanalyzer), which is a microchannel electrophoresis instrument that measures the integrity of the RNA by a laser induced fluorescence detection of the 18S and 28S ribosomal RNA bands. Intact RNA samples will show an elevated 18S and 28S rRNA peaks in addition to small amount of 5S RNA, while degraded samples will show shifts and/or decreased in these peaks (Fleige and Pfaffl, 2006). These peaks are assessed in a software developed by Agilent Technology to classify the RNA Integrity Number (RIN) of eukaryotic total RNA on a number system ranging from 1 to 10, with 1 being the most degraded and 10 being the most intact (Mueller, 2004). We run a select number of RNA samples on the Agilent and found that RIN values were between 9.80 and 10.0. (Fig. 14). This indicates excellent RNA integrity and the assumption was made that this is representative for all samples. Base on that, all samples were recognized as being of acceptable for further analyses.

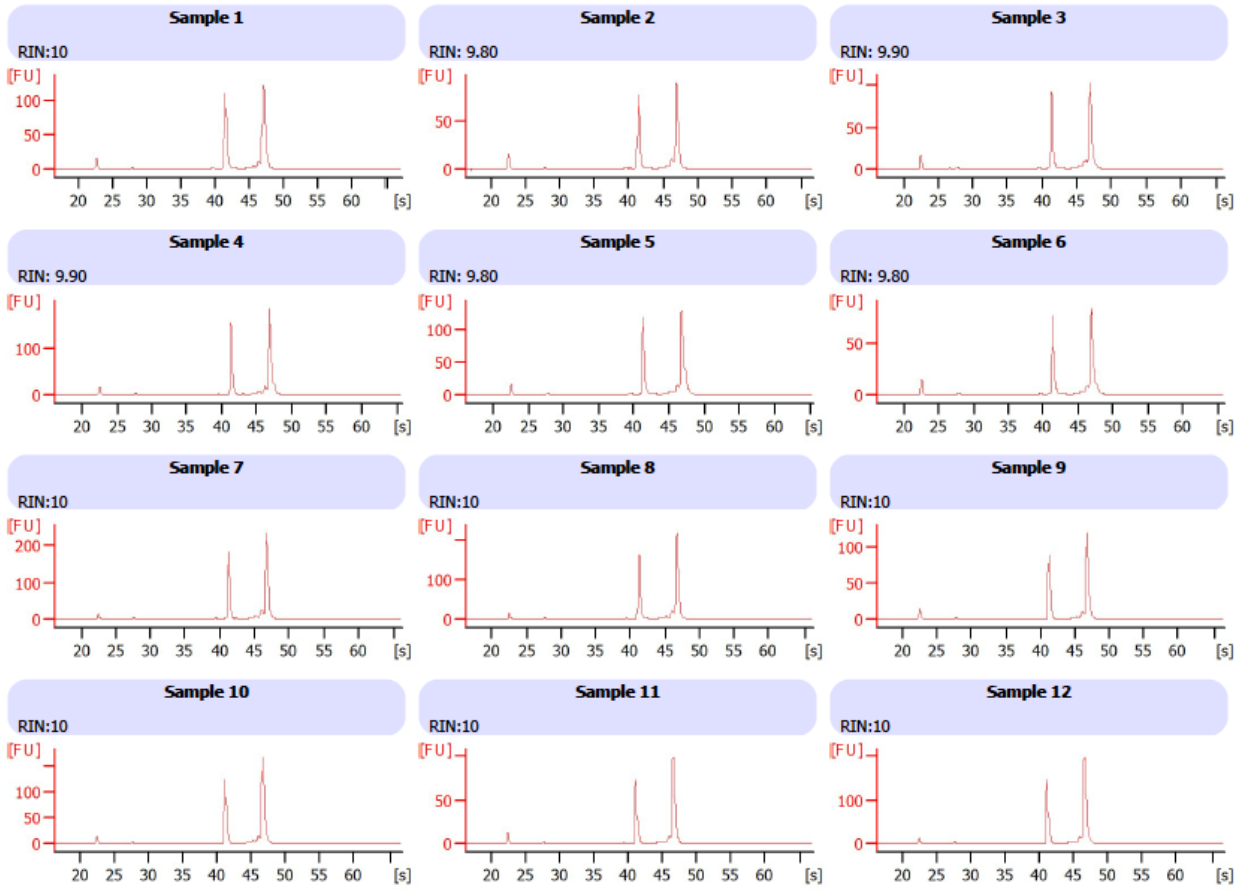

Figure 14: Shows results from one representative RNA 6000 Nano LabChip run with total RNA from randomly chosen telencephalon samples in present study. RIN values are indicated for each sample and the three peaks from the left to the right for each sample indicate 5S, 18S and 28S ribosomal RNA.

cDNA synthesis and Real-Time qPCR

The RT-step (cDNA synthesis) has been identified to be a major source of variability in RT-qPCR experiments (Plaffl, 2004). In the RT-step, complementary DNA copies (cDNA) are produced through reverse transcription (RT) of RNA. This step is very sensitive to different contaminants such as salts, alcohol, phenol or other inhibitors accumulated during RNA isolation (Plaffl, 2004). Furthermore, great care was taken to minimize variability during this RT step; by adding all reagents from the SuperScript® III kit (Invitrogen™, USA) used in this study in Master Mixes (MM). Then we used the Hamilton pipetting robot to dispense the different ingredients to ensure high accuracy and reproducibility (Tom Ole Nilsen, Uni, 2017, personal comment). The SuperScript III kit is widely used for cDNA synthesis applied in both regular PCR and RT-qPCR and have proven to give excellent results in our laboratory. In this study, a two-step RT-qPCR was chosen, which involves creating the cDNA in one separate RT reaction tube before adding a small amount or diluted aliquot of cDNA as template to the RT-qPCR

reaction. This method enhances the flexibility by allowing storage and multiple testing with the same cDNA stock compared to a single-step procedure (Tom Ole Nilsen, Uni, 2017, personal comment). The choice of priming strategy (gene specific, random hexamer or oligo dT) has also proven to be important, as different priming methods have shown to provide different sensitivities and efficiencies (Raja et al., 2000). We chose to use the oligo (dT) primer, which by experience has proven to be a very reliable choice in our laboratory when quantifying gene expression in telencephalon of salmon.

Real time q-PCR is a modern and commonly used method to measure mRNA level according to its high sensitivity, reproducibility, and precision in detecting mRNA transcripts over a wide quantification range (Bustin, 2002). Based on prior experience from screenings of suitable reference gene, the endogenous reference gene S20 was evaluated and found sufficiently stable to use in this study (Olsvik et al., 2005). The working concentrations of cDNA to be used in the q-PCR analysis was determine by generating a pool of cDNA that contained an aliquot from all individual samples. From the pool cDNA a two-fold dilution series was made and used as template to validate each of the gene expression assays used in the present study (Fig. 15). Based on dilution series for each of the assays all genes were found to display a sufficient PCR efficiency and analyzed using a template cDNA diluted 1:40.

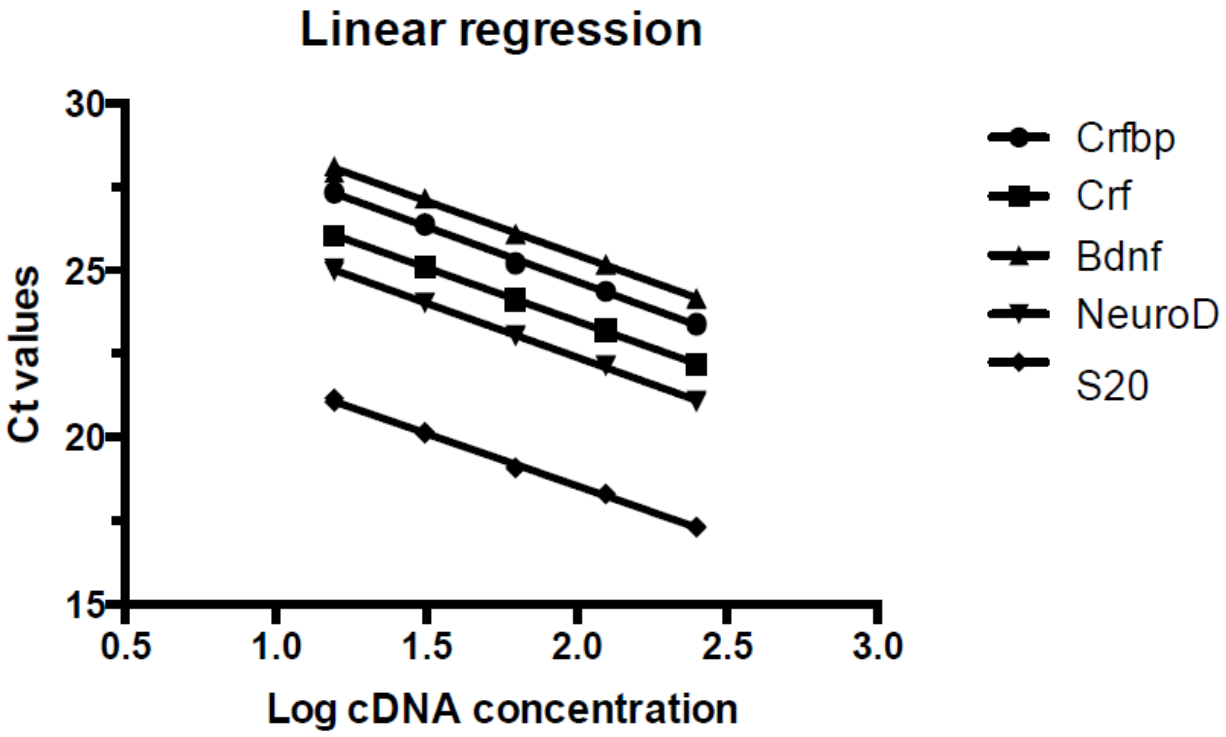

Figure 15: Real-time RT-PCR threshold cycle (Ct) values ± S.E.M. against cDNA dilution series from Atlantic salmon telencephalon for qPCR assay used in present study. Linear regression results Crfbp: y =

- 3.279x + 31.22, R^2 = 0.998; Crf: y = - 3.199x + 29.87, R^2 = 0.999; Bdnf: y = - 3.221x + 31.90, R^2 = 0.999; NeuroD: y = - 3.237x + 28.87, R^2 = 0.999; S20: y = - 3.131x + 24.81, R^2 = 0.998.

SYBRgreen was chosen as dye in this study. This dye binds to the double stranded DNA and emits a detectible fluoresces proportional to the quantity of amplified DNA, which then will be recorded in real-time when it reaches a predetermined threshold set by the operator (Bustin, 2002). However, as it binds to all dsDNA present, specific primers and low genomic DNA contamination are vital to ensure a reliable result (Bustin, 2000). This was addressed by the great care taken in each step prior to this, e.g. RNA integrity, purity tests and screenings as discussed above. Residual genomic DNA will always be present in all samples. Hence, a test using a -RT enzyme cDNA template for all the qPCR assays was performed. This showed no signal and it was concluded that signal from gDNA was negligible in our samples. In addition, NTC wells applied to each plate and these showed no signal and thus no contaminations in our qPCR reactions. It is often recommended to use triplicates for each sample in PCR reactions to reduce variability in addition to detect outliers with a higher precision. However, due to the significant cost associated with carrying out RT-qPCR, compromises had to me made due to limited resources. Hence, we used duplicates reactions for each sample/assay in the present study.

Statistical methods

Statistica 13 (StatSoft, Inc., USA) was used to perform the statistical analysis. Fish from each tank (replicate) were randomly deep netted. Fish with average weight, length and without external damages were chosen for pit tagging. Data of all parameters (weight, length, condition factor, specific growth rate, thermal growth coefficient, plasma cortisol levels and CRF, CRFbp, NeuroD1 and BDNF expression) were tested for normality using Kolmogorov-Smirnov test and homogeneity of variance using Harley F Max. Furthermore, possible difference between replicates were tested with nested design ANOVA and replicates combined in case of non-significant ANOVAs. Therefore, non-parametric analysis (Kruskal-Wallis test) was used only for plasma cortisol and CRF mRNA expression. Nested design ANOVA revealed significant difference between replicates of plasma cortisol levels. When working with plasma cortisol in stressed fish we always expect to observe large variations between individuals within the same group. The assay have a detection limit of 0.4 ng/ml. In several of the individuals cortisol levels was below the detection level of the assay and was therefore assigned a value equal to 0.4 ng/ml. Hence it was concluded that the tank differences was due to detection levels and not a true tank effect. This has been observed in several previous studies where we analyze cortisol levels in

stressed and unstressed fish. It was therefore decided to use one-way ANOVA (Sigurd O. Handeland, Uni, 2017, personal comment).

Appendix B

Table 2: Comparison of end weight of Atlantic salmon between four groups with different temperatures regimes (10, 13,16 and 18^{0}C). Tukey HSD test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

t°C	10	13	16
10	x	0.7	0.9
13	0.7	x	0.9
16	0.9	0.9	x
18	0.9	0.4	0.7

Table 3: Comparison of end length of Atlantic salmon between four groups with different temperatures regimes (10, 13,16 and 18^{0}C). Tukey HSD test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

t°C	10	13	16
10	x	0.7	0.8
13	0.7	x	1
16	0.9	1	x
18	0.9	0.5	0.6

Table 4: Comparison of p-value of condition factor between different sample point (21 May, 11 August, 1 October) significant values are embedded. Univariate Tests of Significance, one–way ANOVA, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Date\Temperature	10C	13C	16C	18C
21 May - 11 Aug	**<0.001**	**<0.001**	**<0.001**	**0.015**
21 May - 1 Oct	**<0.001**	**<0.001**	**<0.001**	**<0.001**
11 Aug - 1 Oct	0.2	0.4	**0.01**	**0.05**

Table 5: Comparison of condition factor within one sample point between all water treatment. For 21 May and 11 August, Univariate Tests of Significance (One –way ANOVA) was used; for 1 October Kruskal-Wallis test was applied. Data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Condition factor	P-value
21 May	0.5
11 August	0.4
1 October	0.3

Table 6: Comparison of end specific growth rate between four groups of Atlantic salmon with different temperatures regimes (10, 13,16 and 18^0C) (sampled 1 October). Tukey HSD test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

t°C	10	13	16
10	x	0.9	**0.02**
13	0.9	x	0.1
16	**0.02**	0.1	x
18	**0.02**	0.1	1

Table 7: Comparison of thermal growth coefficient of Atlantic salmon within thermal groups (10, 13,16 and 18^0C) between two periods: May–August and August-October. Univariate Tests of Significance, one–way ANOVA, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Temperature	P-value
10C	**<0.05**
13C	**<0.001**
16C	**<0.001**
18C	**<0.001**

Table 8: Comparison of thermal growth coefficient between four groups of Atlantic salmon with different temperatures regimes (10, 13,16 and 18^0C) in the period from August to October. Tukey HSD test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

t°C	10	13	16
10	x	0.1	0.06
13	0.1	x	0.9
16	0.06	0.9	x
18	**0.02**	0.9	0.9

Table 9: Comparison of p-value of cortisol blood concentration between different sampling point (1, 7, 45 days' post-transfer and acute stress challenge). Kruskal-Wallis test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Period \ Temperature	10	13	16	18
1 - 7 days	0.7	**<0.001**	**<0.001**	0.8
1 - 45 days	0.7	**<0.001**	**<0.001**	0.5
1 - ACT	**<0.001**	**0.001**	**<0.001**	**<0.001**
7 - 45 days	0.4	0.7	0.1	0.4
7 days - ACT	**<0.001**	**<0.001**	**<0.001**	**<0.001**
45 days - ACT	**<0.001**	**<0.001**	**<0.001**	**<0.001**

Table 10: Comparison of cortisol blood concentration within sampling points. Kruskal-Wallis test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Sampling date	Cortisol concentration
1 day post- transfer	**<0.001**
7 days post- transfer	0.3
45 days post- transfer	0.7
ACT	0.1

Table 11: Cortisol concentration within one-day post transfer. Kruskal-Wallis test, data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Temperature	10	13	16
10	x	**<0.001**	**<0.001**
13	**<0.001**	**x**	0.5
16	**<0.001**	0.5	x
18	0.66	**<0.001**	**<0.001**

Table 12: Comparison of CRF, CRFbp, NeuroD1 and BDNF mRNA concentration within one sample point between all group's treatment. Kruskal-Wallis test was used for CRF analyses, whereas One - way ANOVA was applied for the rest of the genes. Data presented in p-values, significant values ($p < 0.05$) are highlighted in bold.

Sampling date/ Genes	CRF	CRFbp	NeuroD1	BDNF
1 day	0.3	**0.05**	0.3	0.5
7 days	0.3	0.4	0.7	0.9
45 days	**0.02**	0.6	0.09	**0.03**
ACT	0.2	0.9	0.6	**<0.001**

Table 13: CRF mRNA abundance between different sampling dates within one treatment group. One-way ANOVA was used for 10 and 18^0C treatment group, wheares Kruskal-Wallis test was applied for 13 and 16^0C treatment group. Data presented in p-values.

Period / Temperature	10C	13C	16C	18C
1 - 7 days	0.5	0.1	0.06	0.5
1 - 45 days	1	0.08	0.7	0.8
1 - ACT	0.9	1	0.3	0.4

Table 14: CRF mRNA abundance between four treatment's group within 45 days' sampling. Kruskal-Wallis test, data presented in p-values, significant values ($p < 0.05$) are highlighted in bold.

Temperature	10	13	16
10	x	**<0.001**	0.2
13	**<0.001**	x	0.2
16	0.2	0.2	x
18	0.4	**0.01**	0.3

Table 15: CRF mRNA abundance between different sampling dates within one treatment group. One - way ANOVA. Data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Period\Temperature	10C	13C	16C	18C
1 - 7 days	0.3	0.06	0.07	0.1
1 - 45 days	0.9	**0.04**	0.2	0.3
1 - ACT	0.5	0.06	**0.01**	0.3

Table 16: CRFbp mRNA abundance between four treatment's group within 1st day post transfer. Tukey HSD test, data presented in p-values.

Temperature	10	13	16
10	x	0,5	0,6
13	0,5	x	1
16	0,6	1	x
18	0,7	0,07	0,09

Table 17: NeuroD1 mRNA abundance between different sampling dates within one treatment group. One - way ANOVA. Data presented in p-values, significant values ($p<0.05$) are highlighted in bold.

Period\Temperature	10C	13C	16C	18C
1 - 7 days	0.7	0.1	0.6	0.4
1 - 45 days	0.3	0.4	**0.01**	1
1 - ACT	0.2	**0.05**	0.4	0.5

Table 18: BDNF mRNA abundance between different sampling dates within one treatment group. One - way ANOVA. Data presented in p-values, significant values ($p < 0.05$) are highlighted in bold.

Period\Temperature	10C	13C	16C	18C
1 - 7 days	0.8	0.3	1	0.6
1 - 45 days	0.6	0.8	0.06	0.5
1 - ACT	0.4	0.5	0.1	**<0.001**

Table 19: BDNF mRNA abundance between four treatment's group within 45th day post transfer. Tukey HSD test, data presented in p-values, significant values ($p < 0.05$) are highlighted in bold.

Temperature	10	13	16
10	x	0.2	0.7
13	0.2	x	**0.03**
16	0.7	**0.03**	x
18	0.8	0.8	0.2

Table 20: BDNF mRNA abundance between four treatment's group within ACT sampling. Tukey HSD test, data presented in p-values, significant values ($p < 0.05$) are highlighted in bold.

Temperature	10	13	16
10	x	0.4	0.06
13	0.4	x	0.7
16	0.06	0.7	x
18	**<0.001**	**0.01**	0.1