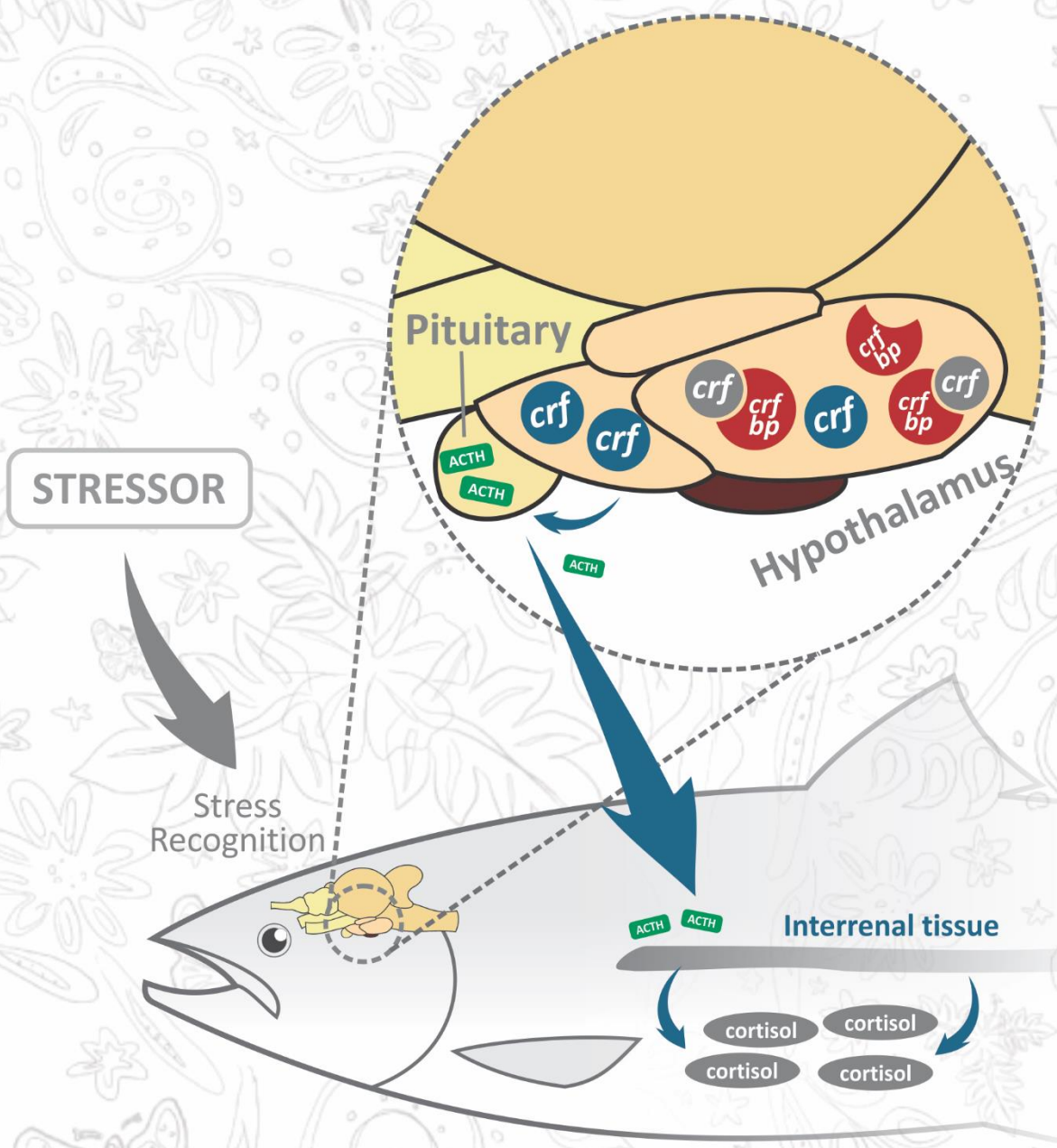
A comparative study of chronic stressors effectivity on the stress response of Atlantic salmon (*Salmo salar* L.) post-smolt

Muhammad Rahmad Royan



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Thesis submitted for partial fulfillment of the degree
Master of Aquaculture Biology

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ABSTRACT

Despite the fact that numerous studies in the literature have explored the effect of chronic or acute stressors on fish stress response, a comparative understanding of how different stressor types affect the Atlantic salmon post-smolt stress response is still not complete, particularly in view of potential paralog genes due to recent whole genome duplication (WGD) in salmonids. In this study we exposed Atlantic salmon post-smolt to chronic chasing, hypoxia and a combination of chasing and hypoxia for 8 days followed by an acute confinement at the end of the experiment. We investigated the stressors effectivity on expression of markers in the stress axis, considering various hypothalamic corticotropin-releasing factor (*crf*) and *crf* binding protein (*crfbp*) paralogs: *crfssa03*, *crfssa14*, *crfssa19*, *crfssa29*, *crfbpssa01* and *crfbpssa11*. The results show that chronic stressors tend to result in a more suppressed weight gain and growth rate for chronically stressed fish and reducing the magnitude of plasma cortisol levels at the end of the chronic stress exposure. In addition, we found that there is a proportional relationship between *crfssa14* gene paralog and plasma cortisol level during chronic stress exposure, despite the presence of an anomaly when the novel stressor was induced. After the novel stressor was exposed, we found no proportional relationship between *crfssa14* gene expression and plasma cortisol level.

We suggest that chasing can be used as an effective and logistically simple method to provoke stress in Atlantic salmon. This was the most pronounced chronic stressor shown by its vigorous effect on the higher magnitude of plasma cortisol level in chasing-exposed fish. We also suggest that *crfssa14* gene paralog can be used as a marker since this was the gene where the expression was best correlated with the stress exposures used in this experiment. However, what is

happening behind the scene of *crfssa14* anomaly and how dynamic relationship between *crf* and *crfbp* needs to be investigated further.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. INTRODUCTION

1.1. Post-smolt Salmon Production

Atlantic salmon (*Salmo salar* L.) has become an economically important fish commodity, and its aquaculture-related activity has been growing substantially throughout northern Europe, particularly Norway as the dominant producer (Bergheim et al., 2009). According to Norwegian Directorate of Fisheries (2018), Salmon production in Norway in recent decade had grown by 66% from 744 125 in 2007 to 1 236 353 ton in 2017. While salmon smolt is termed as newly smoltified salmon juvenile, salmon post-smolt is defined as salmon that have entered the ocean (Thorstad et al., 2012). In Norway, rearing of Atlantic salmon post-smolts to slaughtering normally takes up to 20 months in open sea cages (Aunsmo et al., 2013).

During its life cycle, especially in farming condition, Atlantic salmon might encounter different types of stress episodes. The transformation stage from parr to smolt, for instance, is known to be a typical stress-sensitive phase for Atlantic salmon since many physiological changes occur when the fish attempts to acclimatize in a higher salinity environment (Handeland et al., 1996). Later when the post-smolt have adapted to seawater, the fish will encounter numerous types of stressing conditions in aquaculture settings, such as handling, vaccination, pumping, oxygen shortage or confinement, as part of procedures for treating disease outbreak or sea lice infestation (Sveen, 2018). Not to neglect recent advancements in the technology, especially in semi-closed or closed containment culture systems, the fish also need to deal with potential stressful crowding due to intensification and high densities that are required to be economically feasible (Calabrese, 2017; Kristensen et al., 2012).

Despite the fact that recent advances in technology have somewhat minimized direct anthropogenic stress in fish, physical, mechanical or chemical stress will still inevitably be induced during the rearing process (Sundh et al., 2010). For example, a sea cage environment that has relatively lower current speed will not only result in slower water exchange but also cause waste accumulation that in turn suppresses the oxygen level in the sea cage area (Johansson et al., 2007; Stien et al., 2013). Moreover, when a vaccination procedure needs to be performed, the transport of fish by pumping may stimulate stress response to the fish, or when the vaccine should be administered, netting, handling and exposure to anesthetics are also unavoidable (Iwama, 1998; Kemenade et al., 2009). Quarantining the fish in a relatively small tank as part of the vaccination procedure or bath treatment may also induce confinement as well as hypoxic stressors (Gautam et al., 2017), and these simultaneous stressors can affect the biological and physiological state of the fish (Segner et al., 2012; Sundh et al., 2010). As a consequence of these prolonged stressful conditions, different whole-organism level of stress responses may appear. These include reduced growth, poor disease resistance, immune function impairment or decreased reproduction rate (Sveen, 2018). Taking together, even though better rearing-related techniques have been implemented and improved, several acute and chronic stressful conditions still exist in salmon post-smolt production.

1.2. Stress Conditions in Fish

Stress is defined as a life-threatening circumstance that can stimulate the physiological response of fish because of stressor stimuli perception (Schreck and Tort, 2016). A stressor stimulus is sometimes advantageous by enhancing performance of the fish if perceived as a mild event of stress (eustress), but it can

also be adaptive or maladaptive when leading to a pathological state due to its high intensity (distress) (Bonga, 1997). Since stress is inevitable in salmonids' life, mild or less severe stress may facilitate a positive impact on fish performance by, for instance, enhancing immune system and anabolism of the fish (Dhabhar, 2008; Sadoul and Vijayan, 2016; Yada and Tort, 2016). However, vigorous stressors often lead to impairments in numerous life aspects of the fish, such as reduced growth and appetite, increased susceptibility to disease, poor immune function or high swimming intensity (Noakes and Jones, 2016; Rodnick and Planas, 2016; Schreck and Tort, 2016). The fact that post-smolt salmon encounter numerous types of stressor as part of husbandry activity can be a factor that elicits stress response during the production process in aquaculture system.

Based on the duration of exposure, stress stimuli can be divided into two categories: acute stressor and chronic stressor. Acute stressor is characterized as a typical short-term exposure that lasts from seconds to minutes, and the physiological response to this type of stressor depends on the severity and period of exposure (Gesto et al., 2015, 2013; Sopinka et al., 2016). For example, a study in rainbow trout and zebrafish reveals that a 3-minute chasing with a dip net resulted in 4-fold and 6 times higher plasma cortisol on stressed rainbow trout and zebrafish, respectively, relative to the control groups (Gesto et al., 2015). In a previous study, Gesto et al. (2013) found plasma cortisol of rainbow trout elevated at approximately 2, 6 and 16 times higher than that of control fish after chased for 2, 5 and 15 minutes, respectively. On the other hand, a chronic stressor is basically a prolonged exposure of a stressor during a certain period of time, it can be continuous, sequential or repeated of an acute stressor (Sopinka et al., 2016). Chronic hypoxia (1-3 mg/l O_2), for instance, was found to reduce growth of mummichog (*Fundulus heteroclitus*) after being exposed for 28 days relative to

normoxia group (7 mg/l O_2) (Rees et al., 2012). In another study, the severity of chronic hypoxia is also suggested to affect channel-blue catfish weight in which the more severe the chronic hypoxia (indicated by less saturated O_2), the less the weight that was found (Green et al., 2012).

A typically physical stressor, such as chasing, seems to be more pronounced in eliciting stress response compared to other type of stressors. A study in silver catfish, for instance, shows that 30-second chasing episode stimulated significantly higher plasma cortisol level compared to the exposure to agrichemical compounds, such as methyl-parathion-based insecticide, tebuconazole-based fungicide, glyphosate-based herbicide and atrazine-simazine-based herbicide (Koakoski et al., 2014). However, there are few studies as to how a physical stressor that is chronically induced is compared with other type of chronic stressor. Furthermore, despite the fact that many studies have explored the effects of a single stressor on stress response, either acute or chronic (Burt et al., 2014; Hansen et al., 2015; Madaro et al., 2016b, 2015; Remen et al., 2014, 2012; Vikeså et al., 2017; Vindas et al., 2017b), the understanding of how simultaneous stressors affect stress response, particularly in Atlantic salmon post-smolt, is still very weak. Indeed, stressors never work alone in real aquaculture settings, instead they work in concert with other stressors. Therefore, a comparative study of how different types of stressor alone and in combination with other stressors affect the stress response of Atlantic salmon post-smolt are of importance.

1.3. Stress Response in Salmonids

There are two main stress response pathways in fish: Hypothalamic-Sympathetic-Chromaffin Cell (HSC) axis and Hypothalamic-Pituitary-Interrenal (HPI) axis (**Figure 1.1**). When a stress stimulus is recognized by Central Nervous

System (CNS), hypothalamus will be activated and triggers preganglionic sympathetic nerves that later on stimulate chromafffin cells in the head kidney to secrete catecholamines, as the incipient stress response (Bonga, 1997; Sopinka et al., 2016; Yada and Tort, 2016). HSC pathway only takes seconds until the release of catecholamines. Following the secretion of catecholamines, the production of cortisol through HPI pathway is initiated by the release of corticotropin-releasing factor (*crf*) hormone from the hypothalamus. This hormone will activate the formulation of pro-opiomelanocortin (POMC) in the pituitary gland which in turn will be the precursor of adrenocorticotropic hormone (ACTH) and melanophore-stimulating hormone (α-MSH). Through the blood stream, ACTH will be transported to the interrenal gland and stimulate cortisol production. Unlike catecholamines that are commonly produced within seconds, the secretion of cortisol may take from minutes to hours, thus making it more common to analyze due to the ease of method in laboratory settings (Bonga, 1997; Sopinka et al., 2016; Yada and Tort, 2016). Considering the response period, it is important to decide which pathway to choose in view of the complexity of experimental design.

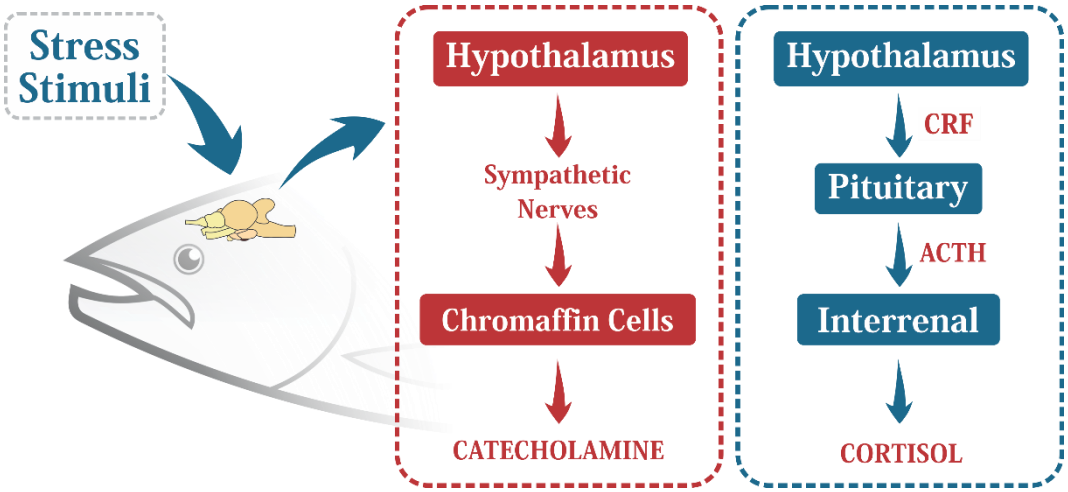

Figure 1.1. Simplified diagram of HSC and HPI axis in response to stressors (Royan, 2019)

The stress response in fish is generally categorized into three phases: primary, secondary and tertiary stress response. As mentioned earlier, after stress stimuli are perceived the primary stress response will be induced as indicated by the upregulated catecholamines and cortisol level (Bonga, 1997; Iwama, 1998). However, there is no fixed term as to how fish can be considered stressed or how fish can be considered in the resting state. For example, Iwama (1998) argued that plasma cortisol level below 10 ng/ml in salmonids can be considered unstressed, whilst it was found that slight and chronic upregulation of cortisol around 5-10 ng/ml was linked to the suppression of Coho salmon immune system (Maule et al., 1993). Moreover, Nilsen et al. (2008) found a relatively high resting level of plasma cortisol (> 50 ng/ml) in Atlantic salmon after being acclimatized to a marine environment for a month. Hence, due to this unstandardized circumstance, comparisons with unstressed fish as a reference can be used to determine the stress status of fish that are exposed to stressors based on the plasma cortisol level variability.

While the primary stress response is often related to hormonal regulation, the secondary response is indicated by physiological alterations occurring in blood or tissues as a result of hormonal effects, i.e. changes in acid-base balance, blood glucose levels, immunological functions or ion balance (Bonga, 1997; Sopinka et al., 2016). For instance, Fanouraki et al. (2011) suggest that the exposure of 5-6 minutes of chasing and 1-1.5 minutes of air exposure resulted in different responses of plasma glucose level in some selected Mediterranean marine fish. Ultimately, the tertiary stress response, also referred to as whole-organism stress response, is obvious when the fish are subjected to severe and prolonged stressors. This can be observed not only in organismal level, but also population level in which there might be effects in growth, body mass, disease resistance, reproduction or immune response of the fish (Naderi, 2018; Sveen, 2018). For

example, not only was the suppression of growth, weight and length found in Atlantic salmon post-smolt after being exposed to certain threshold of chronic hypoxia (Burt et al., 2014; Hansen et al., 2015; Remen et al., 2014, 2012; Vindas et al., 2017b), but similar effects are also normally observed in other fish species, such as Atlantic cod, wild Gulf killifish and channel-blue hybrid catfish (Cheek, 2011; Green et al., 2012; Methling et al., 2010; Sanchez et al., 2011). Thus, it would be interesting to see how the different stages of the stress responses are influenced by different chronic and simultaneous stress exposures.

1.4. Corticotropin-releasing Factor (CRF) and CRF-binding Protein (CRFBP) as the Regulator of Stress Response in Atlantic Salmon

Corticotropin-releasing hormone, often termed as corticotropin-releasing factor (*crf*), is well known for its essential role in regulating corticosteroid secretion by cascade stimulation through HPI axis pathway (Chen and Fernald, 2012; Hauger et al., 2003). After stress stimuli recognition, *crf* is secreted by hypothalamus and activates POMC in the pituitary for ACTH synthesis. Subsequently, cortisol is produced by steroidogenic cells in the interrenal gland after ACTH reaches the head kidney through blood stream (Bernier, 2006; Conde-Sieira et al., 2018; Winberg et al., 2016). The regulation of corticosteroid synthesis in HPI axis is not solely affected by *crf* hormone, but *crf*-binding protein may also have another role. Corticotropin-releasing factor binding protein (*crfbp*) functions to block *crf* from reaching pituitary gland by binding and reducing its bioavailability, thus preventing the secretion of ACTH (Geven et al., 2006; Gorissen and Flik, 2016; Huising et al., 2008; Manuel et al., 2014).

Some studies have revealed that *crf* mRNA expression in the preoptic area (POA) of the brain is directly proportional to the protein level of cortisol in the blood despite not always straightforward, whereas *crfbp* plays a role as *crf* blocker

(Sopinka et al., 2016). For instance, the elevated *crf* mRNA expression in Atlantic salmon post-smolt is followed by the increase in plasma cortisol compared to its resting level (Madaro et al., 2015). In addition, the upregulation of plasma cortisol after fish was being exposed to a novel stressor was confirmed by the higher abundance of *crf* mRNA in the POA of Atlantic salmon parr (Madaro et al., 2016b). This phenomenon also occurs in rainbow trout in which the elevation of *crf* mRNA expression in cortisol-treated and subordinated fish is in line with the upregulation of plasma cortisol level (Jeffrey et al., 2012). Meanwhile, *crfbp* mRNA abundance was found relatively higher compared to *crf* mRNA expression in Atlantic salmon parr (Madaro et al., 2016b) and post-smolt (Madaro et al., 2015), albeit insignificant. Likewise, the inverse relationship between *crf* and *crfbp* mRNA expression was also observed in rainbow trout (Jeffrey et al., 2012) and Senegalese sole (Wunderink et al., 2012). These findings indicate a decrease in *crf* bioavailability as a result of increased *crfbp* peptides. Despite the fact that some studies analyze the POA to assess the expression of *crf* and *crfbp* mRNA (Doyon et al., 2005; Ebbesson et al., 2011; Jeffrey et al., 2012; Madaro et al., 2016a, 2015; Samaras et al., 2018), there are other primary locations of *crf*-related peptide expression in hypothalamus: nucleus lateralis tuberis (NLT) and nucleus recessus lateralis (NRL) (Bernier, 2006). Hence, the analysis of whole hypothalamus is required to get a comprehensive identification of *crf*-related peptide gene expression.

The fact that *crf*-related peptides are not only expressed broadly in different areas of hypothalamus but also in different parts of brain might indicate that these peptides serve different functions, despite having not been completely explored (Alderman and Bernier, 2007; Bernier, 2006; Kovacs, 2013). Interestingly, a study in spotted gar and various vertebrates, such as marsupials, monotremes, lizards,

turtles, birds and fishes shows that there is a duplicated homolog of *crf* gene (*crh2*) as a consequence of the second round of whole-genome duplication (WGD); however the function of the homologs still remain unexplored (Grone and Maruska, 2015a). Due to the loss of this homolog in teleost fish during the third round of WGD, Grone and Maruska (2015b) tried to investigate another option for a possible gene duplication in teleosts, and found two paralogs of *crf* genes: *crha* and *crhb*. They attempted to characterize these gene paralogs in African cichlid and zebrafish, and argued that there is probably neo-functionalization of *crha* paralog because of its diverse localization in different fish species.

In salmonids, as a group of teleost that have undergone the fourth round of WGD, often referred to as Ss4R (salmonids-specific 4^{th} vertebrate whole-genome duplication) event, a comprehensive study concerning the divergence of Ss4R gene duplicates reveals that neo-functionalization normally occurs among Ss4R duplicates (Lien et al., 2016). The Ss4R event appears to open a new chance to evolve a variety of gene duplicates with separate and important functions in stress response, particularly in Atlantic salmon post-smolt. Indeed, in our *in silico* analysis, we found that there are several *crf* and *crfbp* gene paralogs across the Atlantic salmon genome. The fact that many of studies that have been mentioned earlier studied only one *crf* and *crfbp* gene, creates a unique opportunity to characterize hypophysiotropic function among the gene paralogs. In other words, how these diverse gene paralogs are related to stress response in Atlantic salmon post-smolt and how they respond to different types of chronic stress exposures need to be investigated.

1.5. Objectives and Hypotheses

Until recently, there have been numerous studies exploring how an acute or chronic stress exposure affects the stress response in Atlantic salmon post-smolt (Anttila et al., 2013; Burt et al., 2014; Calabrese et al., 2017; Handeland et al., 1996; Hansen et al., 2015; Johansson et al., 2007; Madaro et al., 2016a, 2016b, 2015; Oldham et al., 2019; Olsen et al., 2012; Remen et al., 2014, 2012; Singer et al., 2003; Solstorm, 2017; Sveen, 2018; Vikeså, 2017; Vikeså et al., 2017; Vindas et al., 2017a, 2017b). Nonetheless, there is somewhat limited literature concerning a comparative study of different types of chronic stressors and how stressors that work in concert influence the stress response of Atlantic salmon post-smolt. Moreover, to the best of our knowledge, there is no study so far exploring how the diversity of stress-related gene paralogs resulted from the Ss4R event is linked to the stress response of Atlantic salmon post-smolt. Therefore, this study aims to investigate how different types of chronic stressors affect the stress response of Atlantic salmon post-smolt, considering potential presence of various stress-related gene paralogs. In this study, we evaluate several response parameters, i.e. weight, length, growth, plasma cortisol level, *crf* and *crfbp* gene paralogs, as an effect of different types of stressors.

Based on the aforementioned considerations, we hypothesize that:

$H0_1$: Different types of stressors that are exposed have similar effects on the stress response of Atlantic salmon post-smolt.

$H0_2$: Different gene paralogs of *crf* and *crfbp* have analogous roles in the stress response of Atlantic salmon post-smolt.

II. MATERIALS AND METHODS

2.1. Experimental Units and Facilities

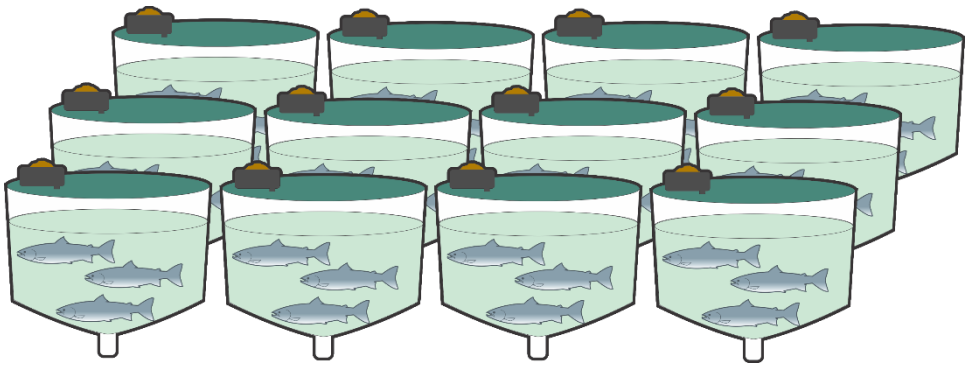

Figure 2.1. Illustration of fish distribution in the rearing tanks (Royan, 2018).

Four weeks prior to the experiment (May 2018) Atlantic salmon (*Salmo salar*, L.) post-smolt of approximately 170 g were distributed into 12 tanks (volume: ca. 600 l) with 40 fish each in Cargill Innovation Center, Dirdal, Rogaland, Norway (**Figure 2.1**). Fish were reared at full light condition (24:0 L:D), and the tanks were supplied with flow through seawater (28 g/l) at 9^{o}C and oxygen saturation 90%. 2.5 dl feed (ca. 180 g; diameter 4 mm, Adapt Marine 80, Dirdal, Norway) was given four times a day (19:00-20:15, 22:00-23:15, 01:00-02:15 and 06:00-07:15) by an automatic feeder (Hølland Teknologi AS Feeder System, Florø, Norway). Salinity, temperature and oxygen saturation were monitored daily.

2.2. Experimental Design

After the acclimation period, on 11th June 2018 (Day 0) tanks were randomly labelled according to one of the four treatments (3 replicates/treatment): control (C), chasing (SA), hypoxia (SB) and the combination of chasing and hypoxia (SC). Stressors were induced twice per day at around 8 am in the morning and around 3 pm in the afternoon for 9 days (day 0 – day 8). On day 9, all groups, including

control, were exposed to a novel stressor in the morning. Due to the complexity of the experiment set-up and the logistics involved with sampling, the protocol was applied from 11th to 20th June for group SA and SB and from 13th to 22nd June for group C and SC (**Table 2.1**).

Table 2.1. The schedule of experiment set-up for stressing and sampling

Date	11/6	12/6	13/6	14/6	15/6	16/6	17/6	18/6	19/6	20/6	21/6	22/6
Stressing	SA SB	SA SB	SA SB SC	SA SB SC	SA SB SC	SA SB SC	SA SB SC	SA SB SC	SA SB SC	SA SB SC	SC	SC
Day-	Day0	Day1	Day0	Day1					Day8	Day9	Day8	Day9
Sampling	SA SB	SA SB	C SC	C SC	-	-	-	-	SA SB	SA SB	C SC	C SC

Details: SA = Stressor A (Chasing); SB = Stressor B (Hypoxia); SC = Stressor C (Chasing + Hypoxia); C = control

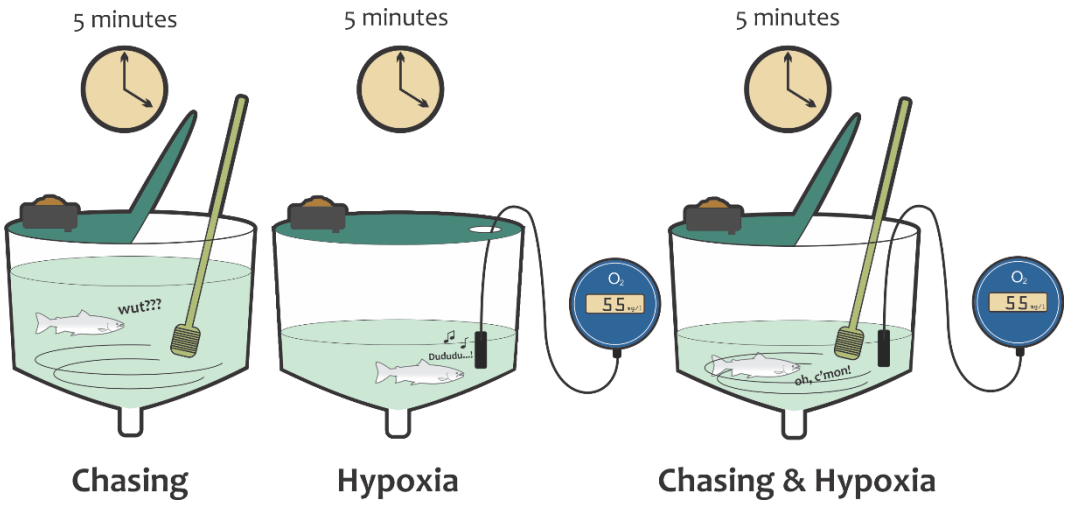

Figure 2.2. Illustration of different types of stress exposures (Royan, 2018).

As illustrated in **Figure 2.2**, fish belonging to group SA were chased with a brush stick for 5 minutes. Hypoxia was applied to group SB by completely closing the water inflow and reducing 2/3 of water in the tank. Once the oxygen saturation reached 55%, 5 minutes were recorded before opening the water inflow again. SC group was treated by combining chasing and hypoxia at the same time. As soon as the oxygen saturation reached 55%, the 5-minute countdown along with the chasing started. On day 9, confinement was performed as a novel stressor by

transferring the fish into a small bucket (40 x 20 x 20 cm) with 12 l water for 15 minutes (**Figure 2.3**), and then the fish were collected after 45 minutes. Oxygen saturation was recorded by using OxyGuard® Dissolved Oxygen Probe (OxyGuard International A/S, Farum, Denmark).

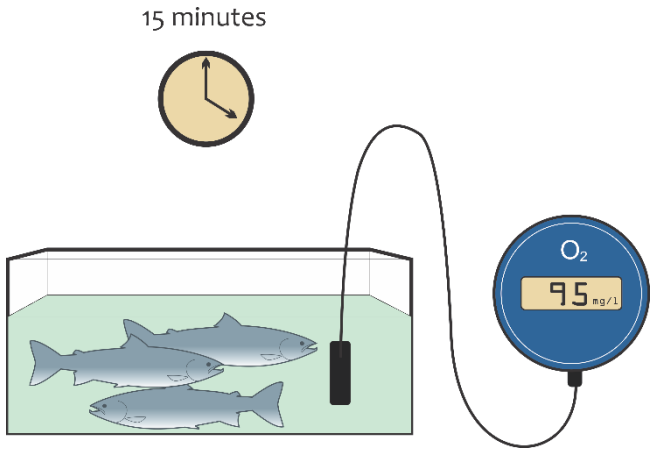

Figure 2.3. Illustration of confinement stress exposure (Royan, 2018).

2.3. Sampling Procedure

Sampling was carried out on day 0, 1, 8 and 9. Two and five fish per tank were sampled before and after stressors respectively on day 0 while five fish were sampled on day 1, 8 and 9 (**Table 2.1**). Fish were anesthetized with 300 mg/l of Tricaine Pharmaq (PHARMAQ Ltd., Hampshire, United Kingdom) in 12 l of seawater, and blood was collected immediately before length and weight were recorded.

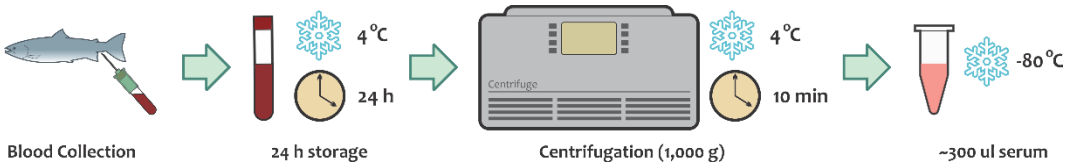

Figure 2.4. Illustration of blood sampling (Royan, 2018).

The blood collection was performed by a caudal venous puncture using a vacuum syringe and BD Vacutainer® set (Ref. 367614, Becton Dickinson, Plymouth, United Kingdom). The blood was then stored overnight at 4 ºC before being centrifuged at 1000 g for 10 minutes (4 ºC) using Hettich Zentrifugen Universal 320R (Hettich®, Tuttlingen, Germany). The supernatant (serum) was collected and stored at 80 ºC until further analysis (**Figure 2.4**). Brain and pituitary were collected (see **Appendix A**) and stored in separated tubes containing RNA later (1.3 ml for Brain; 700 µl for pituitary). Samples were then stored at 4 ºC overnight prior to being transferred to -80 ºC for long-term storage.

2.4. Brain Dissection

Prior to gene expression analysis, brain samples were dissected into 9 parts: olfactory tract, olfactory bulb, telenchepalon, pineal gland, optic lobe, cerebellum, medulla oblongata, saccus vasculosus, hypothalamus and optic nerve (**Figure 2.5**). Considering the primary source of *crf*-related genes in hypothalamus, we decided to study the whole hypothalamus and dissected it referring to the brain dissection procedure in **Appendix B**.

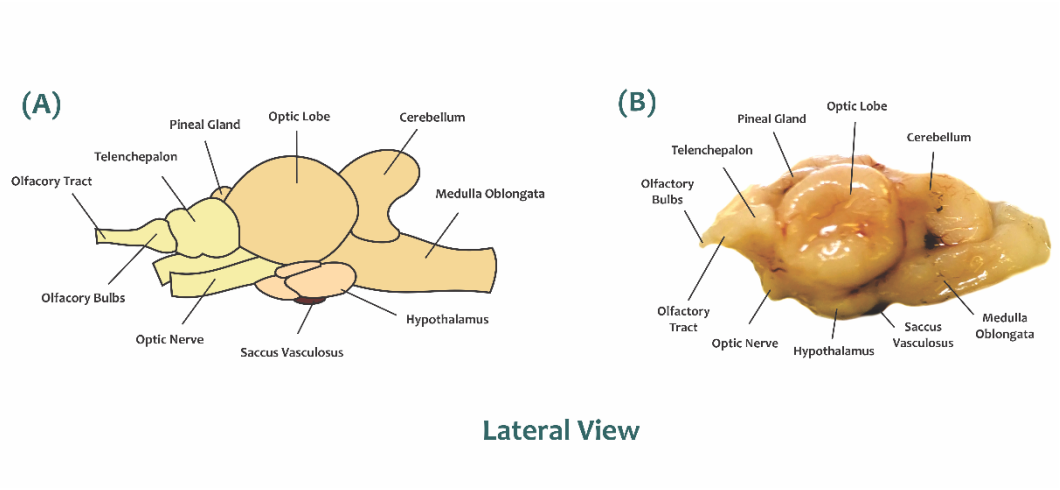

Figure 2.5. Salmon brain. A: schematic drawing; B: real image (Royan, 2018).

To ensure high RNA yield and good tissue integrity, the brain was placed on ice block during dissection. The brain was cleaned from membranes and blood vessels using forceps before cutting a particular part of the brain. Saccus vasculosus was the first part that could be collected easily by forceps. Pineal gland was directly removed using forceps while olfactory bulb and tract were cut using scalpel to separate it from telenchepalon. The next parts that was collected was telenchepalon and cerebellum, respectively. Prior to cutting the hypothalamus, medulla oblongata was removed, and the hypothalamus was separated away from the optic nerve before cutting. After the dissection, the hypothalamus looked like the following figure:

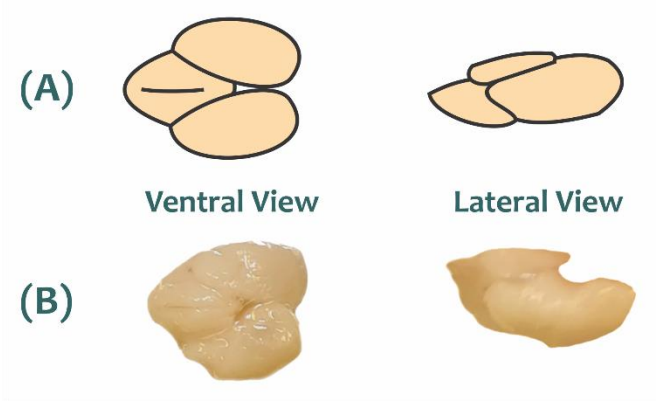

Figure 2.6. Post-dissection hypothalamus. A: schematic drawing; B: real image (Royan, 2018).

2.5. Growth Rate and Condition Factor (K) Calculation

Due to its reliability in comparison to other methods and its suitability for this study, the Relative Growth Rate (RGR) was applied to calculate fish growth rate. RGR is basically the percentage of body mass gain during certain period of time (Lugert et al., 2016). Initial weight from 2 and 5 sampled fish from each tank on day 0 was measured in addition to the final weight from 5 sampled fish from each

tank on day 9. Based on the initial and final weight, RGR was calculated using **Equation 1**.

$$RGR = \frac{w_t - w_i}{w_i} \times 100 \qquad (1),$$

in which w_t is the final weight while w_i is the initial weight (Lugert et al., 2016).

Meanwhile, to demonstrate the fitness of the fish after stress exposures, condition factor (K) was used and quantified using weight and length of the fish by the following equation:

$$100\,\frac{W}{L^3} \qquad (2),$$

where W is the weight (g) and L is the length of the fish (cm) (Froese, 2006).

2.6. Plasma Cortisol Analysis

Plasma cortisol measurement was done by Drs. Marit Espe and Birgitta Norberg at Institute of Marine Research using Enzyme Linked Immunosorbent Assay (ELISA) with Ellman's reagent (see Sokolowska et al., 2013).

2.7. RNA Extraction

Three out of five sampled fish on day 0 before and after stress exposure, 1, 8 and 9 were randomly selected for gene expression analysis. The RNA extraction was done using RNeasy® Mini Kit protocol with On-column DNase Digestion (QIAGEN, Hilden, Germany). The hypothalamus was firstly put into a 2 ml tube containing 600 µl Buffer RLT and 6 µl β-Mercaptoethanol in addition to 0.6-0.7 g of zirconium oxide beads (Bertin Technologies, Versailles, France; diameter 1.4 µm) and then homogenized using Precellys 24 Homogenizer (Bertin Technologies, Versailles, France) for 15 seconds at 5,000 rpm. The other components, such as 70% ethanol, 700 µl Buffer RW1 and 1 ml Buffer RPE, were used in later steps

according to the manufacturer's instruction. Afterwards, the concentration and purity of RNA were checked using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

2.8. cDNA Synthesis

To avoid genomic DNA remnants, TURBO DNase-free Kit® (Ambion Applied Biosystem, Foster City, CA, USA) was used as a treatment for 1.5 µg of RNA sample before performing cDNA synthesis. Afterwards, cDNA synthesis was carried out using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) in which the following components were added in a total reaction volume of 20 µl: 1 µl Oligo(dT)$_{20}$ (50 µM), 1 µl of 10 mM dNTP Mix (10 mM each dATP, dGTP, dCTP and dTTP at neutral pH), 10 pg - 5 µg of total RNA (volume depends on RNA concentration), distilled water and SuperScript™ III RT Master Mix (4 µl of 5x First-Strand Buffer, 1 µl of 0.1 M DTT, 1 µl of RNaseOUT™ Recombinant RNase Inhibitor and 1 µl of SuperScript™ III RT).

2.9. RT-PCR Primer Design

Primers used for Real-Time Polymerase Chain Reaction (RT-PCR) assays in this study, i.e. *crfssa03*, *crfssa14*, *crfssa19*, *crfssa29*, *crfbpssa01* and *crfbpssa11*, were designed by Lai, F. (unpublished sequence) while ***ef1α*** (Valen et al., 2011) and *SsS20* (Olsvik et al., 2005) were used as reference genes. A total of four and two gene-specific RT-PCR primer pairs were designed for *crf* and *crfbp* from Atlantic salmon sequences retrieved from the NCBI data base (https://www.ncbi.nlm.nih.gov/, see **Table 2.3** for accession number). For each amplicon, primers were designed using Primer3 (http://primer3.ut.ee/) and NCBI primer designing tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and

synthesized by Sigma-Aldrich (Saint-Louis, Missouri, USA). In addition, to avoid amplification of genomic DNA, the primers were designed to span between exon-exon junction. The primers had been analyzed for crossing point (Cq), primers efficiency (E) and melting peaks, and the products were run on a gel electrophoresis and sequenced at the sequencing facility at the University of Bergen. Primers had a single melting peak indicating good specificity and good efficiency based on the result shown by the standard curve in RT-PCR test (**Appendix C**). Furthermore, gel electrophoresis test also confirmed that the primers amplify amplicons with corresponding sizes as shown in **Table 2.2**.

Table 2.2. Primer sequences used in the RT-PCR.

Gene	Primer Sequence (5' → 3')	Amplicon (bp)	Accession Number	Reference
ef1α	F: GAGAACCATTGAGAAGTTCGAGAAG R: GCACCCAGGCATACTTGAAAG	71	AF321836	Valen et al. (2011)
SsS20	F: GCAGACCTTATCCGTGGAGCTA R: TGGTGATGCGCAGAGTCTTG	85	NM_001140843.1	Olsvik et al. (2005)
crf ssa03	F: GCACTTGATCCATTCCACAA R: ACCGATTGCTGTTACCGACT	232	NM_001141590.1 XM_014190344.1	Lai, F., unpublished sequence
crf ssa14	F: TGGACATATTCGGGAAATGAA R: GTCAACGGGCTATGTTTGCT	229	XM_014139989.1 XM_014139988.1	Lai, F., unpublished sequence
crf ssa19	F: AACACTTGTCGCGGGTCTTG R: GTCGGGATCAACAGGAATCTTCA	174	XM_014159556.1	Lai, F., unpublished sequence
crf ssa29	F: TCCATCACTCGTGGAAAAGGA R: CAGGGGTTCAACGAGATCTTCA	91	XM_014181363.1	Lai, F., unpublished sequence
crfbp ssa01	F: AATGGCCCCGCCCAGAT R: ATATAGGAGGTGGAGAGATAGAT	197	NM_001173799.1	Lai, F., unpublished sequence
crfbp ssa11	F: AACGGTCCCGCCCAGAT R: TAGGTGGCAGATAGATAAAG	194	XM_014128333.1	Lai, F., unpublished sequence

2.10. Real Time - PCR (RT-PCR)

Each of 20 µl RT-PCR reaction consisted of 10 µl of SYBR Green I Master Mix (Roche Diagnostic, Basel, Switzerland), 0.6 µl forward and reverse primers each (10 mM), 6.8 Ultra-Pure Water (Biochrom, Berlin, Germany) and 2 µl cDNA template. The reaction mixes were run in duplicates and loaded into 96-well plate

(Bio-Rad Laboratories, CA, USA), including non-template control (NTC), no-reverse transcriptase control (NRT) and positive control. The following RT-PCR protocol was performed: 1) 95 ^{o}C for 30 seconds, 2) 95 ^{o}C for 5 seconds, 3) 60 ^{o}C for 25 seconds, 4) repeating step 2-3 for 39 more times. The RT-PCR was performed using C1000 Touch Thermal Cycler, CFX96 Real-Time System (Bio-Rad Laboratories, CA, USA) in connection to CFX Manager Software version 3.1 (Bio-Rad, Laboratories, CA, USA). Since the expression of both reference genes, i.e. *ef1α* and *SsS20*, was assumed to be not stable (**APPENDIX D**), the expression of each target gene, i.e. *crfssa03*, *crfssa14*, *crfssa19*, *crfssa29*, *crfbpssa01* and *crfbpssa11*, represents the copy number of the corresponding target gene.

2.11. Statistical Analysis

Statistical analyses were performed using R Software System version 3.50 (The R Foundation for Statistical Computing, Vienna, Austria). All datasets were tested for the normality using Anderson-Darling Normality Test while Levene's Test was performed to test the homogeneity of variance. In case of very significant normality and/or variance, any unprecedented outliers were removed, and the dataset were subsequently square-rooted transformed before performing the comparison test. The level of significance was set to 0.05. The effect of stressor on RGR was evaluated using One-Way ANOVA test. Meanwhile, the interaction of stressor and observation period in weight, length, plasma cortisol level and gene expression were assessed using Two-Way ANOVA test. Pair-wise multiple comparison test with Bonferroni correction was used to see differences in weight and length. Whereas, multiple comparisons test in RGR, plasma cortisol and gene expression were assessed using Tukey HSD *post hoc* test. All data in tables and

figures are provided as mean ± SEM (Standard Error of Mean) unless otherwise stated.

III. RESULT

3.1. Effect of Stressors on Weight, Length and Growth Rate

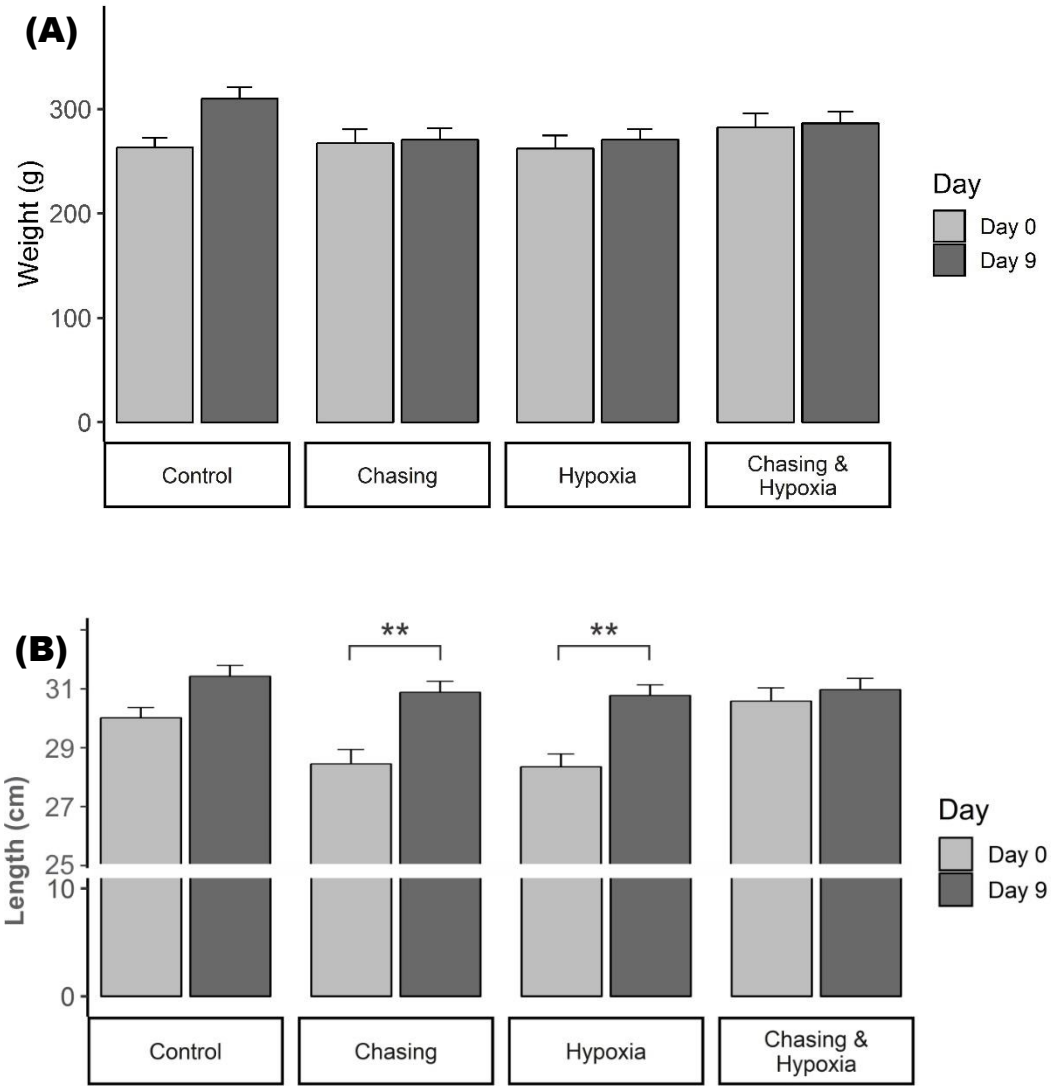

Figure 3.1. Weight (A) and length (B) of Atlantic salmon post smolt at the start (Day 0) and at the end of the experiment (Day 9). Bars represent means ± S.E.M; Number of fish: *N* = 21 on day 0 and *N* = 15 on day 9. Asterisk indicates the degree of significance (Two-way ANOVA followed by pair-wise multiple comparison test with Bonferroni correction; ** $p < 0.01$).

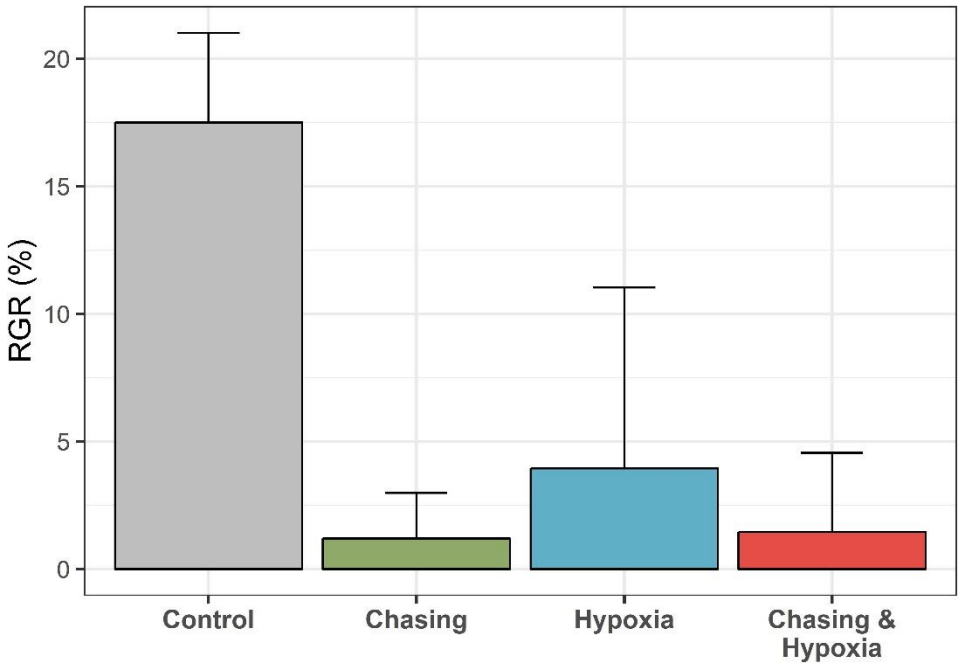

Figure 3.2. Relative Growth Rate (RGR) of the Atlantic salmon post smolt. Relative Growth Rate (RGR) is defined as a percentage of body mass gain during certain period of time. Values represent means ± S.E.M (*N* = 21 for initial weight, *N* = 15 for final weight).

After 9 days, control fish tended to have a higher increase in body mass in contrast to the chronically stressed fish. Fish in the control group grew from 263.38 ± 9.25 g on day 0 to 309.73 ± 11.33 g on day 9. Fish treated with the chasing stressor had grown by 3.10 ± 1.89 g at the end of experiment while fish exposed to hypoxia and the combination of chasing and hypoxia gained 8.82 ± 2.65 g and 3.65 ± 2.38 g, respectively ($F_{1,138}$ = 3.2738, $p_{(day)}$ = 0.0725; **Figure 3.1**). These results are in line with the RGR of the fish, albeit insignificant. Control fish grew around 17.5 ± 3.5 % during the experiment while fish in the chasing, hypoxia and the combination of chasing and hypoxia had grown by around 1.19 ± 1.79 %, 3.95 ± 7.09 %, 1.45 ± 3.1 %, respectively (**Figure 3.2**). With respect to the length, control fish grew from 30.01 ± 0.35 cm on day 0 to 31.43 ± 0.37 cm at the end of experiment; Fish belonging to chasing, hypoxia and the combination of chasing and hypoxia group had grown by 2.43 ± 0.11 cm, 2.42 ± 0.08 cm and 0.39 ± 0.07

cm, respectively ($F_{1,138}$ = 29.3242, $p_{(day)}$ < 0.0001; **Figure 3.1**). There is no tank effect on either weight or length. While chronically stressed fish have reduced condition factor, the control fish shows a slight increase. For 10 days, there is a significant interaction effect of treatment and the observation period on condition factor (K) of the fish ($F_{3,135}$ = 53.475, p < 0.0001). Control fish tended to exhibit an elevation in condition factor from 0.967 ± 0.008 on day 0 to 0.992 ± 0.009 on day 9, albeit insignificant. On the other hand, chronically stressed fish show a significant decline in condition factor for chasing as well as hypoxia group, whereas the combination of chasing and hypoxia group tended to show a reduction in condition factor despite insignificant (**Table 3.1**).

Table 3.1. Condition factor of fish at the start (day 0) and at the end of the experiment (day 9). Condition factor (K) is defined as the fatness of the fish considering its body weight and fork length. Values represent mean ± S.E.M. Asterisk indicates the degree of significance (Two-way ANOVA followed by Tukey's *post hoc* test; ns p > 0.05, **** p < 0.0001).

Treatment	Period	Condition Factor	N	Significance Degree
Control	Day 0	0.967 ± 0.008	21	ns
	Day 9	0.992 ± 0.009	15	
Chasing	Day 0	1.145 ± 0.012	21	****
	Day 9	0.911 ± 0.011	15	
Hypoxia	Day 0	1.134 ± 0.013	20	****
	Day 9	0.925 ± 0.014	15	
Chasing + Hypoxia	Day 0	0.974 ± 0.016	21	ns
	Day 9	0.958 ± 0.013	15	

3.2. Plasma Cortisol

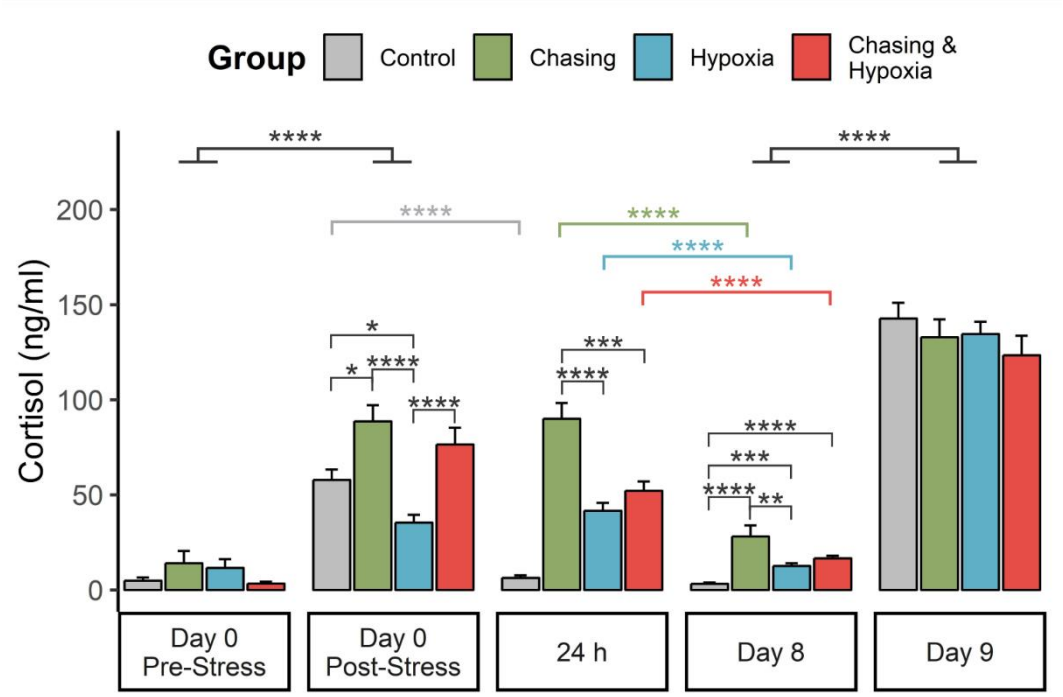

Figure 3.3. Plasma cortisol level of Atlantic salmon post-smolt during the period of the experiment. Fish were exposed to three different types of chronic stressors from Day 0 Post-Stress to Day 8, and on Day 9 all groups were exposed to a novel stressor (confinement). Bars represent mean ± S.E.M. (N = 6 for each group on day 0 before stress; N = 15 for each group on the rest of observation period). A Two Way ANOVA test shows a significant interaction effect (stressors x day of the experiment): $F_{12,226}$ = 12.938, p < 0.0001. Asterisks represent the significance degree quantified by Tukey's *post hoc* test (* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001).

For all groups, plasma cortisol elevation triggered by chronic stressors on day 0 is lower on day 8 and surged on day 9 after a novel stressor. The plasma cortisol begins with no significant difference among groups on day 0 before stressor exposure. Chronic stresses initiated on day 0 appear to elevate the plasma cortisol of stressed groups 1 hour after, but the control group that was left unstressed also shows a rise. Nevertheless, unlike the plasma cortisol of stressed groups that still remains elevated, that of the control group plunges to the basal level after 24 hours. There are significant differences between groups in this period as shown by the comparisons of each stressed group toward control group ($F_{12,226}$ = 12.938, p <

0.0001; **Figure 3.3**). The magnitude of plasma cortisol of the stressed groups decreases on day 8 despite having been continuously exposed to stressors for a week. Meanwhile, the extreme upsurge of plasma cortisol in all groups including control is observed after the novel stressor exposure, in which the control group leads as the highest (142.7 ± 8.31 ng/ml) followed by hypoxia group (134.6 ± 6.45 ng/ml), chasing (132.85 ± 9.46 ng/ml) and the combination of chasing and hypoxia (123.44 ± 10.22 ng/ml). In addition to be significantly different with respect to the interaction effect (treatment x observation period) ($F_{12,226}$ = 12.938, p < 0.0001), the observation period also shows a significant difference in plasma cortisol level of the fish ($F_{4,226}$ = 142.288, p < 0.0001). There is no tank effect on plasma cortisol level.

3.3. Gene Expression

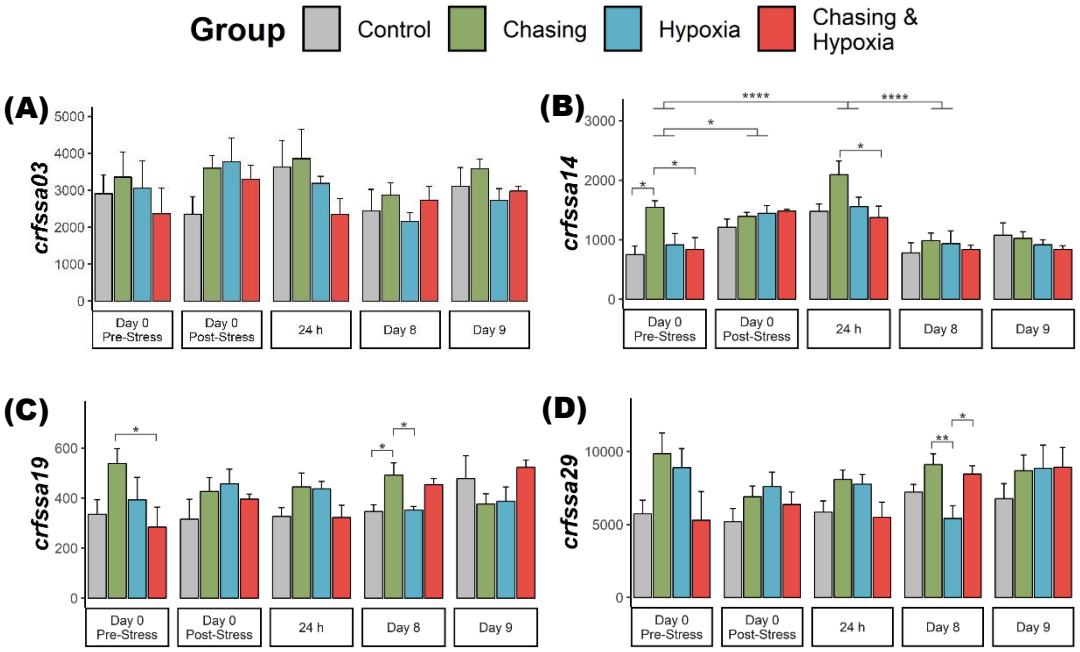

Figure 3.4. The abundance of four *crf* gene paralogs in the hypothalamus of Atlantic salmon post-smolt. Control and three chronically stressed groups (chasing, hypoxia and the combination of chasing and hypoxia) were observed from day 0 to day 8. On day 9, all groups including control were exposed to a novel stressor. Studied gene paralogs were *crfssa03* (A), *crfssa14* (B), *crfssa19* (C) and *crfssa29* (D). Bars represent mean ± S.E.M (*N* = 6 for each group on day 0 before stress; *N* = 9 for each group on the rest of observation period), and the values derive from copy number of the gene. Asterisks show the significance degree (* $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$) as analyzed by Tukey's *post hoc* test.

Figure 3.4 illustrates the expression of four *crf* gene paralogs (*crfssa03*, *crfssa14*, *crfssa19* and *crfssa29*) in the hypothalamus of control and three stressed groups of fish (chasing, hypoxia and the combination of chasing and hypoxia). A significant interaction effect (treatment x observation period) was found in *crfssa19* paralog ($F_{12,147} = 1.842$, $p = 0.046$). Significant differences in treatment (stress exposure) were observed in *crfssa14* ($F_{3,149} = 4.895$, $p = 0.0028$) and *crfssa29* ($F_{3,152} = 4.25$, $p = 0.0065$). In terms of observation period, only *crfssa14* ($F_{4,149} = 16.644$, $p < 0.0001$) paralog exhibits a significant difference considering day 0 before stressor as the reference (day 0 after stress $p < 0.05$; after 24 hours $p <$

0.0001). On the other hand, the abundance of two paralogs of *crf* binding protein gene (*crfbpssa01* and *crfbpssa11*) is depicted in **Figure 3.5**. A significant interaction effect (treatment x observation period) was shown only in *crfbpssa01* paralog ($F_{12,138}$ = 2.084, p = 0.0217) while there is no significant difference in either treatment (stress exposure) or observation period in *crfbpssa11* paralog. There is no tank effect on all gene paralogs expression.

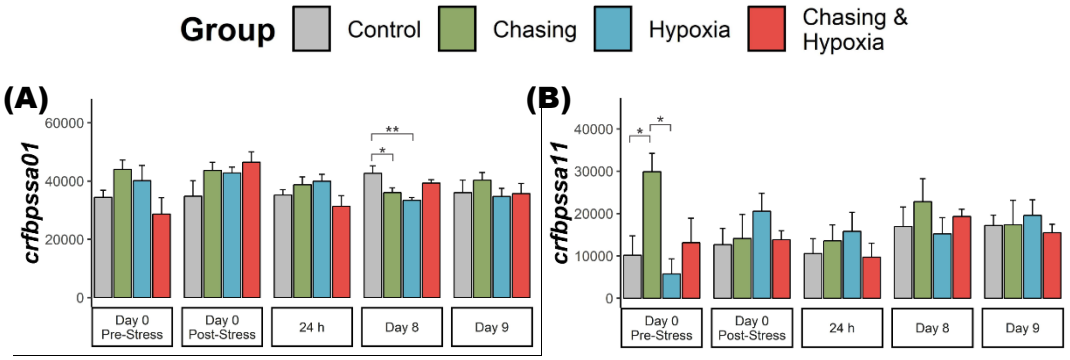

Figure 3.5. The abundance of two *crf* binding protein gene paralogs in the hypothalamus of Atlantic salmon post-smolt. Control and three chronic stressed groups (chasing, hypoxia and the combination of chasing and hypoxia) were observed from day 0 to day 8. On day 9, all groups including control were exposed to a novel stressor. Studied gene paralogs were *crfbpssa01* (A) and *crfbpssa11* (B). Bars represent mean ± S.E.M (N = 6 for each group on day 0 before stress; N = 9 for each group on the rest of observation period), and the values derive from copy number of the gene. Asterisks show the significance degree (* $p < 0.05$, ** $p < 0.01$) as analyzed by Tukey's *post hoc* test.

IV. DISCUSSION

4.1. Discussion of Findings

The aim of this study was to identify and characterize the stress response of Atlantic salmon post-smolt after exposure to chronic stressors for 8 days followed by a novel stressor at the end of experiment. There are three core points that can be deduced from the observations. First, the chronic stress exposures (chasing, hypoxia and the combination of chasing and hypoxia) tend to suppress the growth rate of the chronically stressed fish that resulted in lower weight gain. Second, the response of plasma cortisol level diminishes after 8 days of chronic stress exposure, whereas the introduction of novel stressor at the end of experiment stimulates a higher cortisol response in control fish in contrast to chronically stressed fish. Third, *crf* and *crfbp* gene paralogs in the hypothalamus are expressed diversely throughout the observation period. Only the *crfssa14* gene seems to be linked with exposure to stressors used in this experiment.

4.1.1. Weight, Length and Growth Rate

Stress exposures, i.e. chasing, hypoxia and the combination of both, tend to result in a more suppressed weight gain and growth rate in stressed fish relative to control after 9 days. Despite insignificant, the data indicate that there is a higher growth rate and weight gain in unstressed fish compared to those that have been exposed to the long-term stressors. Earlier study has also shown that repeated chasing leads to reduced body mass of Atlantic salmon post-smolt where fish in the control group gain a significant body mass compared to chasing-exposed fish (Madaro et al., 2016b). The growth rate has also been suggested to be negatively affected by chasing stressor in salmonids and other fish species (Madaro et al., 2015; Pavlidis et al., 2015; Tsalafouta et al., 2015; Vindas et al., 2017b). Similarly,

there have been many studies concerning hypoxic stress toward reduced weight gain and growth rate. Burt et al. (2014), Hansen et al. (2015), Remen et al. (2014, 2012) and Vikeså et al. (2017), for instance, suggested that a hypoxic stressor (in the range from 40 to 70 % O_2 saturation) can inhibit growth of Atlantic salmon post-smolt. Despite having different hypoxic threshold from Atlantic salmon, other species also show suppressed weight and growth after being exposed to hypoxia (Cheek, 2011; Green et al., 2012; Methling et al., 2010; Sanchez et al., 2011). However, we are not aware of any previous studies that have investigated the chronic effect of simultaneous chasing and hypoxia on fish weight and growth. In fact, the study of chronic agrichemical compounds that is in concert with chasing suggests that these simultaneous stressors negatively affect fish weight (Koakoski et al., 2014). Therefore, we suggest that simultaneous exposure of both chasing and hypoxia might be the cause of reduced weight gain and growth rate of the chronically stressed fish.

On the other hand, the trend of relatively higher weight gain and growth rate in control fish appears to be unmatched with the length. While chronically stressed fish tend to have lower weight gain and growth rate, the findings does not demonstrate this tendency with regard to length. Nevertheless, the increase in length does not necessarily indicate that there is no suppressed growth in fish, but condition factor does. Indeed, due to the fact that condition factor of the chronically stressed fish dwindles compared to that of control fish, it shows that there was a suppression of fatness in the fish after 8-day chronic stress exposure. This finding agrees with the study in Atlantic salmon post-smolt (Remen et al., 2014, 2012) showing a decrease in the condition factor of stressed fish. Likewise, a study in rainbow trout suggests that forced swimming resulted in diminished condition factor, shown by lighter weight and leaner body shape (Farrell et al., 1991). Lower

condition factor (K) is not only a sign of poor well-being or fitness in fish, but it is also sometimes tied with bad nutritional status despite the fact that the link is not always straightforward (Blaxter, 1988; Bolger and Connolly, 1989; Kachari et al., 2017).

Weight, length and growth as a part of stress indicators in fish is categorized into the tertiary or whole-organism response to stress (Iwama, 1998; Sopinka et al., 2016). Negative growth of fish as a result of stressors, such as chasing and hypoxia, has previously been investigated in a plethora of papers. It shows a tight connection between stress and reduced weight, length and growth. Bonga (1997) in his review asserted that in connection to stress, reduced growth results from reduced appetite and food intake, impaired food assimilation and suppressed metabolic rate. Indeed, stressors cause negative growth in fish by impairing metabolic pathways and diverting energy allocation (Iwama, 1998; Wang et al., 2009). Diminshed energy of food due to reduced appetite and food intake can cut the energy portion to growth, whereas ineffective food assimilation because of digestive system impairment leads to the increase in faecal energy resulting in decreased growth energy allocation (Wang et al., 2009). Meanwhile, the increase in O_2 consumption and the reduction in heat production are typical markers for metabolic rate suppression in fish (Richards, 2009). Some of above-mentioned aspects, however, are not covered in this study due to the limitation as well as the complexity of experimental design.

4.1.2. Plasma Cortisol

The effect of stressors that were exposed to the fish on the level of plasma cortisol seem in line with the expectation based on exploration of literature data. For instance, the data show that the chronic stress exposure leads to lower levels

of plasma cortisol on day 8 as well as the sudden increase in this corticosteroid after being introduced with a novel stressor on day 9. Started with a basal level in all groups on day 0 before the stress exposure, there was a considerable increase in plasma cortisol in all groups after the fish was exposed to stressors on day 0, including control fish that is supposed to remain at basal levels due to the absence of stressor. The resting cortisol level in the beginning of the experiment is suggested to represent a normal condition since there is no specific stress exposure in this period of time (Conde-Sieira et al., 2018; Kemenade et al., 2009). This agrees with numerous studies finding that plasma cortisol in Atlantic salmon post-smolt stays at the resting level when no specific stressors are induced (Calabrese et al., 2017; Madaro et al., 2016b, 2015; Olsen et al., 2012; Singer et al., 2003).

The event of plasma cortisol elevation in control fish on day 0 after stress exposure appears to contradict with the theory because there was no desired stressor induced to control fish on this day. The hypothesis is that even with the sampling action by netting the salmon, the plasma cortisol will rise to some extent because this might be perceived as a stressor by the control fish. Madaro et al. (2015) argued that sampling may also contribute to disturb HPI axis beside desired stressor that is induced to the fish. The association between brief handling/netting and upregulated plasma cortisol in zebrafish has also been reviewed by Spagnoli et al. (2016). Moreover, reviews in Barton and Iwama (1991) and Bonga (1997) clarify that sampling procedure may also contribute to plasma cortisol elevation. On the other hand, the temporal space of sampling for control fish on day 0 pre-stress and post-stress was only approximately one hour in this experiment. Perhaps, this might also be another reason why plasma cortisol was elevated in control fish, even without exposing the fish to a desired stressor, since upregulated

plasma cortisol normally happens in a relatively short time (Calabrese et al., 2017; Faught et al., 2016). A study by Gesto et al. (2013) in rainbow trout, for example, reveals that the increase in plasma cortisol occurs in a few minutes until one hour, and it returns to resting level in a few hours. Since the sampling interval is still in the range of plasma cortisol response to stress, the upregulation of plasma cortisol in control fish was most probably due to those two factors: sampling action and its short interval.

While the plasma cortisol of chronically stressed fish remains elevated after 24 hours, that of control fish returns to basal level. The downregulation of plasma cortisol at basal level in control fish indicates that there is no stress signal perceived by the fish. Indeed, when no stressor is induced, plasma cortisol level will gradually dwindle and remain at basal level after a few hours. Studies in Coho salmon (Shrimpton and Randall, 1994) and rainbow trout (Jentoft et al., 2005; Yada et al., 2007) point out that plasma cortisol increases significantly approximately one hour after the stressor was induced and returns to basal level after 24 hours. Even plasma cortisol stays back at resting level 8 hours after a stressor was exposed to rainbow trout (Gesto et al., 2013). Furthermore, 24 hours after stress exposure regimes, the plasma cortisol level of chronically stressed fish is significantly higher compared to control fish. Based on those facts, we suggest that the effect of stressors, i.e. chasing, hypoxia and the combination of chasing and hypoxia, on plasma cortisol response is still much more pronounced than that of sampling action. Thus, the variability of plasma cortisol during this experiment is indeed mainly due to the stress treatments.

The effect of chronic stress exposure on the chronically stressed fish is apparent when observing a significant reduction of their plasma cortisol level on day 8. While control fish shows a stable basal plasma cortisol level on day 8, all

stressed groups exhibit a downregulation of plasma cortisol after one-week stress exposure. This downregulation commonly occurs in fish, particularly Atlantic salmon post-smolt, when exposed to chronic stressors. Studies of effects of unpredictable chronic stressors (Madaro et al., 2015) and repeated chasing (Madaro et al., 2016b) on stress response of Atlantic salmon post-smolt show a dwindling level of plasma cortisol within 5 days. Even, the declining trend also happens in other salmonid species, such as Coho salmon (Shrimpton and Randall, 1994) and rainbow trout (Kiilerich et al., 2018) after being exposed to chronic stressors. The decrease in plasma cortisol as a result of chronic stress is suggested due to habituation (Barton et al., 1987; Koolhaas et al., 2011). A study in rainbow trout and Eurasian perch showed that diminished response of plasma cortisol in chronically stressed fish caused by repeated stressor indicates habituation to the stress stimuli (Jentoft et al., 2005). However, chronic downregulation as a result of repeated stressors is sometimes interpreted to connect with impaired HPI axis reactivity due to being exhausted of mounting a proper response of cortisol (Jeffrey et al., 2014; Øverli et al., 1999). Despite having been downregulated after being exposed to chronic stress for a week, the plasma cortisol of chronically stressed fish on day 8 is still significantly higher than that of control, indicating the adverse effect of the chronic stressors on the fish.

To evaluate the effect of habituation due to chronic stress, a novel stressor was subjected to all groups, including control. Consequently, we found an upsurge of plasma cortisol level in all groups. A vigorous stress response after an acute stress exposure normally occurs in unstressed fish, but the assumption of habituation happening in the chronically stressed fish still remains vague. Madaro et al. (2016b, 2015) have clearly described the phenomenon where plasma cortisol level of chronically stressed Atlantic salmon post-smolt surges after exposure to

an acute stress. Besides, not only is the trend observed in Atlantic salmon parr (Madaro et al., 2016b), but it is also consistent in other salmonids as well as in other fish species, such as rainbow trout, brown trout, gilthead sea bream and European sea bass (Barton et al., 2005, 1987; Culbert and Gilmour, 2016; Jeffrey et al., 2014; Pickering et al., 1987; Samaras et al., 2018; Tsalafouta et al., 2015). The studies mentioned above found that plasma cortisol level of control fish is higher than that of chronically stressed fish after being subjected to a novel acute stressor. Consistently, such a circumstance is also found in the current experimental data where fish that were chronically exposed to chasing, hypoxia and the combination of chasing and hypoxia seem to have more suppressed level of plasma cortisol in contrast to control fish, albeit insignificant. In other words, confinement as a novel stressor in this experiment was more pronounced to control fish compared to chronically stressed fish.

There are two arguments as to why the chronically stressed fish has lower plasma cortisol level than control fish after exposed to a novel stressor. First, as a consequence of adaptation, the physiological response of the fish tolerates the subsequent stressor through the negative feedback of HPI axis, thus resulting in reduced response to a novel stressor (Barton et al., 2005; Madaro et al., 2016b, 2015; Pickering et al., 1987). Second, the sub-level plasma cortisol is probably due to the cumulative burden of the prolonged stress that goes beyond the allostatic load of fish as exhibited by the other whole-organism stress responses: growth reduction, inhibition of reproduction and impaired immune response (Barton et al., 1987; Bonga, 1997; Haukenes and Barton, 2004). Owing to the fact that the stressor interval in current experiment is only within a few hours, our findings appear to agree with the latter argument since the cumulative stress response might occur as a result of short interval of stressor. Indeed, while the wider interval

can enhance fish performance in light of compensation and habituation, the short stress interval can lead to maladaptive performance of the fish (Schreck, 2000). According to the current finding with respect to a more suppressed weight gain and growth rate in chronically stressed fish relative to control fish, we suggest that the chronic stress might suppress the HPI axis until surpassing its coping capacity, as accounted for in the latter argument.

As the final product of physiological response to stress in HPI axis pathway, plasma cortisol often becomes the most pivotal aspect in exploring the effect of stressors on fish (Mommsen et al., 1999). Plasma cortisol is a primary circulating corticosteroid that is suggested to be an effective indicator of determining the state of stress in fish, and by which it is able to characterize stress stimuli level (Aluru and Vijayan, 2009; Campbell et al., 2010; Hoffmayer et al., 2015). Overall, chasing stressor appears to be the most pronounced and effective stimuli capable of stimulating vigorous response of the fish to a stressor, indicated by significant difference compared to the other stressors. Despite negligible, the effect of chasing stressor to plasma cortisol level is still more severe than the combination of chasing and hypoxia on day 9 when the novel stressor was induced. The severity of chasing stressor toward plasma cortisol elevation agrees with a study in silver catfish comparing several stressor regimes revealing that chasing stimulates a more pronounced stress response compared to the other stressor types in view of plasma cortisol level (Koakoski et al., 2014). In addition, the study in Gilthead seabream reveals that chasing-added stressor regime have stronger effect on plasma cortisol concentration than a mere confinement or the additional air exposure stressor, albeit the possible presence of cumulative response (Samaras et al., 2018). Nonetheless, as a general remark, it is acknowledged that the magnitude of plasma cortisol level does not necessarily always serve as an

indication of stressor effectivity. Not only may similar stressor elicit the analogous response among different species, but it may also trigger distinct stress reactivity even in family-related species, and vice versa (Donaldson et al., 2014; Fanouraki et al., 2011; Sopinka et al., 2016). Taking a study of large-sized European sea bass as an example, it was found that even though the vigor of stress is different, the response patterns remain identical (Fatira et al., 2014). Therefore, the current conclusion regarding the effective stressor should be taken into consideration with caution and only encompasses in the scope of this experiment.

4.1.3. Gene Expression

Even though most of the *crf* paralogs selected for analysis in this study seem not to link with the stressors, there is one gene paralog that is suggested to connect with the stress exposure in our experiment, namely *crfssa14*. Considering the approximate proportional relationship between cortisol level and *crf* mRNA abundance, it appears *crfssa14* paralog have a connection to the stress regimes used in this study. For instance, the significant increase in *crfssa14* gene abundance after exposure to stressors on day 0 is parallel to the considerable elevation of plasma cortisol level in the same period relative to control. Similarly, this parallel relationship is also observed after 24 hours, where significant upregulation of plasma cortisol level is confirmed by the gene abundance of *crfssa14* paralog, albeit insignificant among groups. Consistently, this approximate proportional trend is also apparent after one week of stress exposure, in which there is a significant reduction of gene abundance on day 8. However, this trend appears to absent after the novel stressor was induced.

The direct proportional relationship between plasma cortisol level and *crf* mRNA abundance has previously been seen in other studies (Carpenter et al.,

2014; Jeffrey et al., 2012; Madaro et al., 2016b, 2015) revealing that this parallel trend indicates the connection between *crf* and plasma cortisol in HPI axis pathway. Meanwhile, the absence of such a parallel trend might perhaps be due to a negative feedback occurring as a consequence of cumulative stress response during the chronic stress exposure (Alderman et al., 2012; Benítez-Dorta et al., 2017; Kiilerich et al., 2018), or it may be in relation to the unprecedented mRNA or protein degradation (Liu et al., 2016; Sopinka et al., 2016) because of failure to predict the optimal time to analyze gene expression. However, the fact that several studies also found no proportional relationship between *crf* abundance and plasma cortisol level (Benítez-Dorta et al., 2017; Jeffrey et al., 2014; Pavlidis et al., 2015) suggests that this is an interesting opportunity to investigate the anomaly further.

Since we found that there is no proportional relationship between *crfssa03*, *crfssa19* and *crfssa29* paralogs and plasma cortisol level, we suggest that there might be no link of these paralogs to the stress regimes used in this experiment. We speculate that there might be a diverging regulation of these paralogs. A study in African cichlid and zebrafish, for instance, argued that probably there might be a regulatory divergence of *crha* paralogs found across those two species because of localization in different tissues (Grone and Maruska, 2015b). By contrast, there might be also a chance for sub-functionalization instead of neo-functionalization as a result of the Ss4R event happening in salmonids. For instance, Lien et al. (2016) found some probability of sub-functionalization among gene duplicates despite the dominance of neo-functionalization occurrence. This finding is also in agreement with a study across vertebrates arguing that the sub-functionalization among paralogs may happen as a consequence of whole genome duplication (Grone and Maruska, 2015a). However, since there is still no supporting evidence yet, our speculation needs to be tested in further investigations.

Based on a stressor perspective, it seems that chasing is the most effective stressor on the stress response of Atlantic salmon post-smolt compared to the other stressors. In *crfssa14*, for instance, the data in **APPENDIX E – 7.5.3. Gene Expression** suggest that gene abundance of *crfssa14* as a result of chasing stressor is more pronounced than that of control and the other groups. This tendency is also confirmed in the other gene paralogs, i.e. *crfssa03*, *crfssa19* and *crfssa29*, in which the gene expression as a result of chasing is more abundant than that of other stressors relative to control in almost the whole observation period. The difference of gene abundance as a consequence of different stressors is also found in the study of European seabass and gilthead seabream depicting that the stressor that is considered severe shows the most abundant *crf* transcript compared to the other groups (Samaras et al., 2018). In short, due to higher abundance of gene expression relative to other groups, it can be deduced that chasing is the most pronounced and effective stressor in eliciting the stress response in Atlantic salmon post-smolt.

On the other hand, according to the findings, it is suggested that there is no link between *crfbp* gene abundance (*crfbpssa01* and *crfbpssa11*) and the stressors used in current experiment. Even though some studies have found an inverse relationship between *crf* and *crfbp* gene expression (Jeffrey et al., 2012; Madaro et al., 2016b, 2015; Wunderink et al., 2012), it is difficult to find such a pattern in this study. An opposite pattern between *crf* and *crfbp* gene expression has previously been displayed in some studies, but no proportional relationship does not necessarily indicate no connection between the *crf* and *crfbp* genes. Indeed, although the inverse relationship of *crf* and *crfbp* appears to be logic since *crfbp* can block *crf* by reducing its bioavailability in the stress response axis, some studies have found no typical relationship of expression between those two genes

(Jeffrey et al., 2014; Vindas et al., 2017b). Moreover, the mechanism of how this bioavailability is reduced are not comprehensively explored.

Since there is no difference in the expression of both *crfbp* gene paralogs, we suggest that the stress regimes used in this experiment do not affect the two *crfbp* gene paralogs. This agrees with previous studies finding that the stress conditions gave no effect on *crfbp* gene expression (Jeffrey et al., 2014, 2012; Madaro et al., 2015). This phenomenon might be rooted from the way the fish perceive stressful conditions where they tend to respond to stress variably when being exposed to the same stressor, or vice versa (Sopinka et al., 2016). Indeed, similar stressors not only might stimulate the same reactivity among different species, but it might also elicit different responses in species that are in the same family (Donaldson et al., 2014; Fanouraki et al., 2011). The fact that there is a significant difference in *crfbpssa11*, particularly on day 0 before stress, seems odd since there are no stressors induced in this period. In fact, we found this anomaly throughout the observation period, in which there are many random samples were found to have too low expression. Nevertheless, this finding might open an opportunity to investigate this phenomenon further.

Due to the fact that gene abundance does not necessarily indicate the level of protein (Maier et al., 2009; Schwanhausser et al., 2011), this study attempts only to emphasize a common framework of gene expression analysis and its relationship with the stress response of Atlantic salmon. Moreover, it is acknowledged that the dynamics in Atlantic salmon gene expression are still not completely understood, particularly in the scope of stress response. Therefore, the results of current study should be taken into consideration with care.

4.2. Discussion of Methods

4.2.1. Experimental Design

In aquaculture settings, Atlantic salmon-post smolts will encounter various stressors as a consequence of rearing activities. Indeed, unlike the other life stages, post-smolts may deal with stress factors more frequently in husbandry conditions, such as handling, crowding, vaccination, pumping, oxygen shortage or confinement (Calabrese, 2017; Kristensen et al., 2012; Sveen, 2018). Despite many studies exploring the effect of stressors on Atlantic salmon stress response, the understanding of how chronic stressors are compared to each other in affecting stress response are not completely understood. In addition, the effect of simultaneous chronic stressors as commonly occurs in a real aquaculture setting on the stress response of Atlantic salmon post-smolt is less studied. Therefore, this study considers three different types of chronic stressors: chasing, hypoxia and the combination of chasing and hypoxia, in addition to a novel stressor (confinement) as a confirmation of the HPI axis responsiveness (Sopinka et al., 2016).

The use of chase as a means of stressor have been performed in several studies (Madaro et al., 2015; Pavlidis et al., 2015; Tsalafouta et al., 2015; Vindas et al., 2017b), and these studies also confirm that chasing is capable of eliciting a stress response in Atlantic salmon post-smolt. Similarly, there have been some studies using hypoxia as a stressor, and this stressor is also found to affect the stress response in Atlantic salmon post-smolt (Burt et al., 2014; Hansen et al., 2015; Remen et al., 2014, 2012; Vikeså et al., 2017). However, to the best of our knowledge, there are no reports on the effect of simultaneous stressors of chasing and hypoxia on the stress response in Atlantic salmon post-smolt. In fact, in aquaculture settings, stressors commonly work in concert with each other to affect

the overall stress response. Hence, this study attempted to investigate how cumulative effect of these simultaneous stressors affects stress response of the fish compared to the other stressors.

Due to complexity of experimental design and the limitation of personnel, this study divides treatment groups into different schedules: chasing and hypoxia group as the first round; control and the combination of chasing and hypoxia group as the second round. Despite the fact that there is no literature, so far, elucidating how different round in experiments gives variance to the result, we believe that different schedules gave no biases to the findings. However, there is another factor that still can affect the stress response in fish, namely disturbance. Even though disturbance may take part in eliciting the stress response in fish (Bonga, 1997), this study ascertains that there was no disturbance factor creating bias to the experiment since the tanks were randomly distributed in such a way that there would be no significant disturbance. Provided the disturbance may take part in affecting the stress response of the fish, it can still be neglected because every tank has similar chances to be exposed to this uncontrolled variable. Nevertheless, we acknowledge that personnel limitation can contribute to result bias during the experiment since 3 people that were attributed to perform the stress procedure can be a source of technical variance. Thus, it is suggested to consider sufficient personnel in the future in order to avoid any bias possibility.

With respect to the stressor types and other practicalities, it is needed to clarify some points. First, even though some studies have performed the chasing stress using a net (Barton et al., 1987; Culbert and Gilmour, 2016; Gesto et al., 2015, 2013; Olsen et al., 2012; Yada et al., 2007), the use of brush stick as a chaser was also found to be effective in eliciting stress response in Atlantic salmon post-smolt (Madaro et al., 2016a). Second, due to its complex practicality, hypoxia stressor

was performed by reducing two-thirds of the water in tank, thereby creating another type of stressor, i.e. high density or crowding. This phenomenon has been depicted in some studies suggesting that crowding or high density is also found to affect the stress response in fish (Calabrese et al., 2017; Frere and Mcdonald, 2013). To avoid these cumulative stressors working in concert, it is suggested to have comparable water volume as the other stressor types. Third, the fact that only two fish were sampled on day 0 before stress compared to five fish in the rest of observation periods may influence data normality and homogeneity in the result. Indeed, the imbalance of data may lead to poor normality and homogeneity of the data in addition to outliers, missing data and other technical interferences (Zhang, 2015). Yet, according to the normality and homogeneity test (**APPENDIX F**), the statistical analysis reveals that most of the data have good normality and homogeneity, despite some that needed transformation. Based on those considerations, the above-mentioned points need to be taken into account in future experiments.

In summary, it is admitted that there were several unideal circumstances that happened during the experiment. Despite some uncontrolled factors may have created some biases, and therefore influencing the result, we believe that the controlled factors in the experimental design are more predominant in view of numerous literature-based justifications. Nevertheless, a more ideal experiment setup is required in the future in order to substantiate the drawn conclusions.

4.2.2. Controlled Variables (Oxygen Saturation, Salinity, Temperature)

In order to get trustworthy justifications concerning the relationship between independent variable (the effect of stressors) and dependent variable (the stress response), the experiment should be set up in such a way that the other variables

are under control. In this study, oxygen saturation, salinity and temperature are variables that were controlled during the experiment. It is noted that oxygen saturation during the experiment was 93.56 ± 0.87% on average. Meanwhile, the recorded data of salinity and temperature showed that these parameters were 28.54 ± 0.04 g/l and 9.2 ± 0.11 ^{o}C on average, respectively (**APPENDIX G**). According to a study concerning welfare in Atlantic salmon post-smolt, water quality in the current experiment is categorized as optimum based on Welfare Index (Stien et al., 2013). In addition, water quality conditions in this experiment is also in line with those of other studies in Atlantic salmon, particularly in the scope of stress response (Calabrese, 2017; Madaro et al., 2015; Solstorm, 2017; Sundh et al., 2010; Sveen, 2018; Vindas et al., 2017a). Therefore, we believe that there are no biases in the current findings resulted from the controlled variables.

4.2.3. Brain Dissection

As far as we are aware of, there are no clear and standardized guidelines in the literatures as to how to dissect Atlantic salmon post-smolt brain. The brain dissection procedure in this study lies around the common way of performing it in the research group as illustrated in **APPENDIX B**. Despite the fact that some studies have analyzed *crf* gene expression in the telencephalon (Pepels et al., 2004; Vindas et al., 2017a, 2017b), this study was designed to encompass only hypothalamic *crf* gene expressions in light of HPI axis pathway. According to some studies that investigated gene expression in Atlantic salmon brain, particularly those studying the stress response (Doyon et al., 2005; Ebbesson et al., 2011; Jeffrey et al., 2012; Madaro et al., 2016a, 2015), such a gene expression analysis is based on the analysis of preoptic area (POA) of the brain. However, Bernier (2006) suggest that the source of corticotropin-related peptides is not only localized in the POA, but also in other areas of hypothalamus, i.e. nucleus lateralis tuberis

(NLT) and nucleus recessus lateralis (NRL). Therefore, it was decided to analyze the whole hypothalamus to obtain more comprehensive result with regard to the gene expression in stress response.

4.2.4. Weight, Length and Growth Rate

The data of weight and length were acquired from the day after the acclimation period ended when the experimental schedule started until the day when the experiment was finished. Despite the fact that there were only approximate records of weight and length before the acclimation period and during the acclimation period, we believe that there would be no biases with this respect since the fish were randomly distributed when the acclimation period was begun. However, the fact that hypoxia group was found to weigh relatively lower in contrast to the others (**APPENDIX E – 7.5.1. Weight, Length and Growth Rate**) would suggest the need of weight and length records even before the acclimation period. To minimalize the variance of fish weight and length, the record of weight and length data before the acclimation period is important to consider in the future. We also realize that due to practical and logistical limitations, the growth rate was not calculated using the whole fish group in each tank at the beginning and at the end of the experiment, but rather using sampled fish. Despite the fact that there is a published study that calculates growth rates based on sampled fish, i.e. Vikeså et al. (2017), there is still the need of a more standardized sampling protocols and calculations of growth rate in future experiments.

The original aim of the design included accurate measurement of food intake. Although automatic feeders were used during the experiment, apparently the practical protocols were somewhat different since the feed was proceeded in the automatic feeder after the field personnel loaded the feed using approximate

measure, thus making the food intake data (**APPENDIX H**) unreliable. In fact, many studies included food intake data as a means of confirming differences in weight, length and growth rates between treatments (Boeck et al., 2000; Hansen et al., 2015; Madaro et al., 2015; McGeer et al., 2000; Sørensen et al., 2012). The correlation of food intake and stress is of key importance in an aquaculture setting and the ingestion rates needs to be recorded in future experiments. Taking together, the improvement of these aspects needs to be done to ensure reliability of the data.

4.2.5. Methodological Consideration in Bio-molecular Assays

Considering the small size of the sample tissues and the possibility of obtaining low RNA concentration, we tested several protocols using test samples prior to deciding which protocol to use in RNA extraction. In this case, there were four protocols included, i.e. full and modified protocol of TRI Reagent® (Sigma-Aldrich, Saint-Louis, Missouri, USA), NucleoSpin® RNA XS (Macherey-Nagel, Düren, Germany) and RNeasy® Mini Kit (QIAGEN, Hilden, Germany). The concentration and purity of RNA were checked using NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). All procedures of extraction were performed according to manufacturer's instructions. Different water volume for diluting the RNA pellet was adjusted according to each protocol consideration.

Test sample tissues were derived from dissected mid brain (optic lobe) weighing from 1 to 2.8 mg. The tissues were firstly put into a tube containing corresponding solution from each protocol in addition to 0.6-0.7 g of zirconium oxide beads (Bertin Technologies, Versailles, France; diameter 1.4 µm) and then

homogenized using Precellys 24 Homogenizer (Bertin Technologies, Versailles, France) for 15 seconds at 5,000 rpm.

The distinction between full and modified protocol of TRI Reagent® is that chemicals used in the modified protocol are a half of those in the full protocol. To extract RNA from 50-100 mg of tissue, the full protocol of TRI Reagent® needs 1 ml TRI Reagent, 200 µl chloroform, 500 µl isopropanol and 1 ml 80% cold ethanol. Unlike the modified protocol, the full protocol also includes additional precipitation with sodium acetate and 100% cold ethanol as well as washing with 130 µl 80% cold ethanol. As a note, the additional precipitation is an added method to the manufacturer's original protocol.

Meanwhile, 200 µl buffer RA1, 4 µl TCEP, 5 µl Carrier RNA Working Solution, 200 µl 70% ethanol, 100 µl MDB Buffer, 25 µl rDNase Reaction Mixture, buffer 100 µl RA2 and 600 µl RA3 were used to extract RNA from each sample in NucleoSpin® RNA XS protocol. To purify RNA using RNeasy® Mini Kit protocol, the following components should be included for each sample: 600 µl Buffer RLT and 6 µl β-Mercaptoethanol, 70% ethanol, 700 µl Buffer RW1 and 1 ml Buffer RPE.

After the RNA concentration and purity of test samples were checked, the result shows that the concentration of RNA ranged from 11.4 to 205.37 ng/µl with optical density (OD) 260/280 ratio between 1.66 and 2.5 and OD 260/230 ratio between 0.01 and 2.11 (**Table 4.1**). Overall, it can be asserted that RNeasy® Mini Kit yielded more and purer RNA compared to the others. With tissues weighing only around 2 mg, this protocol could generate up to 205.37 ng/µl of RNA with 2.082 and 2.038 OD 260/280 and 260/230 ratio, respectively. Therefore, based on the result, we decided to perform the RNA extraction of the experimental samples using RNeasy® Mini Kit.

Table 4.1. The concentration and purity of RNA from various extraction protocols.

Protocol	Sample	Weight (mg)	Concentration (ng/µl)	260/280	260/230	Water (µl)
TRI Reagent® (full)	TestSample_1	2.8	74.2	1.85	1.78	12
	TestSample_2	2.6	82.7	1.73	2.11	12
	TestSample_3	2.5	107.4	1.82	1.46	12
TRI Reagent® (modified)	TestSample_1	2.3	26.8	1.78	0.05	10
	TestSample_2	2	50.2	1.66	0.06	10
	TestSample_3	1	180.2	1.73	0.7	15
NucleoSpin® RNA XS	TestSample_1	2.1	11.4	2.87	0.01	10
	TestSample_2	2	49.1	2.15	0.25	10
	TestSample_3	2.4	12.3	2.5	0.02	10
RNeasy® Mini Kit	TestSample_1	2	132.771	2.104	1.923	15
	TestSample_2	2	205.37	2.082	2.038	15
	TestSample_3	2.2	142.635	2.095	1.366	15

Prior to performing RT-PCR test with the samples, we evaluated *crf* and *crfbp* exon-exon specific assays by testing them using genomic DNA (gDNA) as a template. Based on melting curve analysis shown in **Appendix I**, apparently the primers also amplify gDNA. To confirm the amplification of gDNA, the amplicon sequence and the sequence from the amplified product from the gDNA input was compared by sequencing. First of all, the RT-PCR products were proceeded to PCR (Applied Biosystems^{TM}, Foster City, CA, USA) and 1% agarose gel electrophoresis to see the approximate size of possible gDNA (**Figure 4.1**). PCR was performed using 2.5 µl 10x Thermo Buffer, 0.5 µl dNTPs, 0.5 µl primer F and R of corresponding gene, 0.125 µl Taq Polymerase, 19.875 µl pure water and 1 µl RT-PCR product (Thermofisher, Scientific, Waltham, Massachusetts, USA).

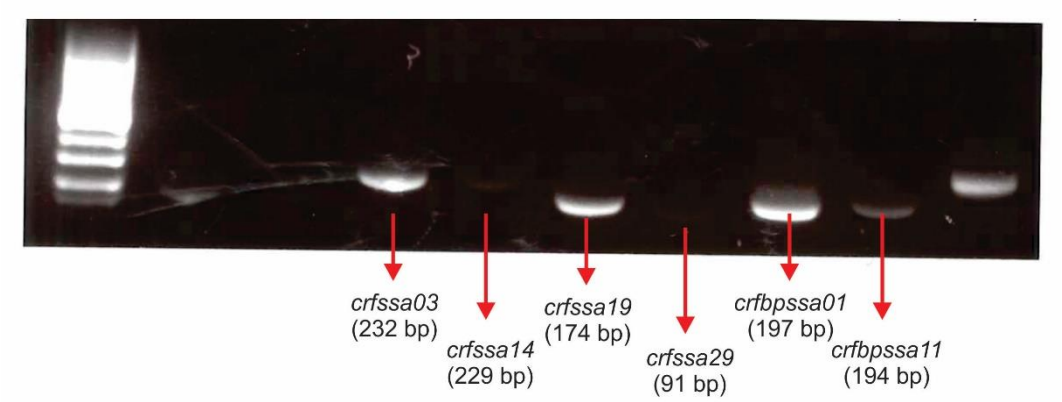

Figure 4.1. Gel electrophoresis of gDNA. The red arrows show gene paralogs amplicon size that corresponds to the gDNA amplicon's approximate size.

To be able to sequence the entire length of the gDNA amplicon, the samples were proceeded to cloning and sequencing. Cloning and sequencing were done together with Dr. F. Lai, by firstly extracting the PCR products from the gel using QIAquick® Gel Extraction Kit (QIAGEN, Hilden, Germany). Afterwards, the templates were ligated into TOPO™ TA Cloning™ Kit (Thermofisher, Scientific, Waltham, Massachusetts, USA), and the resulted plasmids were transformed into TOP10 Chemically Competent *E. coli* (Thermofisher, Scientific, Waltham, Massachusetts, USA). Cells were plated into 10 cm LB agar plate containing the ampicillin antibiotic. 6 colonies were randomly chosen per plate and proceeded to PCR with 2.5 µl of 10x ThermoBuffer, 0.5 µl dNTPs, 0.5 µl Forward F13 Primer, 0.5 µl Reverse F13 Primer, 19.875 µl pure water and 0.125 µl Taq polymerase (all components from: Thermofisher, Scientific, Waltham, Massachusetts, USA). The following PCR protocol was performed: 1) 95 °C for 5 minutes, 2) 95 °C for 5 seconds, 3) 55 °C for 20 seconds, 4) 72 °C for20 seconds, 5) repeating step 2-3 for 35 more times, 6) holding temperature 15 °C. These products were run in 1% agarose gel electrophoresis, and two or three products were randomly selected and proceeded to the sequencing (**Figure 4.2**).

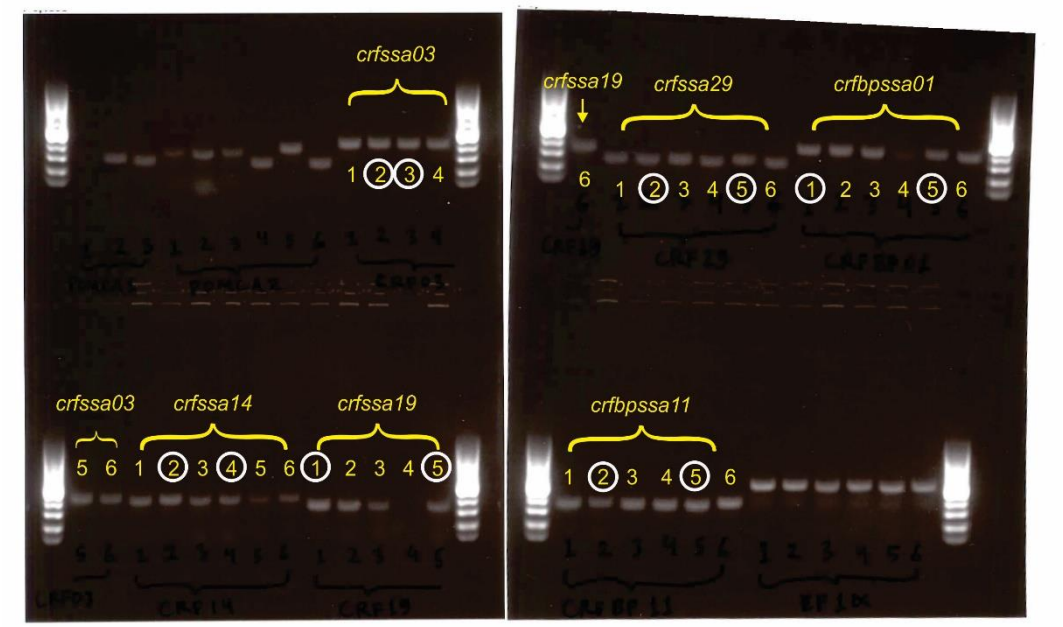

Figure 4.2. Gel electrophoresis of colonies. Number 1 to 6 represents randomly selected colonies. White circle-marked numbers are those who were proceeded to the sequencing.

According to the sequencing results (**Appendix J**), some of gDNA sequences are similar to the target gene while some other partially differ. The hypothesis still remains on the fact that genomic DNA remnants might exist after RNA extraction, so perhaps there might be RNA remnants as well after genomic DNA extraction, thereby amplified in further step. Indeed, based on RT-PCR result in **Appendix I**, the melting curve of some genomic DNA samples have smaller Cq value than those of cDNA samples, indicating the smaller amount of genomic DNA. Despite still being not obvious, it was decided to carry out treatment toward genomic DNA remnant to avoid unreliable data in the further processing of experimental samples.

Considering some previous tests with extraction methods, DNase treatments and cDNA synthesis regimes that remained positive NRTs, we decided to treat the gDNA remnants with other DNase enzymes, and the availability of possible gDNA could be confirmed by performing NRT test. Six salmon hypothalamus samples were treated with three treatment regimes. All samples were extracted using

RNeasy® Mini Kit with On-column DNase Digestion (QIAGEN, Hilden, Germany) treatment. The first two hypothalamuses were directly proceeded to cDNA synthesis (treatment regime 1) while the rest were treated using TURBO DNase-free Kit® (Ambion Applied Biosystem, Foster City, CA, USA) with 1 µl of rDNase I + 3 µl of DNase Inactivation Reagent (treatment regime 2) and 2 µl of rDNase I + 6 µl of DNase Inactivation Reagent (treatment regime 3). Afterwards, they were proceeded to cDNA synthesis (without enzyme reverse transcriptase) and Real Time Polymerase Chain Reaction (RT-PCR).

Based on melting curve analysis from the RT-PCR result (**Appendix K**), it is clear that the On-column DNase Digestion was insufficient to eliminate gDNA remnants as the samples from treatment regime 1 still give signal despite having small Cq value (>33). Meanwhile, treatment regime 2 and 3 succeeded to yield insignificant signals for gDNA remnants. Nonetheless, it was somewhat difficult to see the difference between treatment regime 2 and 3 as both had more or less the same result. In summary, RNA samples should be treated not only with On-column DNase Digestion, but also with TURBO DNase-free Kit® to give no signal on the NRTs. Therefore, in this study, we decided to perform treatment regime 2 to ensure efficiency.

4.2.6. Gene Expression

This study covers two genes to be analyzed, i.e. *crf* and *crfbp*, with four and two paralogs each, respectively. In addition to those, the use of two reference genes, i.e. *ef1α* and *SsS20*, was considered as a means of normalization of the target gene expression. Nonetheless, it turns out that the expression of the reference genes was not stable in this study (**Figure 4.3**), particularly on day 0 before stressors were induced. As a consequence, the normalization appears

impossible to be performed in this study. Hence, we decided to regard the absolute copy number of target genes as a measure of the gene abundance. Besides the relative and comparative methods, the absolute quantification of mRNA is also considered as a valid method of determining gene abundance (Bustin, 2000). Therefore, even though some studies commonly analyze normalized expression of the target gene, the use of absolute copy number as the evaluation of gene expression is still considerable.

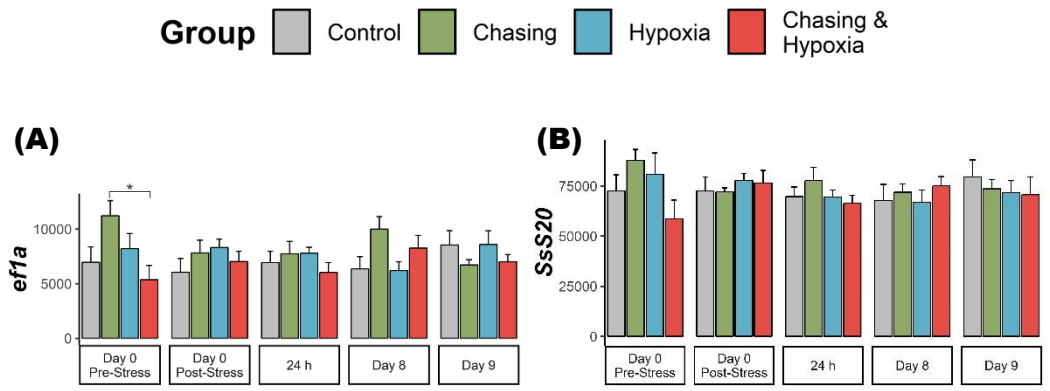

Figure 4.3. The expression of reference genes in the experiment. The variability expression of *ef1α* (A) and *SsS20* (B) was observed throughout treatment differences and the observation period particularly on day 0 before stressors.

It is realized that the study of *crf*, *crfbp* and plasma cortisol level cannot depict a complete picture of stress response in Atlantic salmon post-smolt due to the absence of pituitary gland analysis. In fact, to obtain a comprehensive result of stress response, especially in Atlantic salmon post-smolt, not only is any factor related to HPI axis pathway required, but the other aspects of stress-related endocrinology, including hypothalamic-sympathetic-chromaffin cells (HSC) pathway, also need to be encompassed (Barton and Iwama, 1991; Bonga, 1997; Gorissen and Flik, 2016). At least, the coverage of stress-related peptides in the pituitary gland is required to obtain a full elaboration of stress response through HPI axis pathway. Indeed, the variability in *crf* and *crfbp* expression as well as

plasma cortisol level may result from negative feedback process during the stress response (Barton et al., 1987; Bernier et al., 2009; Schreck, 2000), and the complete details of it can only be elucidated if the whole levels of stress axis are covered. Even though it only covers certain aspects of stress responses in Atlantic salmon-post smolts, we believe that this study provides a new insight into the scope of stress response in Atlantic salmon post-smolts.

V. CONCLUSION AND FUTURE PERSPECTIVES

While the effect of either chronic and acute stress on stress response of Atlantic salmon post-smolt has previously been explored in a plethora of studies, how long-term exposure of stressors and simultaneous stressors affect the stress response of Atlantic salmon post-smolt has been poorly documented. In addition, to the best our knowledge, there are no studies, so far, that have investigated how different *crf* and *crfbp* paralogs are linked to the HPI axis pathway and respond to stressors in Atlantic salmon. Therefore, by exposing Atlantic salmon post-smolt with chronic stressors, this study aimed to observe the effectivity of stressors on the different levels of stress response, in view of the link of HPI axis to various gene paralogs, i.e. *crfssa03*, *crfssa14*, *crfssa19*, *crfssa29*, *crfbpssa01* and *crfbpssa11*. Based on plasma cortisol level, we concluded that different types of stressors used in this experiment give different effects on the stress response of the fish. We suggest that chasing stressor is more pronounced in contrast to the others in view of plasma cortisol level. Despite the fact that we suggest *crfssa14* to be connected with stress regimes in this experiment, we cannot conclude that distinct gene paralogs have a different role in stress response of Atlantic salmon post-smolt. Therefore, a further investigation as to why the other *crf* paralogs do not show a connection to the stress regimes is of importance. In addition, an understanding of *crfssa14* anomaly when the novel stressor was induced as well as the dynamics surrounding *crf* and *crfbp* relationship needs to be explored.

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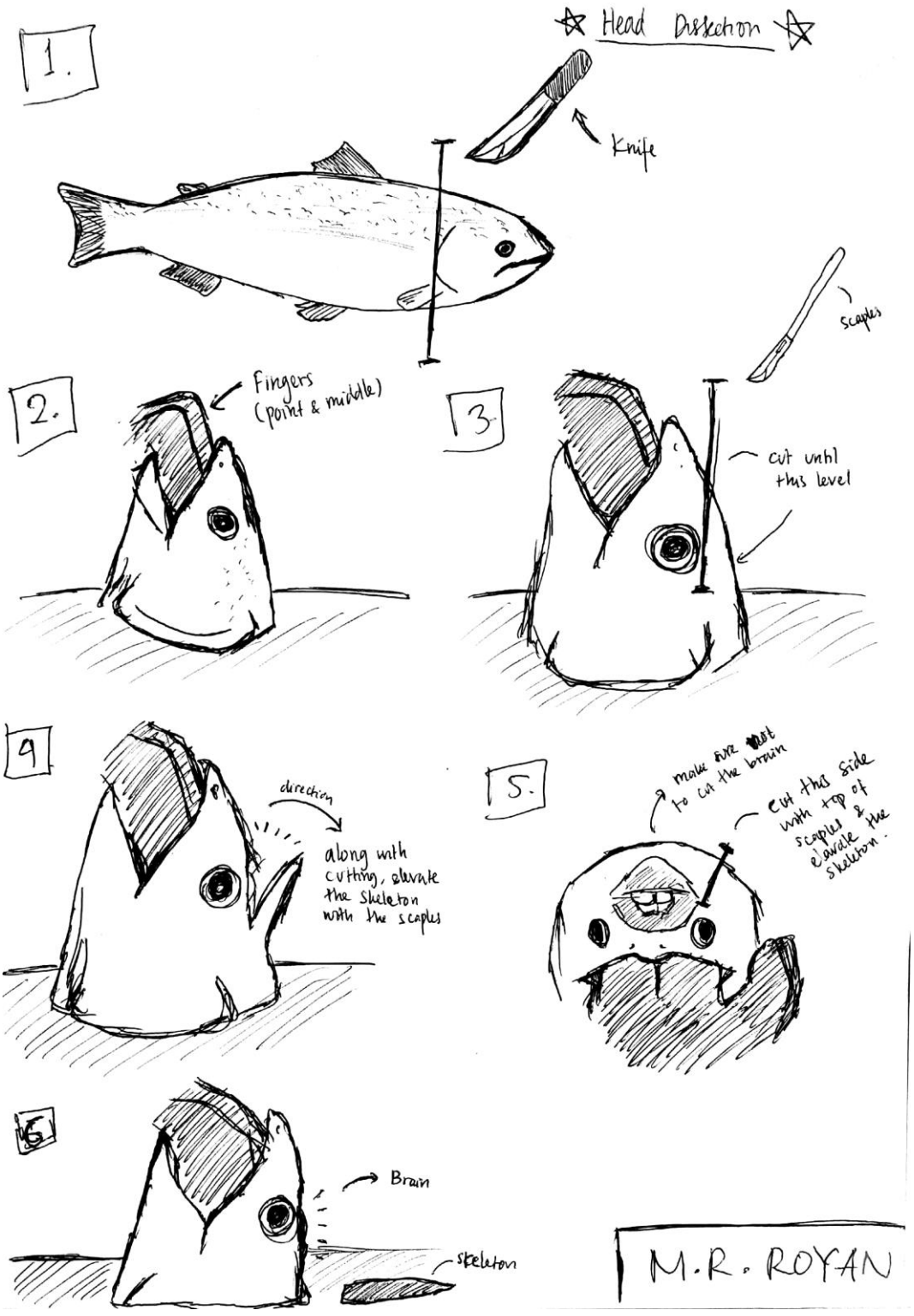
VII. APPENDICES

7.1. APPENDIX A – Head Dissection



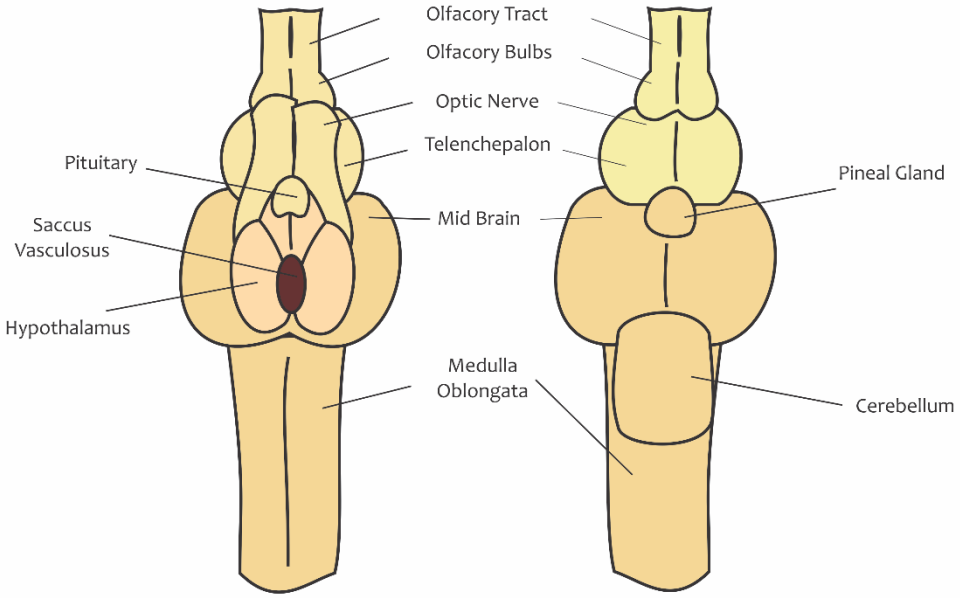

7
cut this (olfactory nerve)
make sure not to cut your finger!!
cut this (medula oblongata)
clean this scelleton if necessary
8
Flip the head
Break the Skeleton with scissors
9.
elevator
Forceps
(use it to elevate the brain slowly from medula)
10
You will see optical nerve (white). cut it by scissors
scissors
11
medula
Carefully take out the brain by pinching the medula with forceps
11.
Brain
VOILA!!!
M. R. ROYAN

7.2. APPENDIX B – Brain Dissection

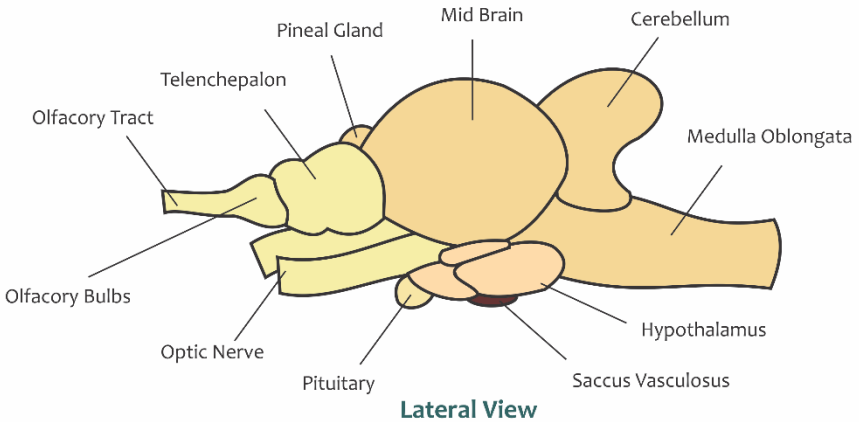
Salmon Brain Dissection

M. R. Royan

Brain Parts

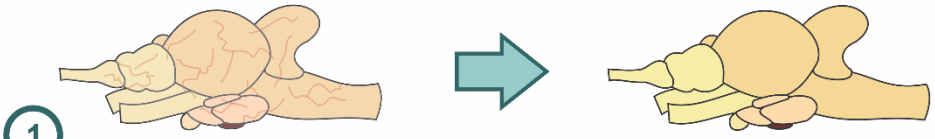


Ventral View Dorsal View



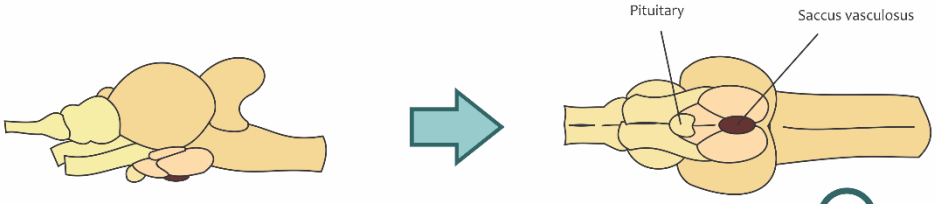
Lateral View

Dissection Procedure



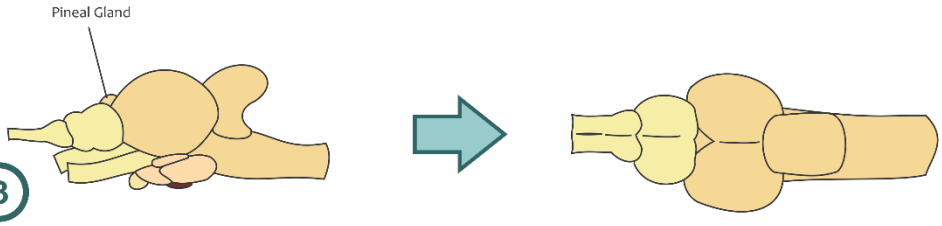
1

Remove the blood vessels and membranes by using forceps



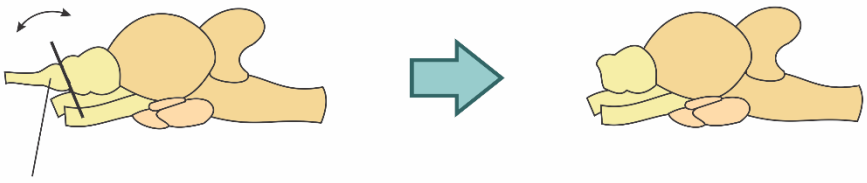
2

Turn the brain facing the ventral view, take the pituitary and saccus vasculosus by using forceps



3

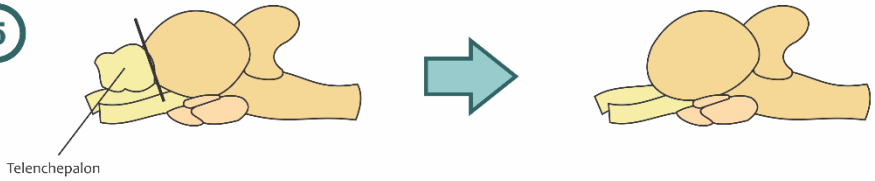
Turn the brain facing dorsal view, remove the pineal gland by using forceps



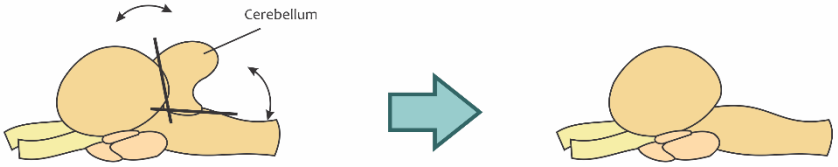
4

Use forceps to shift the olfactory bulbs forward and use scalpels to cut it.

5

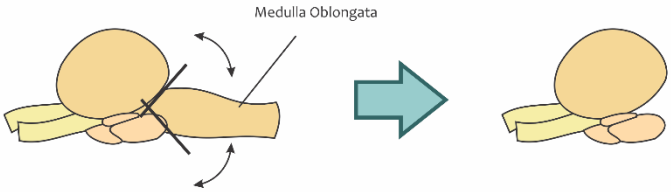


Use forceps to shift the telenchepalon forward and use scalpels to cut it.



6

Use forceps to shift the cerebellum from mid brain and medulla oblongata, and cut it in two direction to get full cerebellum



7

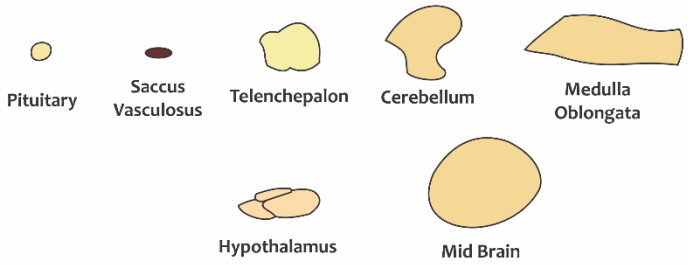
Use forceps to shift the mid brain and hypothalamus away from medulla oblongata, and cut the medulla in two direction

8

Use forceps to shift hypothalamus away from mid brain and optical nerve, and cut it in one direction

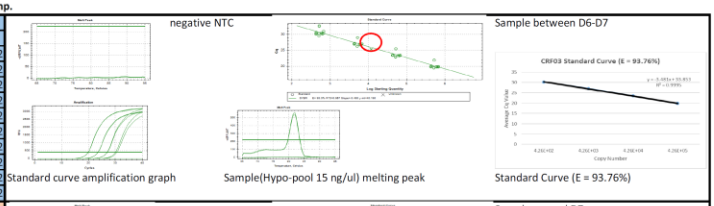
Cut the optical nerve and medulla leftover in three direction to get a full mid brain.

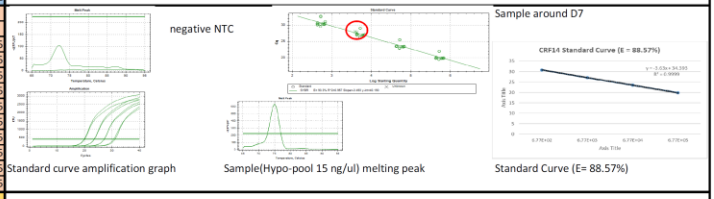
Dissected Parts

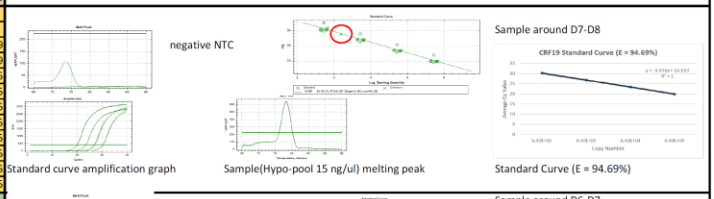


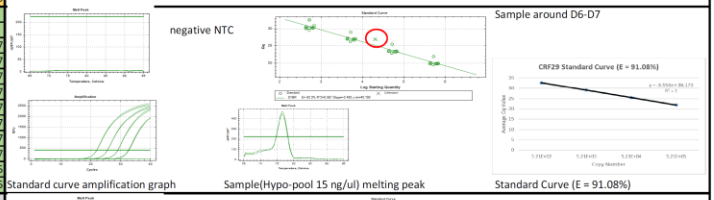
7.3. APPENDIX C – Standard Curve Test

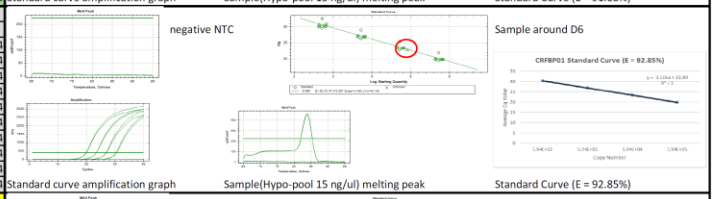
Wells	Target	Content	Sample	Cq	SQ	Melt. Temp.
A01	crf03	NTC		N/A	N/A	None
A02	crf03	NTC		N/A	N/A	None
B01	crf03	Std	d8	30.49	4.26E+02	82
B02	crf03	Std	d8	30.07	4.26E+02	82
B03	crf03	Std	d7	27.02	4.26E+03	82
B04	crf03	Std	d7	26.97	4.26E+03	82
B05	crf03	Std	d6	23.48	4.26E+04	82
B06	crf03	Std	d6	23.47	4.26E+04	82
B07	crf03	Std	d5	19.88	4.26E+05	82
B08	crf03	Std	d5	19.82	4.26E+05	82
B09	crf03	Unkn	15 ng/ul	25.37	1.17E+04	82
B10	crf03	Unkn	15 ng/ul	25.32	1.21E+04	82
A03	crf14	NTC		N/A	N/A	None
A04	crf14	NTC		38.64	N/A	None
C01	crf14	Std	d8	30.54	6.77E+02	75.5
C02	crf14	Std	d8	31.06	6.77E+02	75.5
C03	crf14	Std	d7	27.08	6.77E+03	75.5
C04	crf14	Std	d7	27.12	6.77E+03	75.5
C05	crf14	Std	d6	23.49	6.77E+04	75
C06	crf14	Std	d6	23.42	6.77E+04	75
C07	crf14	Std	d5	20.01	6.77E+05	75
C08	crf14	Std	d5	19.82	6.77E+05	75
C09	crf14	Unkn	15 ng/ul	28.01	3.88E+03	75
C10	crf14	Unkn	15 ng/ul	27.85	4.29E+03	75
A05	crf19	NTC		39.13	N/A	None
A06	crf19	NTC		N/A	N/A	None
D01	crf19	Std	d8	30.24	6.40E+02	79
D02	crf19	Std	d8	30.18	6.40E+02	79
D03	crf19	Std	d7	26.67	6.40E+03	78.5
D04	crf19	Std	d7	26.98	6.40E+03	78.5
D05	crf19	Std	d6	23.33	6.40E+04	78.5
D06	crf19	Std	d6	23.3	6.40E+04	78.5
D07	crf19	Std	d5	19.83	6.40E+05	78.5
D08	crf19	Std	d5	19.89	6.40E+05	78.5
D09	crf19	Unkn	15 ng/ul	28.93	1.52E+03	78.5
D10	crf19	Unkn	15 ng/ul	28.89	1.57E+03	78.5
A07	crf29	NTC		N/A	N/A	None
A08	crf29	NTC		N/A	N/A	None
E01	crf29	Std	d8	32.74	5.21E+02	77
E02	crf29	Std	d8	32.54	5.21E+02	77
E03	crf29	Std	d7	29.02	5.21E+03	77
E04	crf29	Std	d7	29.03	5.21E+03	77
E05	crf29	Std	d6	25.49	5.21E+04	77
E06	crf29	Std	d6	25.52	5.21E+04	77
E07	crf29	Std	d5	21.98	5.21E+05	77
E08	crf29	Std	d5	21.94	5.21E+05	77
E09	crf29	Unkn	15 ng/ul	26.91	2.10E+04	76.5
E10	crf29	Unkn	15 ng/ul	27	1.98E+04	76.5
A09	crfbp01	NTC		N/A	N/A	None
A10	crfbp01	NTC		N/A	N/A	None
F01	crfbp01	Std	d8	30.53	5.94E+02	84
F02	crfbp01	Std	d8	30.22	5.94E+02	84
F03	crfbp01	Std	d7	26.77	5.94E+03	84
F04	crfbp01	Std	d7	27.01	5.94E+03	84
F05	crfbp01	Std	d6	23.39	5.94E+04	84
F06	crfbp01	Std	d6	23.36	5.94E+04	84
F07	crfbp01	Std	d5	19.79	5.94E+05	84
F08	crfbp01	Std	d5	19.93	5.94E+05	84
F09	crfbp01	Unkn	15 ng/ul	22.93	7.97E+04	84
F10	crfbp01	Unkn	15 ng/ul	22.86	8.31E+04	84
A11	crfbp11	NTC		N/A	N/A	None
A12	crfbp11	NTC		N/A	N/A	None
G01	crfbp11	Std	d8	30.15	5.11E+02	84
G02	crfbp11	Std	d8	29.55	5.11E+02	84
G03	crfbp11	Std	d7	26.36	5.11E+03	84
G04	crfbp11	Std	d7	26.3	5.11E+03	84
G05	crfbp11	Std	d6	22.9	5.11E+04	84
G06	crfbp11	Std	d6	23.01	5.11E+04	84
G07	crfbp11	Std	d5	19.54	5.11E+05	84
G08	crfbp11	Std	d5	19.5	5.11E+05	84
G09	crfbp11	Unkn	15 ng/ul	23.83	2.82E+04	84
G10	crfbp11	Unkn	15 ng/ul	24.02	2.49E+04	84
B11	ef1a	NTC		N/A	N/A	None
B12	ef1a	NTC		N/A	N/A	None
H01	ef1a	Std	d8	30.2	4.39E+02	78
H02	ef1a	Std	d8	30.66	4.39E+02	78
H03	ef1a	Std	d7	27.1	4.39E+03	78
H04	ef1a	Std	d7	27.27	4.39E+03	78
H05	ef1a	Std	d6	23.52	4.39E+04	78
H06	ef1a	Std	d6	23.55	4.39E+04	78
H07	ef1a	Std	d5	20.14	4.39E+05	78
H08	ef1a	Std	d5	20.02	4.39E+05	78
H09	ef1a	Unkn	15 ng/ul	16.62	4.43E+06	78
H10	ef1a	Unkn	15 ng/ul	16.72	4.14E+06	78
H11	ef1a	Unkn	1.5 ng/ul	20.79	2.77E+05	78
H12	ef1a	Unkn	1.5 ng/ul	20.95	2.51E+05	78

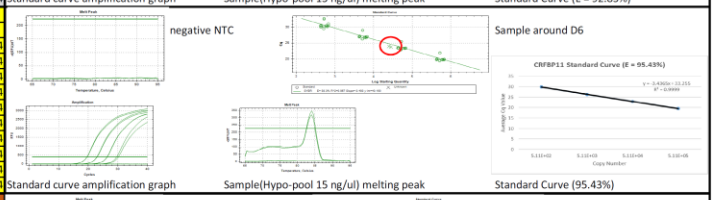


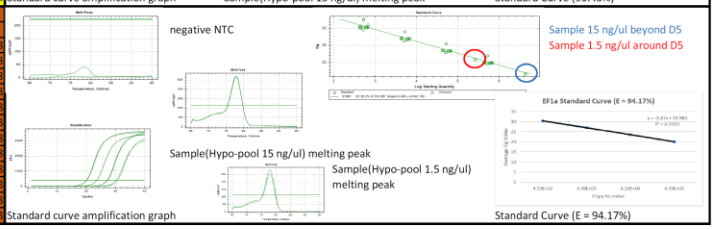






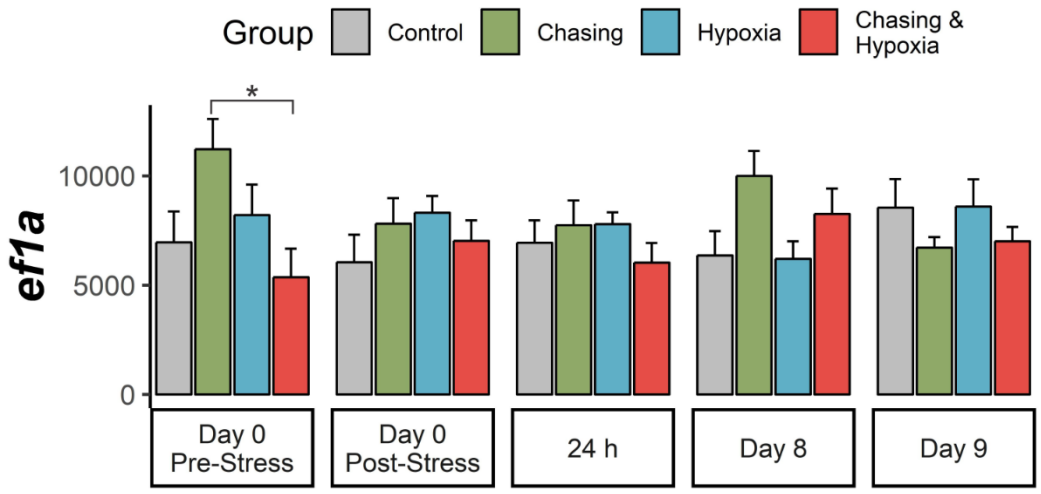




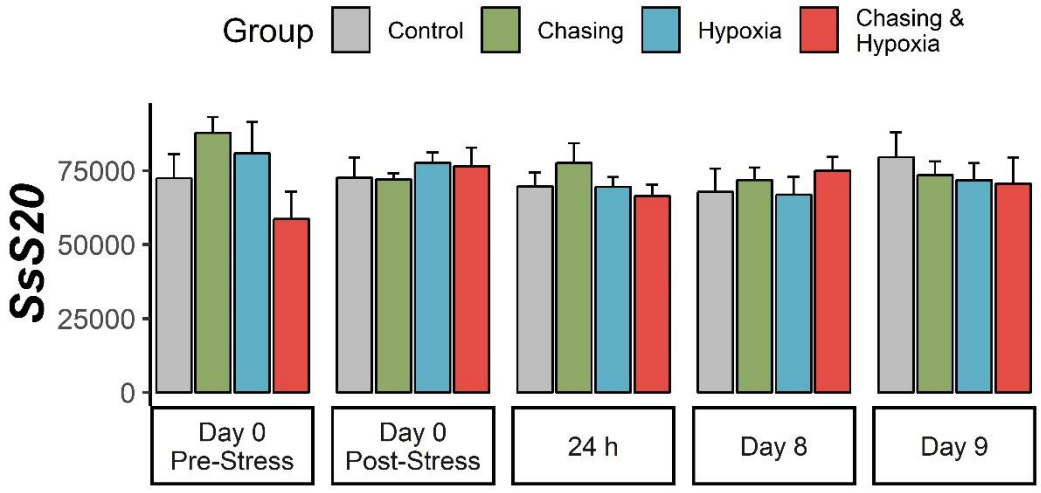


7.4. APPENDIX D – Reference Genes Expression

7.4.1. Elongation Factor 1 Alpha (*ef1α*)



7.4.2. *Salmo salar* S20 (*SsS20*)



7.5. APPENDIX E – Data Structure

7.5.1. Weight, Length and Growth Rate

a. Weight

Treatment	Day	Mean (g)	SD	N	SE
Control	D0	263.381	42.38452	21	9.249061
	D9	309.7333	43.88047	15	11.32989
Chasing	D0	267.4286	59.92877	21	13.07753
	D9	270.5333	43.32744	15	11.1871
Hypoxia	D0	262.05	56.08778	20	12.54161
	D9	270.8667	38.31797	15	9.893657
Chasing + Hypoxia	D0	282.619	61.34287	21	13.38611
	D9	286.2667	42.63042	15	11.00713

b. Length

Treatment	Day	Mean (cm)	SD	N	SE
Control	D0	30.01429	1.596335	21	0.348349
	D9	31.42667	1.438485	15	0.371415
Chasing	D0	28.45238	2.235536	21	0.487834
	D9	30.88	1.456611	15	0.376095
Hypoxia	D0	28.35	1.974175	20	0.441439
	D9	30.77333	1.413439	15	0.364948
Chasing + Hypoxia	D0	30.58095	2.059276	21	0.449371
	D9	30.97333	1.473803	15	0.380534

c. Relative Growth Rate

Treatment	Mean (%)	SD	N	SE
Control	17.5009	6.068843	3	3.503848
Chasing	1.194727	3.09767	3	1.78844
Hypoxia	3.946657	12.27621	3	7.087676
Chasing + Hypoxia	1.450908	5.375395	3	3.103486

7.5.2. Plasma Cortisol

Treatment	Day	Mean (ng/ml)	SD	N	SE
Control	D0bs	4.87	4.207631	6	1.717758
	D0	57.88643	20.38399	14	5.447849
	D1	6.364615	4.981749	13	1.381689
	D8	3.275333	2.393121	15	0.617901
	D9	142.6987	32.19325	15	8.31226
Chasing	D0bs	14.11	12.86008	4	6.43004
	D0	88.715	29.45425	12	8.50271
	D1	90.01714	31.01029	14	8.287849
	D8	28.19857	21.50156	14	5.746533
	D9	132.8527	36.63129	15	9.45816
Hypoxia	D0bs	11.57833	11.28064	6	4.605302
	D0	35.43286	15.47082	14	4.13475
	D1	41.624	16.34783	15	4.220992
	D8	12.646	5.669668	15	1.463902
	D9	134.6043	24.15122	14	6.454684
Chasing + Hypoxia	D0bs	3.295	2.096131	4	1.048066
	D0	76.53692	31.80877	13	8.822166
	D1	52.12667	19.07764	15	4.925824
	D8	16.59385	5.147395	13	1.427631
	D9	123.4407	39.60259	15	10.22534

7.5.3. Gene Expression

a. *crfssa03*

Treatment	Day	Mean (Copy Number)	SD	N	SE
Control	D0bs	2905.114	1227.148	6	500.981
	D0	2352.822	1343.436	8	474.9762
	D1	3618.782	2181.704	9	727.2346
	D8	2434.733	1551.356	7	586.3574
	D9	3098.559	1541.774	9	513.9248
Chasing	D0bs	3350.356	1532.792	5	685.4854
	D0	3594.735	826.1715	6	337.2831
	D1	3854.081	2367.34	9	789.1135
	D8	2865.102	958.1358	8	338.7521
	D9	3585.838	662.727	7	250.4872
Hypoxia	D0bs	3060.847	1794.579	6	732.6337
	D0	3768.579	1808.226	8	639.3045
	D1	3183.144	574.4736	9	191.4912
	D8	2156.798	625.7774	7	236.5216
	D9	2729.89	934.2174	9	311.4058
Chasing + Hypoxia	D0bs	2359.72	1565.832	5	700.2614
	D0	3290.547	1032.496	7	390.2466
	D1	2343.343	1269.79	9	423.2635
	D8	2731.497	1031.126	8	364.5581
	D9	2978.114	313.6356	6	128.0412

b. *crfssa14*

Treatment	Day	Mean (Copy Number)	SD	N	SE
Control	D0bs	750.3677	350.5509	6	143.1118
	D0	1211.025	409.4122	9	136.4707
	D1	1477.602	367.5326	9	122.5109
	D8	779.6931	510.6584	9	170.2195
	D9	1077.954	615.7458	9	205.2486
Chasing	D0bs	1546.273	266.6063	6	108.8416
	D0	1392.156	179.9528	7	68.01576
	D1	2093.573	682.241	9	227.4137
	D8	984.9068	382.6781	9	127.5594
	D9	1023.338	318.1295	8	112.4758
Hypoxia	D0bs	915.6456	463.6303	6	189.2763
	D0	1443.825	369.9765	8	130.8064
	D1	1556.606	447.9978	8	158.3911
	D8	933.9906	647.0579	9	215.686
	D9	918.1335	246.3431	9	82.11437
Chasing + Hypoxia	D0bs	837.9311	486.8497	6	198.7556
	D0	1481.235	65.10415	5	29.11546
	D1	1373.747	574.1144	9	191.3715
	D8	836.9527	205.861	8	72.78286
	D9	838.3598	172.9492	8	61.14677

c. *crfssa19*

Treatment	Day	Mean (Copy Number)	SD	N	SE
Control	D0bs	335.28	142.2806	6	58.0858
	D0	315.9196	238.8175	9	79.60584
	D1	326.8553	105.8772	9	35.2924
	D8	314.5608	118.2803	9	39.42676
	D9	478.1682	273.0165	9	91.00551
Chasing	D0bs	538.3177	144.3943	6	58.94873
	D0	427.1047	166.8352	9	55.61173
	D1	444.8274	166.6021	9	55.53404
	D8	491.9511	146.0886	9	48.6962
	D9	409.1218	136.5976	8	48.29453
Hypoxia	D0bs	392.9918	220.5203	6	90.02705
	D0	456.8726	176.4513	9	58.81708
	D1	466.0442	117.1904	9	39.06348
	D8	316.818	110.5687	9	36.85623
	D9	387.4175	171.5705	9	57.19016
Chasing + Hypoxia	D0bs	240.2533	192.3809	6	78.53915
	D0	376.1371	143.7315	9	47.9105
	D1	307.5299	137.7975	9	45.9325
	D8	434.6677	86.46592	9	28.82197
	D9	481.3959	146.2177	9	48.73923

d. *crfssa29*

Treatment	Day	Mean (Copy Number)	SD	N	SE
Control	D0bs	5733.143	2282.428	6	931.7974
	D0	5194.032	2528.619	8	894.0018
	D1	5854.897	2123.333	8	750.7116
	D8	7222.333	1491.077	8	527.1755
	D9	6771.571	3078.725	9	1026.242
Chasing	D0bs	9853.635	3462.824	6	1413.692
	D0	6906.481	2184.708	9	728.2361
	D1	8080.785	1853.202	8	655.2057
	D8	9108.215	2183.34	9	727.7799
	D9	8687.75	3058.974	8	1081.511
Hypoxia	D0bs	8896.478	3199.057	6	1306.009
	D0	7597.386	2957.931	9	985.9771
	D1	7766.188	1988.352	9	662.7841
	D8	5407.426	2481.549	8	877.36
	D9	8842.603	4785.079	9	1595.027
Chasing + Hypoxia	D0bs	5286.24	4842.903	6	1977.107
	D0	6369.692	2580.726	9	860.2421
	D1	5497.993	2725.574	7	1030.17
	D8	8459.535	1671.752	9	557.2507
	D9	8924.218	4081.569	9	1360.523

e. *crfbpssa01*

Treatment	Day	Mean (Copy Number)	SD	N	SE
Control	D0bs	34475.45	5919.451	6	2416.606
	D0	34808.33	16025.6	9	5341.866
	D1	35271.66	5328.544	9	1776.181
	D8	42715.55	7127.789	8	2520.054
	D9	36007.53	12382.04	8	4377.712
Chasing	D0bs	44002.79	7892.684	6	3222.175
	D0	43695.28	8304.437	9	2768.146
	D1	38764.5	8086.834	9	2695.611
	D8	36074.05	4653.538	8	1645.274
	D9	40329.77	7376.034	8	2607.822
Hypoxia	D0bs	40207.43	12657.21	6	5167.286
	D0	42810.87	5946.442	9	1982.147
	D1	39987.87	6999.412	9	2333.137
	D8	33423.71	2447.051	7	924.8983
	D9	34742.5	7446.403	7	2814.476
Chasing + Hypoxia	D0bs	28698.28	13718.68	6	5600.627
	D0	46509.83	10070.39	8	3560.419
	D1	31379.14	10219.11	8	3613.001
	D8	39333.09	3494.704	9	1164.901
	D9	35735.63	10467.36	9	3489.118

f. *crfbpssa11*

Treatment	Day	Mean (Copy Number)	SD	N	SE
Control	D0bs	10184.56	11231.91	6	4585.408
	D0	12681.2	11427.47	9	3809.158
	D1	10562.18	10538.25	9	3512.75
	D8	16966.07	13783.2	9	4594.401
	D9	17188.19	7438.945	9	2479.648
Chasing	D0bs	29903.09	10658.44	6	4351.289
	D0	14144.53	16983.64	9	5661.213
	D1	13585.31	11246.94	9	3748.981
	D8	22818.06	16289.4	9	5429.798
	D9	17392.77	15237.16	7	5759.104
Hypoxia	D0bs	5775.957	7871.845	5	3520.396
	D0	20553.15	12705.71	9	4235.235
	D1	15820.55	13481.71	9	4493.904
	D8	15212.47	11519.07	9	3839.69
	D9	19586.71	11064.25	9	3688.085
Chasing + Hypoxia	D0bs	13129.54	14226.38	6	5807.897
	D0	13825.22	5695.132	7	2152.557
	D1	9704.568	9912.977	9	3304.326
	D8	19331.52	4552.843	7	1720.813
	D9	15479.74	4863.789	6	1985.634

7.6. APPENDIX F – Data Normality and Homogeneity

7.6.1. Weight and Length

a. Weight

```
Anderson-Darling normality test

data:  residual
A = 0.27405, p-value = 0.6593

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group   7  0.4907   0.84
                          135
```

b. Length

```
Anderson-Darling normality test

data:  residual
A = 0.23762, p-value = 0.7802

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group   7  0.9876  0.443
                          135
```

7.6.2. Plasma Cortisol

```
Anderson-Darling normality test

data:  residual
A = 0.52646, p-value = 0.1779


Levene's Test for Homogeneity of Variance (center = median)
      Df F value  Pr(>F)
group  19  1.4851 0.09201
                                  226
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

7.6.3. Gene Paralogs

a. *crfssa03*

```
Anderson-Darling normality test

data:  residual
A = 0.24544, p-value = 0.7554



Levene's Test for Homogeneity of Variance (center = median)
      Df F value  Pr(>F)
group  19  1.6721 0.04927
                          128
```

b. *crfssa14*

```
Anderson-Darling normality test

data:  residual
A = 0.25912, p-value = 0.7102

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group   3  1.5855 0.1952
                          153
```

c. *crfssa19*

```
Anderson-Darling normality test

data:  residual
A = 0.25073, p-value = 0.7385

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  19  1.2097 0.2573
                          147
```

d. *crfssa29*

```
Anderson-Darling normality test

data:  residual
A = 0.27688, p-value = 0.6504

Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  19  1.2255 0.2456
                               140
```

e. *crfbpssa01*

```
Anderson-Darling normality test

data:  residual
A = 0.28528, p-value = 0.623



Levene's Test for Homogeneity of Variance (center = median)
      Df F value    Pr(>F)
group  19  2.6188 0.0006836
                                     138
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

f. *crfbpssa11*

```
Anderson-Darling normality test

data:  residual
A = 0.75809, p-value = 0.04763



Levene's Test for Homogeneity of Variance (center = median)
      Df F value Pr(>F)
group  19  1.2507 0.2269
                               138
```

7.7. APPENDIX G – Water Quality

7.7.1. Salinity and Temperature in All Tanks

Date	Salinity (g/l)	Temperature (°C)
15-May	28.9	8
16-May	28.3	9.2
18-May	28.1	9.5
22-May	28.3	10.2
23-May	28.3	9.1
24-May	28.7	9.1
25-May	28.7	9.1
28-May	28.3	9.9
29-May	28.6	N/A
30-May	28.6	9.2
31-May	28.6	9.5
1-Jun	28.6	9.3
4-Jun	28.6	9.8
5-Jun	28.7	9.5
6-Jun	28.7	9.1
7-Jun	28.7	9.4
8-Jun	28.7	10
11-Jun	28.6	9
12-Jun	28.7	8.9
13-Jun	28.2	9
14-Jun	28.3	8.7
18-Jun	28.8	8.8
19-Jun	28.6	8.6
22-Jun	28.3	8.6
Mean	**28.54**	**9.20**
SD	**0.21**	**0.50**
N	**24**	**23**
SEM	**0.04**	**0.11**

7.7.2. Oxygen Saturation (%) during the Experiment

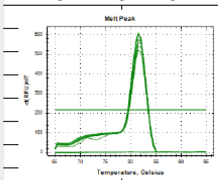
Treatment	Tank	16-May	22-May	23-May	28-May	31-May	4-Jun	7-Jun	12-Jun	14-Jun	18-Jun	Mean	SD	N	SEM
Control	1	96	90	94	86	96	89	89	86	99	102	91.53	5.91	30	1.08
	2	94	89	92	86	93	91	83	76	99	102				
	3	89	94	93	88	93	90	84	87	96	100				
Chasing	4	93	92	95	89	95	91	87	100	101	107	95.33	5.87	30	1.07
	5	91	91	93	89	93	94	89	101	100	106				
	6	96	96	95	88	95	93	88	103	101	108				
Hypoxia	7	91	93	95	91	95	91	85	104	96	104	94.63	5.94	30	1.08
	8	90	94	93	86	93	90	88	103	101	105				
	9	93	88	96	93	95	91	87	102	101	105				
Chasing + Hypoxia	10	94	95	95	88	95	93	90	86	98	107	92.73	6.38	30	1.16
	11	93	93	94	90	94	94	86	84	100	106				
	12	92	89	94	80	89	93	86	83	96	105				

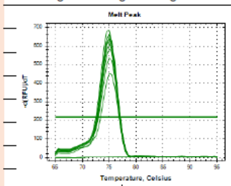
7.8. APPENDIX H – Food Intake Data

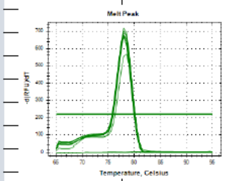
Treatment	Tank	Initial Feed Stock (g)	Final Feed Stock (g)	Given Feed (g)	Uneaten Feed Dry (g)	Moisture (%)	Uneaten Feed (g)	Over Feeding (%)	Feed Intake (g)
Control	1	3200	1596	1604	555	5.2%	585	37%	1011
	2	3200	1624	1576	478	5.2%	504	31%	1120
	3	3200	1290	1910	533	5.2%	562	44%	728
Chasing	4	3200	1282	1918	919	5.2%	969	76%	313
	5	3200	1255	1945	604	5.2%	637	51%	618
	6	3200	1263	1937	641	5.2%	676	54%	587
Hypoxia	7	3200	1263	1937	506	5.2%	534	42%	729
	8	3200	1675	1525	456	5.2%	481	29%	1194
	9	3200	1230	1970	532	5.2%	561	46%	669
Chasing + Hypoxia	10	3200	1650	1550	630	5.2%	665	40%	985
	11	3200	1634	1566	634	5.2%	669	41%	965
	12	3200	1681	1519	366	5.2%	386	23%	1295

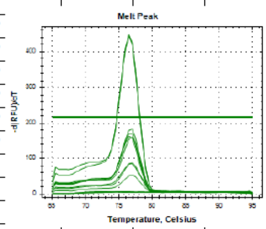
7.9. APPENDIX I – Genomic Contamination Melting Curve

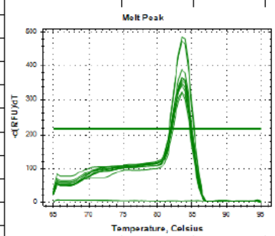
	B	C	D	E	F	G	H	I	J	K	L
26	C01	SYBR	crf03	gDNA 20ng/uL	21.09	81.50	here the genomic signal is higher than the cDNA sample.mmh?				
27	C02	SYBR	crf03	gDNA 20ng/uL	21.12	81.50	here the genomic signal is higher than the cDNA sample.mmh?				
28	C03	SYBR	crf03	gDNA 2ng/uL	24.53	81.50	here the genomic signal is higher than the cDNA sample.mmh?				
29	C04	SYBR	crf03	gDNA 2ng/uL	24.60	81.50	here the genomic signal is higher than the cDNA sample.mmh?				
30	C05	SYBR	crf03	gDNA 0.2ng/uL	29.51	82.00					
31	C06	SYBR	crf03	gDNA 0.2ng/uL	29.98	81.50					
32	C07	SYBR	crf03	QMAR whole brain p	27.00	82.00					
33	C08	SYBR	crf03	QMAR whole brain p	27.10	81.50					
34	C09	SYBR	crf03	NTC		None					
35	C10	SYBR	crf03	NTC		None					
36	C11	SYBR	crf03	NRT QMAR Br23		None					
37	C12	SYBR	crf03	NRT QMAR Br33		None					
38	D01	SYBR	crf14	gDNA 20ng/uL	21.51	75.00	here the genomic signal is higher than the cDNA sample.mmh?				
39	D02	SYBR	crf14	gDNA 20ng/uL	21.43	75.00	here the genomic signal is higher than the cDNA sample.mmh?				
40	D03	SYBR	crf14	gDNA 2ng/uL	25.06	75.00	here the genomic signal is higher than the cDNA sample.mmh?				
41	D04	SYBR	crf14	gDNA 2ng/uL	25.10	75.00	here the genomic signal is higher than the cDNA sample.mmh?				
42	D05	SYBR	crf14	gDNA 0.2ng/uL	29.57	75.00					
43	D06	SYBR	crf14	gDNA 0.2ng/uL	29.29	75.00					
44	D07	SYBR	crf14	QMAR whole brain p	28.49	75.00					
45	D08	SYBR	crf14	QMAR whole brain p	28.50	75.00					
46	D09	SYBR	crf14	NTC		None					
47	D10	SYBR	crf14	NTC		None					
48	D11	SYBR	crf14	NRT QMAR Br23	32.43	75.00					
49	D12	SYBR	crf14	NRT QMAR Br33	33.43	75.00					
50	E01	SYBR	crf19	gDNA 20ng/uL	30.04	78.00					
51	E02	SYBR	crf19	gDNA 20ng/uL	30.01	78.00					
52	E03	SYBR	crf19	gDNA 2ng/uL	29.96	78.00					
53	E04	SYBR	crf19	gDNA 2ng/uL	29.40	78.00					
54	E05	SYBR	crf19	gDNA 0.2ng/uL	29.26	78.00					
55	E06	SYBR	crf19	gDNA 0.2ng/uL	29.39	78.00					
56	E07	SYBR	crf19	QMAR whole brain p	28.51	78.00					
57	E08	SYBR	crf19	QMAR whole brain p	28.38	78.00					
58	E09	SYBR	crf19	NTC		None					
59	E10	SYBR	crf19	NTC		None					
60	E11	SYBR	crf19	NRT QMAR Br23	31.10	78.00					
61	E12	SYBR	crf19	NRT QMAR Br33	31.09	78.50					
62	F01	SYBR	crf29	gDNA 20ng/uL	37.55	None	low genomic sigal here. Why?				
63	F02	SYBR	crf29	gDNA 20ng/uL	38.99	None	low genomic sigal here. Why?				
64	F03	SYBR	crf29	gDNA 2ng/uL	39.80	None	low genomic sigal here. Why?				
65	F04	SYBR	crf29	gDNA 2ng/uL	37.21	None	low genomic sigal here. Why?				
66	F05	SYBR	crf29	gDNA 0.2ng/uL	37.57	None	low genomic sigal here. Why?				
67	F06	SYBR	crf29	gDNA 0.2ng/uL	38.85	None	low genomic sigal here. Why?				
68	F07	SYBR	crf29	QMAR whole brain p	25.27	76.50					
69	F08	SYBR	crf29	QMAR whole brain p	25.32	76.50					
70	F09	SYBR	crf29	NTC		None					
71	F10	SYBR	crf29	NTC		None					
72	F11	SYBR	crf29	NRT QMAR Br23	37.40	None					
73	F12	SYBR	crf29	NRT QMAR Br33		None					
74	G01	SYBR	crfbp1	gDNA 20ng/uL	30.78	83.50					
75	G02	SYBR	crfbp1	gDNA 20ng/uL	30.21	83.50					
76	G03	SYBR	crfbp1	gDNA 2ng/uL	30.12	83.50					
77	G04	SYBR	crfbp1	gDNA 2ng/uL	29.85	83.50					
78	G05	SYBR	crfbp1	gDNA 0.2ng/uL	30.28	83.50					
79	G06	SYBR	crfbp1	gDNA 0.2ng/uL	30.17	83.50					
80	G07	SYBR	crfbp1	QMAR whole brain p	23.02	83.50					
81	G08	SYBR	crfbp1	QMAR whole brain p	22.91	83.50					
82	G09	SYBR	crfbp1	NTC		None					
83	G10	SYBR	crfbp1	NTC		None					
84	G11	SYBR	crfbp1	NRT QMAR Br23	31.56	83.50					
85	G12	SYBR	crfbp1	NRT QMAR Br33	31.91	83.50					
86	H01	SYBR	crfbp11	gDNA 20ng/uL	37.95	None	low genomic sigal here. Why?				
87	H02	SYBR	crfbp11	gDNA 20ng/uL	36.03	None	low genomic sigal here. Why?				
88	H03	SYBR	crfbp11	gDNA 2ng/uL		None	low genomic sigal here. Why?				
89	H04	SYBR	crfbp11	gDNA 2ng/uL		None	low genomic sigal here. Why?				
90	H05	SYBR	crfbp11	gDNA 0.2ng/uL	35.20	None	low genomic sigal here. Why?				
91	H06	SYBR	crfbp11	gDNA 0.2ng/uL		None	low genomic sigal here. Why?				
92	H07	SYBR	crfbp11	QMAR whole brain p	25.47	84.00					
93	H08	SYBR	crfbp11	QMAR whole brain p	25.37	84.00					
94	H09	SYBR	crfbp11	NTC		None					
95	H10	SYBR	crfbp11	NTC		None					
96	H11	SYBR	crfbp11	NRT QMAR Br23		None					
97	H12	SYBR	crfbp11	NRT QMAR Br33		None					

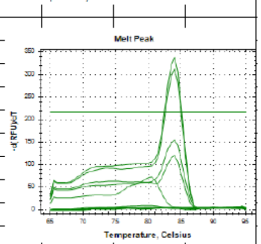












7.10. APPENDIX J – Sequencing Result

FL188 = *crfssa03* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)

NNNNNNNNNNNNGGGCGANNGNNTTAGCGGCCGCGAATTCGCCCTTACCGATTG
CTGTTACCGACTTTACCTTGCAGAAGACGCTGCGTAAACTGAAGTAAAGCCCTGTTG
ACCGCTGTTGACCGCGCAGCAGCTCCTGGAGATTTATTCGACAATGAGGACTGGGG
CGAATTTTGATTGGAGTTGTCAAGCCGAATGAAGTACTCCTCTCCTAGTCGCAGAAG
AATAGGGAGTTGCTGTTGCAGCTCTGCCTGAAGATTGTGGAATGGATCAAGTGCAA
GGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGC
TTGGCGTAATCATGGTCATAGCTGGTTTCCNN

FL189 = *crfssa03* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)

NNNNNNNNNNNNGGGCGANTGATTTAGCGGCCGCGAATTCGCCCTTGCACTTGAT
CCATTCCACAATCTTCAGGCAGAGCTGCAACAGCAACTCCCTATTCTTCTGCGACTA
GGAGAGGAGTACTTCATTCGGCTTGACAACTCCAATCAAAATTCGCCCCAGTCCTCA
TTGTCGAATAAATCTCCAGGAGCTGCTGCGCGGTCAACAGCGGTCAACAGGGCTTT
ACTTCAGTTTACGCAGCGTCTTCTGCAAGGTAAAGTCGGTAACAGCAATCGGTAAGG
GCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTT
GGCGTAATCATGGTCATANNGGTTTCCNN

FL190 = *crfssa14* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)

NNNNNNNNNNNNNNNNNNGGNNANTGATTTAGCGGCCGCGAATTCGCCCTTGTCAA
CGGGCTATGTTTGCTTCTCATCAAACAATGTAATAACTATACAGCGAATTACAACTCG
ATTTTACAGCTCTCGTTTAAATAAATACAAATTATAAATAAAATAACGAAAGTTAACCA
ATTAAAGAGTAATACAGAAATGGAATAGTAGCGTACACTTTGTGCAAGATGTAAACAA
ATTATTTGGCAAATGTATCTCTAACACTTTCATTTCCCGAATATGTCCAAAGGGCGAA
TTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGT
AATCATGGTCATAGCTGTTTCCNN

FL191 = *crfssa14* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)

NNNNNNNNNCNNNNGGGCNANTGANTTTAGCGGCCGCGAATTCGCCCTTTGGAC
ATATTCGGGAAATGAAAGTGTTAGAAATACATTTGCCAAATAATTTGTTTACATCTTGC
ACAAAGTGTACGCTACTATTCCATTTCTGTATTACTCTTTAATTGGTTAACTTTCGTTA
TTTTATTTATAATTTGTATTTATTTAAACGAGAGCTGTAAAATCGAGTTGTAATTCGCT
GTATAGTTATTACATTGTTTGATGAGAAGCAAACATAGCCCGTTGACAAGGGCGAAT
TCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTA
ATCATGGTCATAGCTGTTTCCNN

FL192 = *crfssa19* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)

NNNNNNNNNNNNNGGGNNANTGNNTTAGCGGCCGCGAATTCGCCCTTAACACTTGT
CGCGGGTCTTGGCTATATAAATCCAAACTGCCGTCCTTTCTTTGAAGAACACCTTATA
ACAATTTCTTGAACAACACTACTGGAAGAGGAAGGCAGCTCTCAACTAATAACTAAAA
TCTTCCAAGACACACAACGGCTCAACTGAAGATTCCTGTTGATCCCGACAAGGGCGA
ATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCG
TAATCATGGTCATAGCTGTTTCCNNN

FL193 = *crfssa19* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)
NNNNNNNNNNNNNNGGGNGATTGATTTAGCGGCCGCGAATTCGCCCTTGTCGGGA
TCAACAGGAATCTTCAGTTGAGCCGTTGTGTGTCTTGGAAGATTTTAGTTATTAGTTG
AGAGCTGCCTTCCTCTTCCAGTAGTGTTGTTCAAGAAATTGTTATAAGGTGTTCTTCA
AAGAAAGGACGGCAGTTTGGATTTATATAGCCAAGACCCGCGACAAGTGTTAAGGG
CGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTG
GCGTAATCATGGTCATAGCTGTTTCCTGA

FL194 = *crfssa29* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)
NNNNNNNNNNNNNNGGGCGANTGATTTAGCGGCCGCGAATTCGCCCTTTCCATCAC
TCGTGGAAAAGGAAGAGAGTTCTCAACAAATACCTAAAATCCAGGGACACAACGACT
CAACTGAAGATCTCGTTGAACCCCTGAAGGGCGAATTCGTTTAAACCTGCAGGACTA
GTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCN
NNN

FL195 = *crfssa29* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)
NNNNNNNNNNNNNNGGGCGATTGANTTAGCGGCCGCGAATTCGCCCTTCAGGGGT
TCAACGAGATCTTCAGTTGAGTCGTTGTGTCCCTGGATTTTAGGTATTTGTTGAGAAC
TCTCTTCCTTTTCCACGAGTGATGGAAAGGGCGAATTCGTTTAAACCTGCAGGACTA
GTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCC
NNN

FL196 = *crfbpssa01* (Primers amplify genomic DNA sequence that is exactly similar to the gene target sequence)
NNNNNNNNNNNNNNGGGCGANTGATTTAGCGGCCGCGAATTCGCCCTTATATAGGA
GGTGGAGAGATAGATAGAGAGAGCTCAGCCAGTGAAGCAGAAATCCTCCACACTGT
TCACCTTGATCCTCTGCAGCTCCTGCCGGTCCAGCAGCCGGTACTGGAACGCCACC
CGGTTGACAAACCTACCGCTGGACACCATTCTCACCACCGTGTTGTCACAACCTATC
TTCATCTGGGCGGGGCCATTAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCC
TTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCNNN

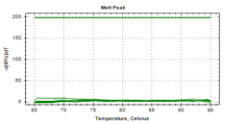
FL197 (This sequence partially blast a sequence that is different from *crfbpssa01*)
NNNNNNNNNNNNNNGGGCNANTGATTTAGCGGCCGCGAATTCGCCCTTATATAGG
AGGTGGAGAGATAGATAGAGAGATAGAGACCGAGAAACAAAGAAAGATGGATATAG
TAGAAACGAGGACTGAAGGCTAAACTAGGTGTACTGAATACCTAAAGAGACTCTTCA
TCCATACTGTACCTGAGATGCAGATGAAGCCTGAGGCGTAGAAGGCTTCGTTGTAG
GAGGCCAGGGAAGCCATCTGGGCGGGGCCATTAAGGGCGAATTCGTTTAAACCTG
CAGGACTAGTCCCTTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAG
CTGTTTCCTGA

FL198 (This sequence partially blast a sequence that is different *crfbpssa11*)
NNNNNNNNNANNNNNNGGGNNANNNANTTAGCGGCCGCGAATTCGCCCTTAACGG
TCCCGCCCAGATCACAGAGAAGGTGGTGGAGCTCTTCAGGAGTAAAAGCGAATTTA
CCTTCCTGGCCTCCATTCAGCAGAAGTCCTCTACGTCAGGAGTCATCTTCTCCATCC
ATGAATCTGAACACAGGTAATGCATATTTAATAATTTTATTACTGCCTTTTCAGCAGCT
ACTTTATCTATCTGCCACCTAAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCC
TTTAGTGAGGGTTAATTCTGAGCTTGGCGTAATCATGGTCATAGCTGTTTCCNNN
FL199 (This sequence partially blast a sequence that is different from *crfbpssa11*)
NNNNNNNNNNNNNGGGNNANNGANTTAGCGGCCGCGAATTCGCCCTTTAGGTGGC
AGATAGATAAAGATACAGGATACAGAAAATACAGGAGATAGGAAATGAGGAAGAGGT

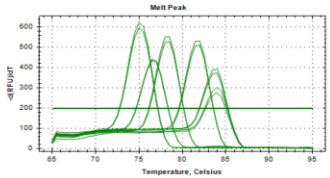
GTAAAAAAAACGCACCATCTAGTGGAGGAAAGAGACACTACACCACGTAGCTTTGGC
CCAGGGCCCAGTTTCCCAAAAGCATCTTAAGCCTAGTTTCATCTGGGCGGGACCGT
TAAGGGCGAATTCGTTTAAACCTGCAGGACTAGTCCCTTTAGTGAGGGTTAATTCTG
AGCTTGGCGTAATCATGGTCATAGCTGTTTCCN

7.11. APPENDIX K – NRT Melting Curve

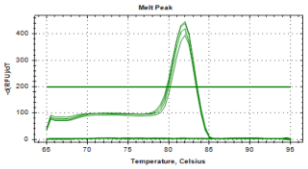
Well	Content	Sample	Cq	Melt.Temp	
A01	NTC	crf03	N/A	None	
A02	NTC	crf03	N/A	None	
A03	NTC	crf14	N/A	None	
A04	NTC	crf14	N/A	None	
A05	NTC	crf19	N/A	None	
A06	NTC	crf19	N/A	None	
A07	NTC	crf29	N/A	None	
A08	NTC	crf29	N/A	None	
A09	NTC	crfbp01	N/A	None	
A10	NTC	crfbp01	N/A	None	
A11	NTC	crfbp11	N/A	None	
A12	NTC	crfbp11	N/A	None	
B01	cDNA	crf03	25.04	82	
B02	cDNA	crf03	25.08	82	
B03	cDNA	crf14	29.28	75	
B04	cDNA	crf14	29.28	75	
B05	cDNA	crf19	31.14	78.5	
B06	cDNA	crf19	30.68	78.5	
B07	cDNA	crf29	27.97	76.5	
B08	cDNA	crf29	27.91	76.5	
B09	cDNA	crfbp01	24.43	84	
B10	cDNA	crfbp01	24.4	84	
B11	cDNA	crfbp11	24.74	84	
B12	cDNA	crfbp11	24.72	84	
C01	1.1	crf03	33.13	82	
C02	1.1	crf03	33.14	82	
C03	1.2	crf03	34.7	82	
C04	1.2	crf03	34.26	82	
C05	2.1	crf03	N/A	None	
C06	2.1	crf03	N/A	None	
C07	2.2	crf03	N/A	None	
C08	2.2	crf03	N/A	None	
C09	3.1	crf03	N/A	None	
C10	3.1	crf03	N/A	None	
C11	3.2	crf03	N/A	None	
C12	3.2	crf03	N/A	None	
D01	1.1	crf14	36.05	75.5	1 cycle different, pipetting error?
D02	1.1	crf14	37.49	76	
D03	1.2	crf14	34.47	75.5	
D04	1.2	crf14	N/A	None	mistake
D05	2.1	crf14	N/A	None	
D06	2.1	crf14	N/A	None	
D07	2.2	crf14	N/A	None	
D08	2.2	crf14	36.81	75.5	pipetting error?
D09	3.1	crf14	N/A	None	
D10	3.1	crf14	N/A	None	
D11	3.2	crf14	N/A	None	
D12	3.2	crf14	N/A	None	



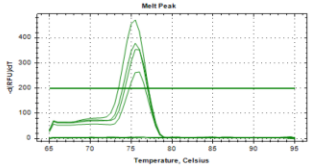
NTCs are negative for all (MM not contaminated)



cDNA control samples work



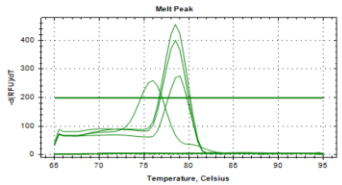
No ambion treatment give signal (1.1, 1.2)
Ambion treated (2.1 - 3.2) no signal



No signal for ambion treated samples

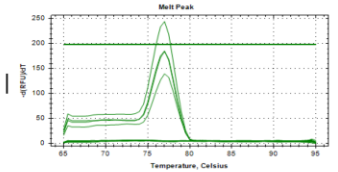
Well	Content	Sample	Cq	Melt.Temp	
E01	1.1	crf19	37.17	79	which one is error?
E02	1.1	crf19	N/A	None	
E03	1.2	crf19	N/A	None	
E04	1.2	crf19	N/A	None	
E05	2.1	crf19	N/A	None	
E06	2.1	crf19	N/A	None	
E07	2.2	crf19	N/A	None	
E08	2.2	crf19	35.55	78.5	error?
E09	3.1	crf19	N/A	None	
E10	3.1	crf19	36.22	78.5	error?
E11	3.2	crf19	N/A	None	
E12	3.2	crf19	36.47	76	error (different peak)
F01	1.1	crf29	37.93	None	
F02	1.1	crf29	N/A	None	
F03	1.2	crf29	38.12	None	
F04	1.2	crf29	37.16	77	
F05	2.1	crf29	N/A	None	
F06	2.1	crf29	38.69	None	error?
F07	2.2	crf29	N/A	None	
F08	2.2	crf29	N/A	None	
F09	3.1	crf29	N/A	None	
F10	3.1	crf29	N/A	None	
F11	3.2	crf29	N/A	None	
F12	3.2	crf29	N/A	None	
G01	1.1	crfbp01	35.41	84	
G02	1.1	crfbp01	35.11	84	
G03	1.2	crfbp01	N/A	None	
G04	1.2	crfbp01	N/A	None	
G05	2.1	crfbp01	N/A	None	
G06	2.1	crfbp01	N/A	None	
G07	2.2	crfbp01	N/A	None	
G08	2.2	crfbp01	N/A	None	
G09	3.1	crfbp01	N/A	None	
G10	3.1	crfbp01	N/A	None	
G11	3.2	crfbp01	N/A	None	
G12	3.2	crfbp01	N/A	None	
H01	1.1	crfbp11	N/A	None	
H02	1.1	crfbp11	N/A	None	
H03	1.2	crfbp11	N/A	None	
H04	1.2	crfbp11	N/A	None	
H05	2.1	crfbp11	N/A	None	
H06	2.1	crfbp11	N/A	None	
H07	2.2	crfbp11	N/A	None	
H08	2.2	crfbp11	N/A	None	
H09	3.1	crfbp11	35.77	80.5	error?
H10	3.1	crfbp11	N/A	None	
H11	3.2	crfbp11	N/A	None	
H12	3.2	crfbp11	N/A	None	

Maybe none of them
give no signal as
the untreated one (1.1)
has 37.17 Cq value

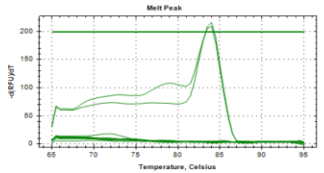


error pattern the same

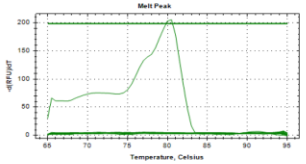
At least 1 ambion treated give no signal (2.1)



Assumption:
no signal for treated samples
since the untreated ones
give very low signal



No signal for treated samples



No signal for both treated and untreated samples

7.12. APPENDIX L – Statistical Analysis Result

7.12.1. Weight, Length and Growth Rate

a. Weight

```
Anova Table (Type II tests)

Response: weight
          Sum Sq  Df F value  Pr(>F)
treatment   9601   3  1.2500 0.29413
day         8382   1  3.2738 0.07257 .
Residuals 353311 138
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

b. Length

```
Anova Table (Type II tests)

Response: length
          Sum Sq  Df F value    Pr(>F)
treatment  56.97   3  5.8029 0.0009152 ***
day        95.96   1 29.3242 2.642e-07 ***
Residuals 451.58 138
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Pairwise comparisons using t tests with pooled SD

data:  C.df$length and C.df$day

   D0
D9 0.01

P value adjustment method: bonferroni
```

```
Pairwise comparisons using t tests with pooled SD

data:  SA.df$length and SA.df$day

   D0
D9 0.00081

P value adjustment method:
bonferroni
```

```
Pairwise comparisons using t tests with pooled SD

data:  SB.df$length and SB.df$day

   D0
D9 3e-04

P value adjustment method:
bonferroni
```

```
Pairwise comparisons using t tests with pooled SD

data:  SC.df$length and SC.df$day

   D0
D9 0.53

P value adjustment method: bonferroni
```

c. Growth Rate

```
Anova Table (Type III tests)

Response: RGR
            Sum Sq Df F value   Pr(>F)
(Intercept) 918.84  1 16.2608 0.003775 **
treatment   540.81  3  3.1902 0.084186 .
Residuals   452.05  8
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

d. Condition Factor

```
Anova Table (Type III tests)

Response: cf
               Sum Sq  Df   F value Pr(>F)
(Intercept)   19.6370   1 6949.2952 <2e-16 ***
treatment      0.5955   3   70.2450 <2e-16 ***
day            0.0053   1    1.8674  0.174
treatment:day  0.4533   3   53.4750 <2e-16 ***
Residuals      0.3815 135
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Tukey multiple comparisons of means
    95% family-wise confidence level
```

```
Fit: aov(formula = cf ~ treatment * day, data = cf.df)

$treatment
             diff         lwr         upr     p adj
SA-C   0.07004503  0.03745201  0.10263805 0.0000007
SB-C   0.06733296  0.03450796  0.10015796 0.0000023
SC-C  -0.01026414 -0.04285716  0.02232887 0.8453054
SB-SA -0.00271207 -0.03553707  0.03011293 0.9964771
SC-SA -0.08030917 -0.11290219 -0.04771616 0.0000000
SC-SB -0.07759710 -0.11042210 -0.04477210 0.0000000

$day
            diff       lwr         upr p adj
D9-D0 -0.1079193 -0.125734 -0.09010457     0

$`treatment:day`
                     diff         lwr            upr     p adj
SA:D0-C:D0   0.177600148  0.12708889  0.2281114014 0.0000000
SB:D0-C:D0   0.167100648  0.11596190  0.2182393946 0.0000000
SC:D0-C:D0   0.006572721 -0.04393853  0.0570839748 0.9999195
C:D9-C:D0    0.024557410 -0.03077490  0.0798897161 0.8706940
SA:D9-C:D0  -0.055974726 -0.11130703 -0.0006424196 0.0452565
SB:D9-C:D0  -0.041815366 -0.09714767  0.0135169404 0.2869623
SC:D9-C:D0  -0.009278344 -0.06461065  0.0460539626 0.9995617
SB:D0-SA:D0 -0.010499500 -0.06163825  0.0406392470 0.9983627
SC:D0-SA:D0 -0.171027427 -0.22153868 -0.1205161729 0.0000000
C:D9-SA:D0  -0.153042738 -0.20837504 -0.0977104316 0.0000000
SA:D9-SA:D0 -0.233574873 -0.28890718 -0.1782425673 0.0000000
SB:D9-SA:D0 -0.219415513 -0.27474782 -0.1640832072 0.0000000
SC:D9-SA:D0 -0.186878491 -0.24221080 -0.1315461851 0.0000000
SC:D0-SB:D0 -0.160527927 -0.21166667 -0.1093891800 0.0000000
C:D9-SB:D0  -0.142543238 -0.19844895 -0.0866375246 0.0000000
SA:D9-SB:D0 -0.223075374 -0.27898109 -0.1671696604 0.0000000
SB:D9-SB:D0 -0.208916014 -0.26482173 -0.1530103003 0.0000000
SC:D9-SB:D0 -0.176378991 -0.23228470 -0.1204732782 0.0000000
C:D9-SC:D0   0.017984689 -0.03734762  0.0733169951 0.9736219
SA:D9-SC:D0 -0.062547447 -0.11787975 -0.0072151407 0.0151245
SB:D9-SC:D0 -0.048388087 -0.10372039  0.0069442194 0.1338885
SC:D9-SC:D0 -0.015851065 -0.07118337  0.0394812415 0.9872182
SA:D9-C:D9  -0.080532136 -0.14029786 -0.0207664143 0.0014829
SB:D9-C:D9  -0.066372776 -0.12613850 -0.0066070542 0.0183338
SC:D9-C:D9  -0.033835754 -0.09360147  0.0259299679 0.6590816
SB:D9-SA:D9  0.014159360 -0.04560636  0.0739250815 0.9959558
```

```
SC:D9-SA:D9  0.046696382 -0.01306934  0.1064621036 0.2471384
SC:D9-SB:D9  0.032537022 -0.02722870  0.0923027436 0.7024597
```

7.12.2. Plasma Cortisol

```
Anova Table (Type III tests)

Response: cortisol_trans
              Sum Sq  Df  F value    Pr(>F)
(Intercept)    24.47   1  12.8226 0.0004192 ***
treatment       8.94   3   1.5615 0.1995535
day          1086.12   4 142.2888 < 2.2e-16 ***
treatment:day  296.27  12  12.9380 < 2.2e-16 ***
Residuals      431.28 226
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
 Tukey multiple comparisons of means
    95% family-wise confidence level

 Fit: aov(formula = cortisol_trans ~ treatment * day, data = copy_new.df)

 $treatment
           diff       lwr       upr     p adj
 SA-C   2.8674597  2.2197019  3.515218 0.0000000
 SB-C   0.8235389  0.1889819  1.458096 0.0050486
 SC-C   1.7907352  1.1457709  2.435699 0.0000000
 SB-SA -2.0439209 -2.6892267 -1.398615 0.0000000
 SC-SA -1.0767245 -1.7322670 -0.421182 0.0001816
 SC-SB  0.9671963  0.3246947  1.609698 0.0007388



 $day
             diff       lwr       upr     p adj
 D0-D0bs  5.0333965  4.036493  6.0302996 0.0000000
 D1-D0bs  3.5864309  2.599158  4.5737042 0.0000000
 D8-D0bs  0.8035193 -0.183754  1.7907926 0.1695671
 D9-D0bs  8.6964347  7.713517  9.6793521 0.0000000
 D1-D0   -1.4469656 -2.171844 -0.7220867 0.0000011
 D8-D0   -4.2298772 -4.954756 -3.5049983 0.0000000
 D9-D0    3.6630382  2.944103  4.3819731 0.0000000
 D8-D1   -2.7829116 -3.494489 -2.0713344 0.0000000
```

```
  D9-D1    5.1100038  4.404483  5.8155249 0.0000000
  D9-D8    7.8929154  7.187394  8.5984365 0.0000000
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ treatment, data = D0.df)

$treatment
          diff       lwr        upr     p adj
SA-C   1.8095865  0.1818972  3.43727574 0.0238744
SB-C  -1.6601904 -3.2240236 -0.09635711 0.0335144
SC-C   1.0736566 -0.5199667  2.66727978 0.2895548
SB-SA -3.4697768 -5.0974661 -1.84208760 0.0000044
SC-SA -0.7359299 -2.3922611  0.92040120 0.6411854
SC-SB  2.7338469  1.1402237  4.32747013 0.0001948
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ treatment, data = D1.df)

$treatment
          diff       lwr        upr     p adj
SA-C   7.0004164  5.5779756  8.4228573 0.0000000
SB-C   3.9952907  2.5958654  5.3947161 0.0000000
SC-C   4.7519904  3.3525650  6.1514158 0.0000000
SB-SA -3.0051257 -4.3775152 -1.6327362 0.0000022
SC-SA -2.2484260 -3.6208155 -0.8760365 0.0003593
SC-SB  0.7566997 -0.5918203  2.1052197 0.4515897
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ treatment, data = D8.df)

$treatment
          diff       lwr        upr     p adj
SA-C   3.3436078  2.2903678  4.3968478 0.0000000
SB-C   1.7952078  0.7602864  2.8301293 0.0001526
SC-C   2.3434677  1.2694790  3.4174564 0.0000023
SB-SA -1.5483999 -2.6016400 -0.4951599 0.0015161
SC-SA -1.0001401 -2.0917920  0.0915119 0.0836561
SC-SB  0.5482599 -0.5257288  1.6222486 0.5333280
```

```
Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ day, data = C.df)

$day
              diff       lwr       upr     p adj
D0-D0bs  5.4644929  3.914781  7.014205 0.0000000
D1-D0bs  0.3169809 -1.250511  1.884473 0.9789974
D8-D0bs -0.3355822 -1.869719  1.198555 0.9720000
D9-D0bs  9.8578879  8.323751 11.392025 0.0000000
D1-D0   -5.1475121 -6.370781 -3.924243 0.0000000
D8-D0   -5.8000751 -6.980301 -4.619849 0.0000000
D9-D0    4.3933950  3.213169  5.573621 0.0000000
D8-D1   -0.6525630 -1.856039  0.550913 0.5497545
D9-D1    9.5409070  8.337431 10.744383 0.0000000
D9-D8   10.1934701  9.033772 11.353169 0.0000000
```

```
Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ day, data = SA.df)

$day
               diff        lwr       upr     p adj
D0-D0bs  5.88874985  3.1358838  8.641616 0.0000015
D1-D0bs  5.93206772  3.2288069  8.635329 0.0000008
D8-D0bs  1.62269606 -1.0805648  4.325957 0.4461690
D9-D0bs  8.01896014  5.3357982 10.702122 0.0000000
D1-D0    0.04331786 -1.8324442  1.919080 0.9999958
D8-D0   -4.26605380 -6.1418158 -2.390292 0.0000004
D9-D0    2.13021029  0.2835316  3.976889 0.0160940
D8-D1   -4.30937166 -6.1115455 -2.507198 0.0000001
D9-D1    2.08689242  0.3150093  3.858776 0.0132995
D9-D8    6.39626408  4.6243810  8.168147 0.0000000
```

```
Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ day, data = SB.df)

$day
              diff        lwr       upr     p adj
D0-D0bs  2.7446161  1.1482934  4.340939 0.0000934
D1-D0bs  3.2525851  1.6723063  4.832864 0.0000028
D8-D0bs  0.3999392 -1.1803396  1.980218 0.9529185
```

```
D9-D0bs  8.4770898  6.8807671 10.073412 0.0000000
D1-D0    0.5079690 -0.7077541  1.723692 0.7650372
D8-D0   -2.3446769 -3.5604000 -1.128954 0.0000110
D9-D0    5.7324737  4.4959674  6.968980 0.0000000
D8-D1   -2.8526459 -4.0472244 -1.658067 0.0000001
D9-D1    5.2245047  4.0087815  6.440228 0.0000000
D9-D8    8.0771506  6.8614274  9.292874 0.0000000
```

```
Tukey multiple comparisons of means
    95% family-wise confidence level

Fit: aov(formula = cortisol_trans ~ day, data = SC.df)

$day
             diff        lwr       upr     p adj
D0-D0bs  6.821449  4.4043170  9.238580 0.0000000
D1-D0bs  5.352271  2.9733567  7.731184 0.0000004
D8-D0bs  2.291185 -0.1259469  4.708317 0.0711717
D9-D0bs  9.234076  6.8551624 11.612990 0.0000000
D1-D0   -1.469178 -3.0710924  0.132736 0.0869251
D8-D0   -4.530264 -6.1884026 -2.872125 0.0000000
D9-D0    2.412628  0.8107133  4.014542 0.0007734
D8-D1   -3.061086 -4.6629999 -1.459172 0.0000147
D9-D1    3.881806  2.3381626  5.425449 0.0000000
D9-D8    6.942891  5.3409773  8.544806 0.0000000
```

7.13.3. Gene Paralogs

a. *crfssa03*

```
Anova Table (Type II tests)

Response: crf03_trans
          Sum Sq  Df F value Pr(>F)
treatment   975.8   3  1.9809 0.1196
day         901.7   4  1.3728 0.2465
Residuals 22988.3 140
```

b. *crfssa14*

```
Anova Table (Type II tests)

Response: crf14_trans
          Sum Sq  Df F value   Pr(>F)
treatment  656.5   3  4.8953 0.002834 **
```

```
day       2976.0   4 16.6436 2.69e-11 ***
Residuals 6660.5 149
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crf14_trans ~ treatment + day, data = crf14_new.df)

$day
              diff        lwr        upr     p adj
D0bs-D0  -5.8844880 -10.979362 -0.7896139 0.0147668
D1-D0     3.0060033  -1.630143  7.6421493 0.3829679
D8-D0    -8.2021532 -12.838299 -3.5660071 0.0000258
D9-D0    -6.1008735 -10.767811 -1.4339363 0.0037751
D1-D0bs   8.8904913   3.997364 13.7836185 0.0000145
D8-D0bs  -2.3176652  -7.210792  2.5754621 0.6866900
D9-D0bs  -0.2163855  -5.138697  4.7059256 0.9999505
D8-D1   -11.2081565 -15.621636 -6.7946769 0.0000000
D9-D1    -9.1068768 -13.552690 -4.6610636 0.0000008
D9-D8     2.1012796  -2.344534  6.5470928 0.6884238
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crf14_trans ~ treatment, data = D0bs.df)

$treatment
           diff        lwr       upr     p adj
SA-C   12.472196   1.588108 23.356284 0.0211472
SB-C    2.632036  -8.252051 13.516124 0.9046354
SC-C    1.298340  -9.585747 12.182428 0.9867835
SB-SA  -9.840160 -20.724247  1.043928 0.0853335
SC-SA -11.173856 -22.057943 -0.289768 0.0428969
SC-SB  -1.333696 -12.217784  9.550392 0.9857114
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crf14_trans ~ treatment, data = D1.df)

$treatment
           diff        lwr        upr     p adj
SA-C   7.0907565  -1.202813 15.3843257 0.1151761
SB-C   0.9685584  -7.580257  9.5173738 0.9897123
SC-C  -1.8480239 -10.141593  6.4455453 0.9297881
```

```
SB-SA -6.1221981 -14.671014  2.4266173 0.2312325
SC-SA -8.9387804 -17.232350 -0.6452112 0.0307915
SC-SB -2.8165822 -11.365398  5.7322332 0.8078729
```

c. *crfssa19*

```
Anova Table (Type III tests)

Response: crf19
               Sum Sq  Df F value    Pr(>F)
(Intercept)    674476   1 25.5920 1.24e-06 ***
treatment      280314   3  3.5454  0.01615 *
day            174449   4  1.6548  0.16364
treatment:day  582514  12  1.8419  0.04646 *
Residuals     3874178 147
---

Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crf19 ~ treatment, data = D0bs.df)

$treatment
           diff        lwr       upr     p adj
SA-C   203.03766  -84.60109 490.67640 0.2300842
SB-C    57.71184 -229.92691 345.35059 0.9422573
SC-C   -95.02670 -382.66545 192.61205 0.7920780
SB-SA -145.32582 -432.96456 142.31293 0.5054912
SC-SA -298.06436 -585.70311 -10.42561 0.0405672
SC-SB -152.73854 -440.37729 134.90020 0.4637804
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crf19 ~ treatment, data = D8.df)

$treatment
           diff        lwr       upr     p adj
SA-C   177.390351   27.57862 327.20209 0.0152514
SB-C     2.257187 -147.55455 152.06892 0.9999750
SC-C   120.106947  -29.70479 269.91868 0.1528384
SB-SA -175.133164 -324.94490 -25.32143 0.0168821
SC-SA  -57.283404 -207.09514  92.52833 0.7298420
SC-SB  117.849760  -31.96197 267.66149 0.1649988
```

d. *crfssa29*

```
Anova Table (Type II tests)

Response: crf29
            Sum Sq  Df F value   Pr(>F)
treatment  110193332   3  4.2500 0.006473 **
day         67641568   4  1.9566 0.103969
Residuals 1313686458 152
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crf29 ~ treatment, data = D8.df)

$treatment
          diff       lwr       upr     p adj
SA-C   1885.883  -747.3911  4519.1569 0.2304712
SB-C  -1814.907 -4524.5234   894.7098 0.2834894
SC-C   1237.203 -1396.0711  3870.4769 0.5838978
SB-SA -3700.790 -6334.0636 -1067.5157 0.0033056
SC-SA  -648.680 -3203.3310  1905.9710 0.8999360
SC-SB  3052.110   418.8357  5685.3836 0.0182169
```

e. *crfbpssa01*

```
Anova Table (Type III tests)

Response: crfbp01
                 Sum Sq  Df F value  Pr(>F)
(Intercept)   7.1313e+09   1 89.4242 < 2e-16 ***
treatment     8.0714e+08   3  3.3737 0.02032 *
day           3.7358e+08   4  1.1711 0.32627
treatment:day 1.9941e+09  12  2.0838 0.02168 *
Residuals     1.1005e+10 138
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

```
Tukey multiple comparisons of means
   95% family-wise confidence level

Fit: aov(formula = crfbp01 ~ treatment, data = D8.df)

$treatment
          diff       lwr       upr     p adj
```

```
SA-C  -6641.498 -13172.5618  -110.4334 0.0451476
SB-C  -9291.836 -16052.1296 -2531.5422 0.0042634
SC-C  -3382.461  -9729.5140  2964.5930 0.4771057
SB-SA -2650.338  -9410.6320  4109.9554 0.7099859
SC-SA  3259.037  -3088.0164  9606.0906 0.5086553
SC-SB  5909.375   -673.3186 12492.0694 0.0904571
```

f. *crfbpssa11*

```
Anova Table (Type II tests)

Response: crfbp11_trans
          Sum Sq  Df F value Pr(>F)
treatment   6294   3  0.5212 0.6683
day        25016   4  1.5536 0.1897
Residuals 603829 150
```

```
Tukey multiple comparisons of means
  95% family-wise confidence level

Fit: aov(formula = crfbp11 ~ treatment, data = D0bs.df)

$treatment
           diff       lwr       upr     p adj
SA-C   19718.532   1251.444 38185.620 0.0338608
SB-C   -4408.598 -23777.043 14959.847 0.9177013
SC-C    2944.984 -15522.104 21412.072 0.9691317
SB-SA -24127.130 -43495.575 -4758.685 0.0116716
SC-SA -16773.548 -35240.636  1693.540 0.0830835
SC-SB   7353.582 -12014.863 26722.027 0.7127402
```