Stress, cortisol, and feed intake in rainbow trout (*Oncorhynchus mykiss*)

Can you stomach the stress?

Maren Høyland

Thesis submitted for the degree of
Master of Science in Molecular Bioscience
60 credits

Department of Bioscience
Faculty of Mathematics and Natural Sciences

UNIVERSITY OF OSLO

February 2018

© Maren Høyland

2018

Stress, cortisol, and feed intake in rainbow trout (*Oncorhynchus mykiss*)

Maren Høyland

http://www.duo.uio.no/

Trykk: Reprosentralen, Universitetet i Oslo

Stress, cortisol, and feed intake in rainbow trout (*Oncorhynchus mykiss*)

Can you stomach the stress?

Acknowledgements

The first two studies for this thesis was conducted at the Section for Physiology and Cell Biology, Department of Biosciences, Faculty of Mathematics and Natural Sciences at the University of Oslo and the third was conducted at Hirtshals, Denmark.

Firstly, I would like to thank my amazing supervisors, Ida Beitnes Johansen and Øyvind Øverli, for taking me in and nursing my fascination and curiosity of science. Without them I'd still be a master's student without a master's project. I am very grateful to have been given this opportunity to be a part of their small research group, for taking me to winter meetings and other trips. I would also thank Marco Vindas for not making too big of a fuss when I stole his desk in the last months of writing this. In addition, a thanks to the knowledgeable Siri and Helene, for showing me the ropes around the lab at NMBU Adamstuen and always taking the time to answer my questions.

Secondly, a thank you is due to Karoline S. Nørstrud, who donated rainbow trout hypothalamus and plasma from her master's project to me when my own study didn't go as planned, thus basically saving this thesis. And also, a thanks to Patricia Da Silva, for conducting study #3 in this thesis and providing me the data.

Thirdly, a huge thanks to all my friends, specially Vebjørn, Maria and Halvor for spending time in the cold and damp cellar to feed the fishes when I had to go away. It was worth it in the end (right?).

And lastly, a huge thanks to my mom and boyfriend, where mom listened to my complaining in the daytime and my boyfriend took the nightshift. For being supportive and always cheering me on no matter how negative my attitude was.

Oslo, February 2018
Maren Høyland

Abstract

Fish in aquaculture is known to ingest less feed after exposure to stressors such as handling, crowding, vaccination and transportation. In scientific studies, this phenomenon has been ascribed to an appetite-reducing effect of stress, namely the stress hormone cortisol, or other aspects of the physiological stress response. However, preliminary findings have suggested that during recovery from stress fish has not necessarily lost the will to feed, but rather the ability to feed.

To test the hypothesis that decreased feed intake in salmonids subjected to stressful situations is at least in part caused by other factors than a reduction in appetite, feed intake and attempts at feed intake in isolated rainbow trout was closely monitored after transfer to a novel environment. In a follow-up study known appetite markers were measured in the hypothalamus and plasma in rainbow trout receiving exogenous cortisol through feed. Furthermore, I also investigated alteration of the gastrointestinal (GI) tract in rainbow trout, by measuring stomach volume after exogenous cortisol exposure.

From these studies I found that the anorectic stage following subjection to stress is, at least partly, mediated through some physical obstruction in the upper GI tract. I saw that nearly all fish went through three distinct phases of feeding behavior subsequent to stress exposure: Passive/anorectic, active but unable, and active and able. The second stage is an intermediate phase between being completely passive and successfully ingesting feed. The intermediate phase of feeding behavior has not been reported earlier. Here, fish are seen actively trying to ingest feed, but appearing unable to swallow, and thus repeatedly spitting grabbed pellets back out. I also noted that reduced feed intake in response to cortisol treatment was not caused by the alteration of neither corticotropin-releasing factor, cocaine- and amphetamine-regulated transcript, neuropeptide Y, leptin or arginine vasotocin, all which are known appetite-regulating factors. When examining stomach volume of rainbow trout intraperitoneally injected with cortisol, I found that stomach volume was significantly reduced ($p<0.05$) compared to control.

In conclusion, the sum of these findings indicates that the observed reduction of feed intake in stressed salmonids is not caused by the lack of appetite *per se*, but rather a physical obstruction somewhere along the upper GI tract, rendering the fish willing but unable to ingest feed. The mechanisms contributing to this behavior are still to be elucidated.

Table of contents

1 Introduction 10
1.1 Cortisol and stress 12
1.2 Appetite markers 13
1.2.1 Leptin 14
1.2.2 Arginine vasotocin 14
1.2.3 Neuropeptides 15
1.3 Aims of study 17
2 Materials and methods 18
2.1 Study 1: Feeding behavior in isolation after transfer to novel environment 18
2.1.1 Experimental animals 18
2.1.2 Feeding regime for rearing in isolation for 39 days 19
2.2 Study 2: Effect on chronic cortisol administration on neuroendocrine appetite markers 20
2.2.1 Experimental animals 20
2.2.2 Exogenous cortisol administration and signaling peptides in plasma 21
2.2.3 Appetite markers in the hypothalamus 22
2.3 Study 3: Gut volume after exogenous cortisol exposure 26
2.3.1 Experimental animals 26
2.3.2 Assessment of stomach volume 26
2.3.3 Leptin analysis 27
2.4 Statistical analysis 28
3 Results 29
3.1 Study 1: Feeding behavior in isolation after transfer to novel environment 29
3.2 Study 2: Effect on chronic cortisol administration on molecular appetite markers .. 31
3.2.1 Feed intake and growth in respect to cortisol treatment 31
3.2.2 Signaling peptides in plasma 34
3.2.3 Effects of cortisol treatment on mRNA levels of hypothalamic appetite markers 35
3.3 Study 3: Gut volume after exogenous cortisol exposure 36
3.3.1 Feed intake during acclimation and treatment 36
3.3.2 Cortisol and spitting behavior 37

3.3.3 Growth and plasma leptin 38
3.3.4 Stomach volume 39
4 Discussion 40
4.1 Feeding behavior can be separated into three phases 40
4.2 None of the studied appetite markers indicated inhibition of appetite 41
4.3 Reduction of stomach volumes 43
4.4 Methodical considerations 44
4.5 Future perspectives 45
4.6 Summary and conclusion 46
5 Appendices 47
5.1 List of abbreviations 47
5.2 Functions and packages in R 48
5.3 Supplementary tables 49
6 References 51

Figure 1 Number of pellets in the esophagus of a small cohort of juvenile rainbow trout Each data point represents one individual fish, and individuals no. 5-8 received exogenous cortisol at 4 µg/g body weight for 4 days prior to sampling 11
Table 1 A summary of the different appetite markers assessed in this thesis and their effect on appetite. AVT: arginine vasotocin, CRF: corticotropin-releasing factor, NPY: neuropeptide Y, CART: cocaine- and amphetamine-regulated transcript. 16
Figure 2 Feeding criteria for the 39 days in isolation The fish were first offered 5 pellets, dropped in the water one by one. Feeding continued only for fish that reacted on 3 or more pellets. In such cases, feeding continued until one of the following end points were achieved: 1) If a total number of 10 pellets had been intercepted but spat out, feeding was terminated 2) If 20 pellets was actually consumed, feeding was terminated 3) In intermediate cases, i.e. fish that started to spit before achieving to swallow 20 pieces feeding was terminated after spitting 5 pellets. 4) Feeding was also terminated if 5 or more pellets were ignored in succession. 19
Table 2 RNA concentration measured by Nanodrop™ 1000 (**A**), and RNA integrity number (RIN) measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) (**B**). n=30 for both **A** and **B**. Mean with ± s.e.m. 23
Table 3 Nucleotide sequence of primers used for quantitative real-time PCR EF 1-α: elongation factor 1-α, NPY: neuropeptide Y, CRF: corticotropin releasing factor, CART: cocaine- and amphetamine-regulated transcript, F: forward primer, R: reverse primer, bp: base pairs. 24
Table 4 Cycling conditions for qPCR 25
Figure 3 Stomach volume was measured by filling with ringer solution 27

Figure 4 Over time more fish would attempt to eat, ingest more feed and spit less The number of fish reacting to feeding increased with time after transfer to rearing in isolation (**A**). Feed intake increased as more of the fish reacted and successfully ingested feed (**B**). Spitting behavior (as number of pellets spitted out of all pellets reacted upon, in percent) reached a peak after 10 days before declining (**C**). N = 20. Data in **A** presented as number of fish reacting to feed for any given day and as mean ± s.e.m in **B** and **C**. 30
Figure 5 Cortisol treatment reduced feed intake in fish fed cortisol for 7 days Percent of daily ration eaten per day, with n=14 in control and n=16 in cortisol group in **A**, and n=7 in control and n=8 in cortisol group in **B**. Upward pointing arrows indicate sampling time points of 7 control and 8 cortisol treated fish in both A and B. The sampling was subsequent to feeding on the indicated days. Data presented as mean ± s.e.m. 32
Figure 6 Cortisol treatment increased plasma cortisol levels but had no significant effect on specific growth rate (SGR) Cortisol levels were higher in the cortisol treated fish compared to control groups (**A**). Specific growth rate (percent growth per day) did not change significantly during the treatment period (**B**). All control groups n=7 and all cortisol groups n=8. Data presented as mean ± s.e.m. Tukey HSD post-hoc test: *** = p<0.001 versus treatment control 33
Figure 7 Cortisol treatment significantly affected levels of leptin, while treatment period affected levels of vasotocin Plasma leptin was significantly lowered of cortisol treatment compared to the control groups, p<0.05 (**A**). Plasma vasotocin significantly increased over time, p<0.05 (**B**). Bars for a given parameter that do not share a common letter are significantly different from one another. All control groups n=7 and all cortisol groups n=8. Data presented as mean ± s.e.m. 34
Figure 8 Cortisol treatment significantly decreased expression of corticotropin-releasing factor (CRF) Corticotropin-releasing factor (CRF) was significantly decrease after cortisol treatment, p<0.05 (**A**). Neither neuropeptide Y (NPY) (**B**) nor cocaine- and amphetamine-regulated transcript (CART) (**C**) expression was significantly altered by cortisol treatment. Bars for a given parameter that do not share a common letter are significantly different from one another. All control groups n=7 and all cortisol groups n=8. Data presented as mean ± s.e.m. 35
Figure 9 Cortisol treatment had no significant effect on feed intake Percent of daily ration ingested per day (**A**), and mean percent feed ingested per day during treatment period (**B**). Control group (n=12), cortisol group (n=12). Data presented as mean ±s.e.m....................... 36
Figure 10 Cortisol treatment increased plasma cortisol levels, but had no significant effect on spitting behavior Cortisol treated fish had significantly higher levels of plasma cortisol compared to the treatment control, p<0.01 (**A**). Spitting behavior showed as mean percent of daily ration spitted during treatment period (**B**). Control group (n=8), cortisol group (n=8) in **A**, and control group (n=12), cortisol group (n=12) in **B**. Data presented as mean ±s.e.m. T-test: ** = p<0.01 37
Figure 11 Growth rate was significantly reduced by 7 days of exogenous treatment There was a significant reduction in specific growth rate (SGR) in cortisol treatment group, p<0.001 (**A**). There was also a significance in difference in condition factor (CF) between start of treatment and end of treatment, p<0.05 (**B**). Plasma leptin levels were not altered by treatment

(**C**). Control group (n=12) and cortisol group (n=12) for graph **A** and **B**, and control group (n=8) and cortisol group (n=8) for graph **C**. Data presented as mean ±s.e.m. T-test: * = p<0.05, *** = p<0.001 38
Figure 12 Stomach volume was significantly lower in the cortisol treatment group There was a significant correlation between stomach volume and body weight, Pearson's p=0.001 (not shown, see text), but when separated into groups, the correlation was no longer significant for the cortisol group (Pearson's p=0.09) (**A**)**.** Stomach volume was significantly lower in the cortisol treated group (**B**). Control group (n=11) and cortisol group (n=11). r^2 signifies the Pearson correlation coefficient and p signifies the significance level. Data presented as one dot/triangle per individual with regression line in **A**, and as mean ± s.e.m in **B**. T-test: * = p<0.05 39
Table 5 Physiological information about the fish in study #1 Data presented as mean ± s.e.m. 49
Table 6 Physiological information about the fish in study #2 CONT2 = control treatment 2 days, CORT2 = cortisol treatment 2 days, CONT7 = control treatment 7 days and CORT7 = cortisol treatment 7 days. Data presented as mean ± s.e.m. 49
Table 7 Physiological information about the fish in study #3 Parenthesis represent data from 8 individuals in each group. * represents data from 11 individuals from each group. Data presented as mean ± s.e.m. 50

1 Introduction

Teleost fish are currently emerging as alternatives to rodents in biomedical and behavioral research due to easy maintenance, short generation times, and increasingly mapped genomes[1, 2]. In addition to being a much used comparative model [3, 4], members of the family Salmonidae also dominate the rapidly growing aquaculture industry [5, 6], in which the utilization of feed resources is an important constraining factor. Impaired fish growth is commonly observed in aquaculture [5] as well as in small-scale experimental rearing and it is widely accepted that stress is an important contributor to this costly predicament. Common fish culture-associated stressors such as crowding [7-9], vaccination [10] and handling [11, 12] have been shown to decrease growth in fish. The transportation in itself acts as a stressor and increases plasma cortisol even after several hours has passed [13, 14]. A number of later studies have shown that administration of the stress hormone cortisol have similar growth impairing effects, and it is generally accepted that the negative effects of stress on growth are largely mediated by cortisol [15-17].

It has been postulated that stress and the stress hormone cortisol reduces appetite in fish [15], resulting in decreased feed intake and growth rate. Although it may be correct that these factors impair feed intake, it might not be as correct to claim that it is because they simply reduce appetite. According to the Merriam-Webster dictionary, the medical definition of appetite is *any of the instinctive desires necessary to keep up organic life; especially: the desire to eat* [18]. It is however disputable whether the actual desire for food is affected by cortisol or glucocorticoids. Humans who received a synthetic glucocorticoid had significantly increased intake of calories compared to untreated controls [19], implying appetite stimulatory effects of glucocorticoids. Cortisol is also a potent inducer of obesity in humans [20]. In fish however, the effect appears to be the opposite. Several studies claim that stress and cortisol reduces appetite in fish [15, 16, 21]. The life cycle of anadromous fish such as Atlantic and Pacific salmon (i.e. several *Oncorhynchus* species) involves one or more extended phases of homing migration [22, 23], during which levels of cortisol and other steroids increase dramatically [24-26]. Notably, at least in anadromous species, food intake appears to decrease or cease completely during upstream migration prior to spawning. Kadri *et al.* (1995) found that Atlantic salmon (*Salmo salar*) became anorectic preparing for return to the home river to spawn [27], and ceased eating several weeks before their estimated migrating timepoint. Calderwood (1907) refers to two studies of the stomach content of adult

Atlantic salmon, in which only a few stomachs (9 of 4162) contained food items [28]. Any sports fisherman can however testify to the fact that migrating salmonid fish, including salmon, will at least sometimes readily bite on prey-like fishing lures and baits. Hence it would appear that even a close to completely anorectic life-stage does not imply a complete inhibition of feeding instincts and behavior.

In view of the above, even if the onset of anorexia in migrating as well as stressed salmonids certainly seem to be mediated through cortisol and the hypothalamus-pituitary-interrenal (HPI) axis (the fish homolog to the mammalian hypothalamus-pituitary-adrenal (HPA) axis), the possibility that cortisol inhibits food intake through other mechanisms than the reduction of appetite exists. In the following, I will explore the possibility that stressful circumstances (specifically, the transfer between two different rearing environments) and exogenous cortisol treatment inhibits the intake of food through other means than by affecting the drive to eat.

Impetus for the proposition above comes from a small pilot study done by Johansen *et al.* (unpublished), where it was discovered that 3 out of 4 fish that had been exposed to cortisol (668.48 ± 225.32 ng/ml, mean ± s.e.m.) had pellets stuck in their esophagus at the time of sampling (Figure 1), while the presence of engulfed but undigested pellets was not recorded in an untreated control group.

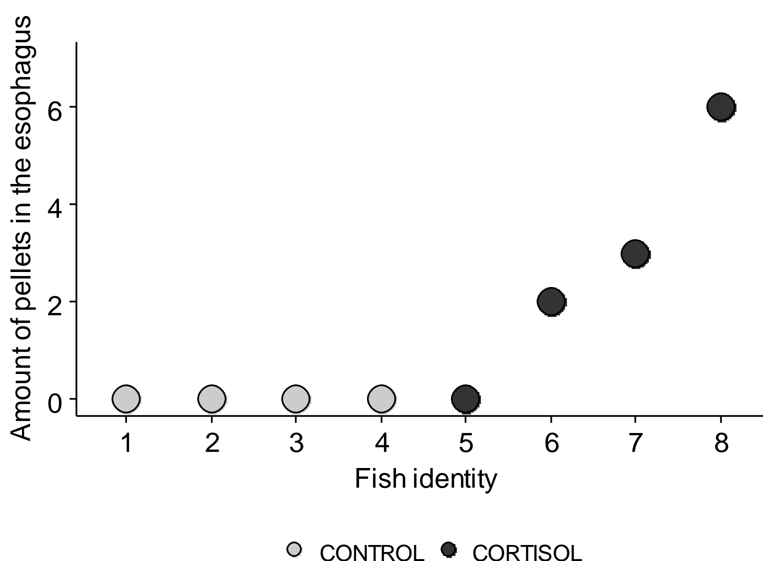

Figure 1 Number of pellets in the esophagus of a small cohort of juvenile rainbow trout
Each data point represents one individual fish, and individuals no. 5-8 received exogenous cortisol at 4 µg/g body weight for 4 days prior to sampling

Although there is little point in doing statistical analysis on such small data set (c.f. Figure 1), these results are intriguing. Why did three out of the four cortisol treated fish have food stuck in their throat? The possibility emerges that rather than bran mediated appetite inhibition, physical or physiological changes in the gut and/or esophagus simply prevents food from being ingested during stress or cortisol induced anorexia. Such a mechanism would also be in line with the common observation that migrating anadromous salmon ceases food intake but continues to react to lures, artificial flies, and even live-bait such as earthworms.

Previous observations done by this research group and mentioned by others [16], has shown that fish in stressful situations often will attempt to eat when food is presented, but food items will be spit out rather than ingested, where after new attempts are made.

This behavior has however never been quantified. Records of individual feeding behavior is not easily obtained from groups of fish, which may explain why previous studies have concluded that stressed fish lose their appetite, based on the finding that feed intake is reduced. In order to lay forward further studies as to the exact mechanism behind stress-induced anorexia in comparative vertebrate models, a primary aim of this thesis is to quantify food spitting behavior of rainbow trout after transfer to rearing in isolation in a novel rearing environment. Additionally, I will describe the time-course of the transition to normal feed intake at ordinary rearing temperatures in freshwater. Furthermore, I will test the tentative hypothesis that the corticosteroid "stress hormone" cortisol induces a physical constriction of gut volume, but does not affect neuroendocrine control of appetite *per se*. Testable hypotheses are stated after a brief review of these physiological systems.

1.1 Cortisol and stress

The stress response is comprised by many factors and several of the contributors are also important in other signaling pathways, making it a less straight forward condition to study. Elevated plasma cortisol is however the most established indicator of stress in salmonids. Cortisol is a steroid hormone and is the main corticosteroid in fish. It is an end-product of the HPI axis and is regulated by a negative feedback loop, both to the hypothalamus, the pituitary and the interrenal cells. Cortisol binds to intracellular glucocorticoid receptors (GR) which are found throughout the body. Binding of cortisol to GR will translocate the ligand-receptor complex to the cell nucleus where it will exert its function as a transcription factor, either by

inducing transcription of genes or inhibiting them. Cortisol is also known to bind to membrane receptors and exert rapid non-genomic actions, like producing more forceful contractions [29] and inducing harmful reactive oxygen species in muscle tissue [30]. These rapid non-genomic effects are also affecting behavior and brain function [31]. Hence, the outcome of the GR activation will depend on what type of cell and tissue the receptor is activated in.

Cortisol is involved in several important processes in the fish body, ranging from the circadian rhythm [32], energy metabolism and balance [33], osmoregulation [34], and of course, the involvement in the stress response [21], which is the main interest in this thesis.

Cortisol as an indicator of stress is widely accepted throughout the academic community, and prolonged elevations in plasma cortisol levels indicate that the organism is having trouble adapting to whatever is causing the stress. While acute exposure to stress has proven to be beneficial to an organism (eustress), prolonged exposure to elevated levels over longer periods can be detrimental (distress) [35]. Acute stress will for example stimulate neurogenesis and neural plasticity i.e. the ability to make new neural connections in the brain to be able to adapt. Extended periods of stress, on the contrary, is inhibiting on these processes [4]. Further, stress is linked to a variety of gastrointestinal dysfunctions in humans [36], and there has also been reported direct effects on the gut wall in fish, where the permeability of enterocytes in the intestine is increased [37, 38].

Cortisol does not only affect the physiology during times of stress, but also the behavior. The stress response is known to increase appetite in mammals [39] yet decrease the feed intake in fish. Madison *et al.* (2015) found that rainbow trout given osmotic cortisol pumps over 14 or 28 days, had significantly reduced feed intake in addition to alterations of several appetite markers [40]. The measurement of certain appetite markers can help elucidate an animal's appetite.

1.2 Appetite markers

Appetite is not the same as feed intake. The term 'appetite' describes the will and urge to consume food items, not the specific act of eating. Appetite marker is a collective term used for molecules that influence appetite in either direction, and comprises both brain derived neurohormones and neurotransmitters in addition to molecules produced by or enacted upon

peripheral tissues. Inducers of appetite are called orexigenic and inhibiters are called anorexigenic factors. The hypothalamus is considered the ‘appetite center’ of the brain, which regulates hunger and satiety, while receiving peripheral signals from the gastrointestinal (GI) tract and adipose tissue [41]. Appetite markers can therefore be assessed by measuring the amount of mRNA of a certain gene in the hypothalamus [42]. Appetite markers that are produced outside of the brain are often peptides and can be measured in plasma.

1.2.1 Leptin

Fish leptin peptide is mainly expressed in the liver [43] and has receptors in the hypothalamus where it exerts its function through the Janus kinase (JAK) coupled with signal transducers and activators of transcription (STAT) and phosphatidylinositol-3-kinase (PI3K) signaling pathways [44, 45]. The leptin peptide both suppresses and induces several other appetite-regulating molecules. It downregulates neuropeptide Y (NPY) mRNA expression (see section 1.2.3) [43], while stimulating the expression of cocaine- and amphetamine-regulated transcript (CART) mRNA [46] (see section 1.2.3). Rainbow trout injected with recombinant leptin intraperitoneally has previously shown significant reduction in feed intake and expression of NPY mRNA [43]. Dragogo-Jack *et al.*(2005) found that leptin was significantly lowered when blocking cortisol biosynthesis in humans [47], indicating that cortisol is a direct inducer of leptin production.

1.2.2 Arginine vasotocin

Arginine vasotocin (AVT) is the non-mammalian homolog to vasopressin. It is an oligopeptide produced in the hypothalamus and is involved in several processes, e.g. regulation of the circadian rhythm, osmotic regulation, reproduction, and social hierarchy formation among others [48]. High doses lead to decrease in aggression and loss in fights for domination resulting in social subordination, which in turn is associated with a chronic stress response [49]. It is also a potent anorexigen, resulting in significantly lower feed intake if injected intracerebroventricularly, as well as significant increase in expression of proopiomelanocortin (POMC) and CART in the hypothalamus, both of which are known anorexigens [50]. AVT also work in synergy with corticotropin-releasing factor (CRF) to stimulate the secretion of adrenocorticotropic hormone (ACTH) in the sustained stress response mediated through the HPI axis [51].

1.2.3 Neuropeptides

Neuropeptide Y (NPY) is a potent orexigenic factor and an important stress-coping hormone [52], which is produced mainly in the central nervous system and is stimulated by increased levels of cortisol [53]. NPY peptide is highly upregulated in obese rats [54] and is also significantly increased in fasted rats who were anticipating food [55] indicating that NPY has a close relationship to feed intake.

Corticotropin-releasing factor (CRF) is the first product of the HPI axis. It is released from the hypothalamus during stressful conditions and is potentiated by adrenaline [56]. CRF binds to corticotropin-releasing-factor receptors (CRFRs) in the pituitary, facilitating the release of adrenocorticotropic hormone (ACTH). ACTH binds to receptors in the interrenal cells in the head kidney and triggers the release of cortisol into the blood stream. CRF release is under a negative feedback loop from cortisol, meaning elevated levels of cortisol causes lower levels of CRF, which in turn causes lower levels of cortisol. Elevated levels of CRF however, is known to evoke anxiety responses and decrease feed intake [57, 58], implying anorexigenic properties.

Cocaine- and amphetamine-regulated transcript (CART) has a complex role in appetite regulation, and it has been shown to function both as an anorexigenic and orexigenic factor [59]. The CART peptide is transcribed as a pro-peptide, and is cleaved by prohormone convertase (PC) to CART(55-102) [60], which is the most studied fragment in relation to appetite regulation. In a study done by Kristensen *et al.* (1998) an intracerebroventricular (ICV) injection of CART(55-102) in rat brains inhibited feeding in both fed and fasted rats while also suppressing the orexigenic effect of NPY [46]. In another study, CART(55-102) stimulated the release of NPY in hypothalamus explants [61]. It has been demonstrated that CART(55-102) delays gastric emptying though the HPA axis, specifically CRF when injected in to rat brains [62], and injection of CRF significantly increased plasma CART immunoreactivity [63]. These studies connect CART directly to CRF and the stress response mediated through the HPI axis.

A brief summary of the different appetite markers is given in Table 1.

	Effect on appetite	**Affected by stress/cortisol**	**Source**
Leptin	Decreased	Upregulated	Madison *et al.*, 2015 [40]
AVT	Decreased	Upregulated	Backström & Winberg, 2009 [49], Gesto *et al.*, 2014 [50]
CRF	Decreased	Upregulated	Backström *et al.*, 2011 [64], Doyon *et al.*, 2005 [53]
NPY	Increased	Upregulated	Doyon *et al.*, 2005 [53], Madison *et al.*, 2015 [40]
CART	Decreased	Upregulated [a] Downregulated [b]	Cortés *et al.*, 2018 [65][a], Conde-Sieira et al., 2010 [66][b]

Table 1 A summary of the different appetite markers assessed in this thesis and their effect on appetite. AVT: arginine vasotocin, CRF: corticotropin-releasing factor, NPY: neuropeptide Y, CART: cocaine- and amphetamine-regulated transcript.

1.3 Aims of study

- Test the hypothesis that salmonid fish in a stressful situation (specifically: after transfer to novel environment) will engulf but not ingest food items, and that the frequency of this behavior will decrease with time after the stressor.

- Investigate if cortisol alters the physical attributes of the upper gastrointestinal tract by measuring stomach volume in cortisol-implanted fish.

- Investigate the effect of exogenous cortisol given in the feed on appetite markers in the hypothalamus and in plasma.

2 Materials and methods

2.1 Study 1: Feeding behavior in isolation after transfer to novel environment

The aim of this study was to quantify specific components of feeding behavior in rainbow trout under stress, i.e. after transfer to social isolation in a novel rearing environment. Cessation of feed intake is a time-honored indicator of stress in teleost fish [67-69]. Hence, it is reasonable to assume that the transfer process which involves physical capture with a dip net, anaesthetizing and weighing followed by a change in both the physical and social environment would induce a stress response. Indeed, the resumption of feed intake after transfer to a novel environment has frequently been used as an indicator of stress reactivity, or coping style, in fish [70-72]. Explicitly, the hypothesis to be tested was that the frequency of spitting behavior was high directly after transfer but decreased with time as fish got adapted to their rearing environment.

2.1.1 Experimental animals

The research animals for this study was obtained at Valdres Ørretoppdrett (Røn Gård, Valdres, Norway), when the fish were 10 months old. The rainbow trout were transported by car in bags of oxygenated water and a small amount of diluted AQUI-S to calm them during the transportation. The fish were kept at the fish holding facilities of the Department of Biosciences at the University of Oslo for 10 weeks, in a circular holding tank with circulatory system and continuous water exchange. They received dechlorinated Oslo tap water held at approximately 6-8 °C. After the 10-week acclimation period, 20 fish were moved to isolation. The fish were distributed into 5 aquaria, with dimensions of 100x50x50 cm, holding roughly 240 liters of water. Four fish shared one aquarium divided by three PVC walls. This gave approximately 60 liters of water to each fish. The fish were kept on a 12:12 light:dark cycle. The body weight at the time of isolation was 217.8 grams ± 56.6 grams (mean ± standard deviation, SD). In isolation, fish were hand fed to a maximum of approximately 1% of body weight with commercial rainbow trout pellets (Spirit Trout 300-40A 4,5 Se, Skretting AS, Stavanger, Norway) once a day. This study was done in the springtime, from April 30th to June 7th at the University of Oslo, Blindern, Norway.

2.1.2 Feeding regime for rearing in isolation for 39 days

As feeding behavior was the primary interest in this study, a set of feeding criteria had to be implemented, as illustrated in Figure 2 and explained more detailed in the figure text. The argument for using feeding criteria was to assure uniform feeding opportunities for all fish. From experience, fish who did not respond to pellets or had started to spit during feeding, was not going to change behavior that day. In addition, it appeared as fish who did eat would continue to eat until it became full followed by spitting of the pellets it could not ingest, possibly due to congestion in the stomach. Thus, based on earlier observations from the pilot study, at set number of pellets for each category of reaction was determined.

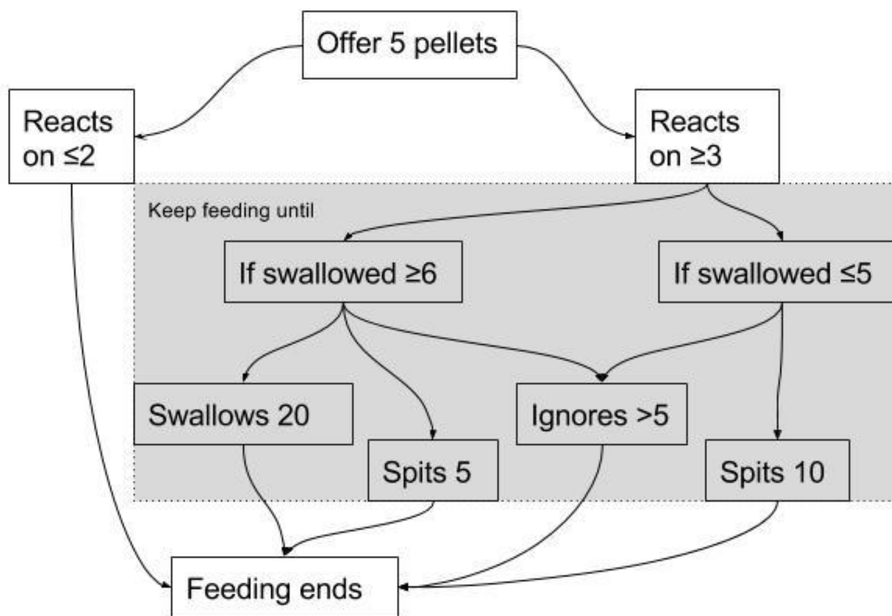

Figure 2 Feeding criteria for the 39 days in isolation
The fish were first offered 5 pellets, dropped in the water one by one. Feeding continued only for fish that reacted on 3 or more pellets. In such cases, feeding continued until one of the following end points were achieved: 1) If a total number of 10 pellets had been intercepted but spat out, feeding was terminated 2) If 20 pellets was actually consumed, feeding was terminated 3) In intermediate cases, i.e. fish that started to spit before achieving to swallow 20 pieces feeding was terminated after spitting 5 pellets. 4) Feeding was also terminated if 5 or more pellets were ignored in succession.

"Reaction" to distributed feed was defined as a successfully engulfed pellet. Fish looking at or nibbling at pellets without actually taking the item into its mouth were not counted in this category. After a fish had reached one of the end points, it was observed until it had stopped moving to ascertain if it would try to eat feed from the bottom of the aquaria or spit any of the pellets counted as ingested. The fish were fed in the same order every day. All uneaten pellets, either they were ejected after intake or entirely disregarded, were removed by a suction tube after all fish were fed. Which meant that the fish fed first, would have its discarded or ignored pellets in the aquaria for quite some time while the other fish were fed, and had the chance to eat them without being observed. Food eaten in this manner was not counted.

2.2 Study 2: Effect on chronic cortisol administration on neuroendocrine appetite markers

After establishing the pattern of recovery of feed intake during acclimation to rearing in a novel environment an attempt was made to use cortisol implants to study the effect of this hormone on feeding behavior and appetite markers. For reasons unknown the implants (made according to the method describe by Gregory and Wood [16]) proved ineffective to provide exogenous cortisol levels, resulting in plasma cortisol levels at 11.47 ± 2.76 ng/ml for control group (n=9) and 9.58 ± 1.08 ng/ml for cortisol group (n=7, mean ± s.e.m.). Thus, the materials analyzed in this study had to be taken from a study performed in 2013 in relation to a previous project [73].

2.2.1 Experimental animals

To examine peptide levels in plasma and mRNA expression on signaling peptides known to be involved in appetite regulation, analysis was performed on hypothalamus and plasma obtained from a previous MSc project at UiO-IBV. A short summery will be given here. For more details refer to MSc thesis by Karoline Sletbak Nøstrud [73].

Rainbow trout were obtained from Valdres Ørretoppdrett (as above). The fish were held at the same fish holding facilities as utilized above, and acclimated for three weeks before transfer to rearing in isolation. In this period, the fish were fed a diet corresponding to 1% body

weight with commercial trout pellets (EFICO, Enviro, 920, Biomar, Brande, Denmark). The holding tank was aerated and supplied with dechlorinated Oslo tap water, at 5-7 °C. During the whole length of the experiment the fish were kept in a 12:12 light:dark cycle, and fed once a day with the same feed as during acclimation.

30 fish weighing 161.6 grams ± 24.5 grams (mean ± SD) were acclimated for 12 days in isolation before treatment began. Four fish shared one aquarium with the same dimensions as in study #1, and was divided with PVC walls to separate the fish. The fish who were in the same aquarium received the same treatment. Experimental groups were established in a 2x2 design as follows: Cortisol treatment vs. control with a duration of 2 or 7 days. Fish who did not eat during the acclimation period were removed from the analysis, which resulted in one fish from 2-day control group and one fish from 7-day control group being removed.

2.2.2 Exogenous cortisol administration and signaling peptides in plasma

Cortisol was administered through the feed, as described by Nøsterud [73], i.e. 15 mg rape seed oil containing 500 mg hydrocortisone powder (Sigma-Aldrich) was applied to one kg commercial rainbow trout feed inside a vacuum coater. Control feed was coated with pure rape seed oil. The fish were fed daily to satiation or to a maximum of 0.8% of body weight, with either a control diet or a cortisol-enriched diet for two or seven days. Consumption of the entire available ration would then correspond to a cortisol dose of 4 µg/g body weight per day Notably, however, control fish and fish treated with cortisol only consumed about 2/3 of the available food (approximately 0.6 % B.W. / day), while food intake dropped to about 50% of the available ration (0.4 % B.W. / day) after 7 days of cortisol treatment. Circulating plasma cortisol levels were thus lower in the 7 day treatment group than in the 2 day treatment group (Figure 4 below). The concentration of plasma cortisol was determined by radio immune assay (RIA) based on the assay described by Pottinger and Carrick (2001) [74] performed by Nøsterud and colleagues in 2013. See [73] for more details.

Leptin analysis

Plasma was stored at -20 °C between Nøsterud's study and the current study. Analysis of plasma leptin was done using a commercially available ELISA kit for detecting leptin in salmon, manufactured by CUSABIO (College Park, MD, USA, catalog #CSB-EL012870FI).

The plasma was diluted 1:250 by adding 1.0 µl of plasma to 249 µl Sample Diluent. Enclosed protocol was followed, and concentration from optic density (OD) was calculated by using the four-parameter logistic curve software found on *www.myassays.com*

Vasotocin analysis

Analysis of plasma arginine vasotocin was done using a commercially available species-independent ELISA kit for detecting vasopressin, manufactured by Nordic BioSite (Täby, Sweden, catalog #EKX-98E17C). The plasma was not diluted. Enclosed protocol was followed, and concentration from OD was calculated with the same software as for the leptin analysis.

2.2.3 Appetite markers in the hypothalamus

Experimental fish were sampled after two days and at seven days of treatment, and anesthetized in 1 mg MS-222 (tricaine methanesulfonate, Sigma-Aldrich, St. Louis, MO, USA) per liter water, followed by cutting the spinal cord. The hypothalamus was dissected out following protocol developed by Øverli *et al.*[75] The hypothalamus was thereafter stored at -80 °C in RNAlater until analysis of mRNA expression of select candidate genes involved in appetite regulation.

RNA extraction from tissue

Hypothalami were thawed and homogenized in 1 ml TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) using lysing matrix D beads from MP medicals (1 bead/mg tissue) in 2 ml reaction tubes and shook in a homogenizing machine (FastPrep®-24 Classic Instrument, MP Biomedicals, LLC, OH, USA) on 4.0 m/s in 20 seconds. The homogenizing step was repeated after an incubation period for 2 minutes on ice. 750 µl homogenate was then transferred to new RNase-free tubes and incubated in room temperature (RT) for 10 minutes. Chloroform was added in a 1:5 ratio (150 µl chloroform to 750 µl homogenate) and mixed by flipping the tubes up-side-down ten times. The samples were allowed to incubate for another 5 minutes in RT, and then centrifuged (Mega Star 600R, VWR, Germany) at 4 °C, 12000g for 20 minutes. 200 µl of the supernatant was transferred to new nuclease free tubes and 200 µl isopropanol was added. The samples were vortexed and incubated in RT for 10 minutes followed by incubation at -20 °C for 20 minutes. For the RNA to be pelletized, the samples were

centrifuged in at 12000g at 4 °C for 10 minutes. The supernatant was removed, the pellet washed with 500 µl 75% cold ethanol 2 times, followed by centrifugation for five minutes at 7500g at 4 °C. After removing as much ethanol as possible the pellet was centrifuged one more time for one minute using the same setting as in the previous step. The pellets were then allowed to dry until they became transparent followed by dissolving the pellets in 30 µl of DEPC water. All steps were performed on ice, if nothing else was noted.

DNase treatment

After RNA was extracted from the tissues, 3.6 µl of DNase buffer and 2 µl DNase was added to each tube using TURBO DNA-free Kit by Invitrogen™ (catalog number: AM1907). The samples were incubated at 37 °C for 30 minutes, followed by 1 µl DNase inactivation reagent and 2 minutes incubation in RT. After incubation the samples were centrifuged for one minute at 12000g at 4 °C and 30 µl of the supernatant was transferred to new tubes. 3 µl of the samples were saved for quality- and concentration analysis (Table 2A and B). All RNA samples were stored at -80 °C.

RNA quantity and quality

A	RNA concentration (ng/µl)
Mean	126.69 ± 4.53
Median	128.87
Highest	166.09
Lowest	39.34

B	RNA integrity number
Mean	8.81 ± 0.12
Median	8.95
Highest	9.60
Lowest	7.30

Table 2 RNA concentration measured by Nanodrop™ 1000 (**A**), and RNA integrity number (RIN) measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) (**B**). n=30 for both **A** and **B**. Mean with ± s.e.m.

cDNA synthesis

The concentration of the extracted RNA was determined using Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) (Table 2A) and the amount of RNA to make 200 ng/µl was calculated. cDNA was made following the enclosed protocol in the iScript™ cDNA Synthesis Kit by Bio-Rad Laboratories Inc. (catalog #1708891SP). The PCR cycling program contained a priming step for 5 minutes at 25 °C, a reverse transcription step for 20 minutes at 46 °C, and a reverse transcriptase inactivation step for 1 minute at 95 °C on a T100 Thermal cycler by Bio-Rad (Hercules, CA, USA).

qPCR

Primers sequences for the appetite markers neuropeptide Y (NPY), corticotropin releasing factor (CRF) and cocaine- and amphetamine-regulated transcript (CART) and for the internal reference gene elongation factor 1-α (EF1-α) were previously published [40]. Primers were ordered from Invitrogen (Thermo Fisher Scientific Inc., MA, USA) with the sequences stated in Table 3.

Gene name	Primer sequence (5'-3')	Predicted amplification product (bp)	Accession number
EF 1-α	F: CCATTGACATTTCTCTGTGGAAGT R: GAGGTACCAGTGATCATGTTCTTGA	106	AF498320
NPY	F: CGGTCAAACCCGAAAATCC R: TCTTCCCATACCTCTGCCTTGT	111	AF203902
CRF	F: ACAACGACTCAACTGAAGATCTCG R: AGGAAATTGAGCTTCATGTCAGG	54	AF296672
CART	F: CCTCGACACAAGAAGTGTGAGAGA R: TGTAGTGCTCCAAGCAGTTGCT	81	CA380644

Table 3 Nucleotide sequence of primers used for quantitative real-time PCR
EF 1-α: elongation factor 1-α, NPY: neuropeptide Y, CRF: corticotropin releasing factor, CART: cocaine- and amphetamine-regulated transcript, F: forward primer, R: reverse primer, bp: base pairs.

10 µl qPCR reaction contained 5 µl Power SYBR Green PCR master mix (Applied Biosystems), 2 µl 10 µM primer mix (1:1 forward and reverse primer (Invitrogen)) and 3 µl 5-fold diluted cDNA. The samples were run in duplicates together with a standard curve, made from the sample with the highest concentration of RNA. The standard curve was made by five-fold dilution, to get a 5-point curve with concentration ranging from 1:1 to 1:625.

Real-time quantitative PCR (qPCR) was performed on the LightCycler 96 by Roche Diagnostics GmbH (Mannheim, Germany), using a 2-step amplification program (Table 4).

Program	Cycles	Acquisition mode	Temperature (°C)	Time (s)	Ramp (°C/s)
Preincubation	1x	None	95	600	4.4
Amplification	45x	None	95	10	4.4
		Single	60	30	2.2
Cooling	1x	None	37	30	2.2

Table 4 Cycling conditions for qPCR

2.3 Study 3: Gut volume after exogenous cortisol exposure

To investigate whether cortisol induced any anatomical changes in the upper GI, a new experiment was initiated. The fish in this study were injected with a cortisol implant in the abdomen which insured a steady increase of cortisol in the bloodstream, so that any effects could not be attributed to direct effects of locally high cortisol concentrations on the gut wall. Furthermore, to make sure that any observed changes in gut volume was not due to that cortisol treated fish ate less than the control, the fish were sampled as soon as the cortisol fish showed signs of struggling to eat their daily ration, but before they started to eat less than the controls.

2.3.1 Experimental animals

24 juvenile rainbow trout from Store Restrup Fiskeri (Nibe, Denmark) weighing 74.1 grams ± 11.2 grams (mean ± SD) were isolated from a holding tank kept at 15 °C, and allowed to acclimate for four days before treatment started. The fish were fed to 0.8% of their body weight, kept at a 13:11 light:dark cycle, with water temperature at 12 ± 1 °C.

Cortisol administration and doses was given as elucidated by T. R. Gregory and C.M. Wood (1999) [16]: A cortisol pellet was made by dissolving 25 mg cortisol per ml in melted coconut oil, at a ratio of 10 ml per gram of fish, giving a cortisol dose of 250 µg/g body weight (cortisol as 11,17,21-trihydroxypregn-4-ene-3,20-dione, Sigma-Aldrich). The cortisol-coconut oil solution was injected peritoneally into 12 fish, while a control group was injected with pure coconut oil. When the injection came in contact with the cold water it solidified, making a sustained-release pellet. The animals were anesthetized in 1 mg MS-222 per liter water, and killed by cutting the spinal cord. Cortisol analysis was done at the laboratory of Danish Technical University, Hirtshals, Denmark by Dr. Patricia Da Silva.

2.3.2 Assessment of stomach volume

Freshwater rainbow trout saline (ringer solution) was made by mixing 7.25g NaCl, 0.186g KCl, 0.23g $MgSO_4$-$7H_2O$, 0.37g $CaCl_2$-$2H_2O$, 1.0g D-Glucose and 1.0g $NaHCO_3$ per 1 liter MiliQ water. The stomachs were excised by cutting the esophagus as far up as possible and cutting below the lower stomach sphincter separating the stomach from the intestines. The

stomach was emptied before being fastened on a 50 ml burette with surgical thread, as illustrated in Figure 3. The burette was filled with ringer solution and the stomach was filled for 150 seconds with the ringer solution. The volume was noted, and the burette closed before refilling the burette with ringer solution. Thereafter the burette was re-opened, and the stomach was allowed to fill for another 150 seconds.

Figure 3 Stomach volume was measured by filling with ringer solution

2.3.3 Leptin analysis

Analysis of plasma leptin was done using the same ELISA kit as in study #2. The plasma was diluted 1:100 by adding 10 µl of plasma to 40 µl Sample Diluent followed by 15 µl from this solution to 285 µl of Sample Diluent. Enclosed protocol was followed, and concentration from OD was calculated with the same software as for the previous ELISA analysis.

2.4 Statistical analysis

Effects of time after transfer to novel environment on feeding behavior in study #1 were assessed by repeated measure analysis of variance (RM ANOVA). In study #2 effects of feed intake were analyzed by RM ANOVA followed by Mauchly's Test for Sphericity with Greenhouse-Geisser (GG) sphericity correction when assumption for sphericity was not met, and effects of chronic cortisol administration on molecular appetite markers was assessed by two-way ANOVA followed by Tukey HSD post-hoc testing. In study #3 gut volume after exogenous cortisol exposure was analyzed by unpaired t-tests. Outliers were determined using Bonferroni outlier test. Statistical significance level for all tests was $p<0.05$.

In all studies where cortisol was measured, plasma cortisol levels had to be log transformed prior to analysis, to achieve homogenous variance. The log transformation yielded a $p=0.45$ using Levene’s homogeneity test in study #2 and $p=0.99$ for study #3. All data are presented as mean ± standard error of mean (s.e.m.) if nothing else noted.

In studies #2 and #3, specific growth rate (SGR) was calculated using body weight by treatment start (BW_0), body weight by sampling point (BW_1) and the length of treatment period (t, in days), as SGR = $\frac{(BW_1 - BW_0)}{t}$. Condition factor (CF) was calculated as CF_n = $(\frac{BW_n}{FLn^3}) \times 100$, where n is the given time-point (start or end). To determine an individual’s stomach volume in study #3, the volume was calculated as stomach-body weight-ratio = $(\frac{stomach\ volume}{BW_1}) \times 100$, rendering the relative volume.

All analysis and production of graphs were performed using R programming language [76] in RStudio (RStudio Inc., Boston, USA). For detailed description of functions and packages, please refer to appendix section 5.2.

3 Results

3.1 Study 1: Feeding behavior in isolation after transfer to novel environment

To examine feeding behavior during and after adaptation to a stressful situation in rainbow trout, ingested and spitted feed was registered over a period of 39 days after transfer from communal rearing to isolation in a novel environment. An absolute majority of the experimental fish would show complete anorexia for a variable period of time after transfer to rearing in isolation. In the current study, I focus on the fact that after a variable number of days of complete feeding inhibition, fish would begin to express a spitting behavior when attempting to feed, where the fish would try to ingest the food, but spit it back out, sometimes with such force they would recoil backwards. This behavior was characterized by flaring of the gills, opening and closing of the mouth, and a form of twitching of the body in a motion reminiscent of coughing in humans. In an intermediate period before the fish successfully ingested all of its daily ration, the fish would often spit the pellet out and try to ingest it again. This could go on for several minutes. In combination with the initial preliminary observation that cortisol treated fish had pellets stuck in their throat, this indicates an anatomical obstruction of the upper GI tract in stressed fish.

Although the spitting behavior was notably reduced the last 10 days of the study, it did not disappear completely. This was due to the fact that some fish became full before the feeding endpoint was met, and to make sure that the fish actually were full it was offered more pellets until it had spat 5 consecutive times. Figure 4 shows the detailed time course of the behavioral transition described above. Apart from the expected increase in food consumption after transfer, it was apparent that spitting was more extensive directly after the first feeding attempts, whereafter this behavior decreased significantly. Figure 4A shows that most of the fish were passive during feeding the first few days, however after approximately 14 days 100% of the fish reacted to feeding. A highly significant increase in amount of feed ingested was detected with time after isolation (Repeated measure ANOVA; $F_{(38,779)} = 46.03$, $p<0.001$) depicted in Figure 4B. Regarding amounts of feed spitted the effect of time in isolation was also highly significant (Repeated measure ANOVA; $F_{(38,779)} = 4.39$, $p<0.001$), as seen in

Figure 4C. Spitting behavior in 4C closely overlap with the number of fish reacting to feeding in the first days (4A).

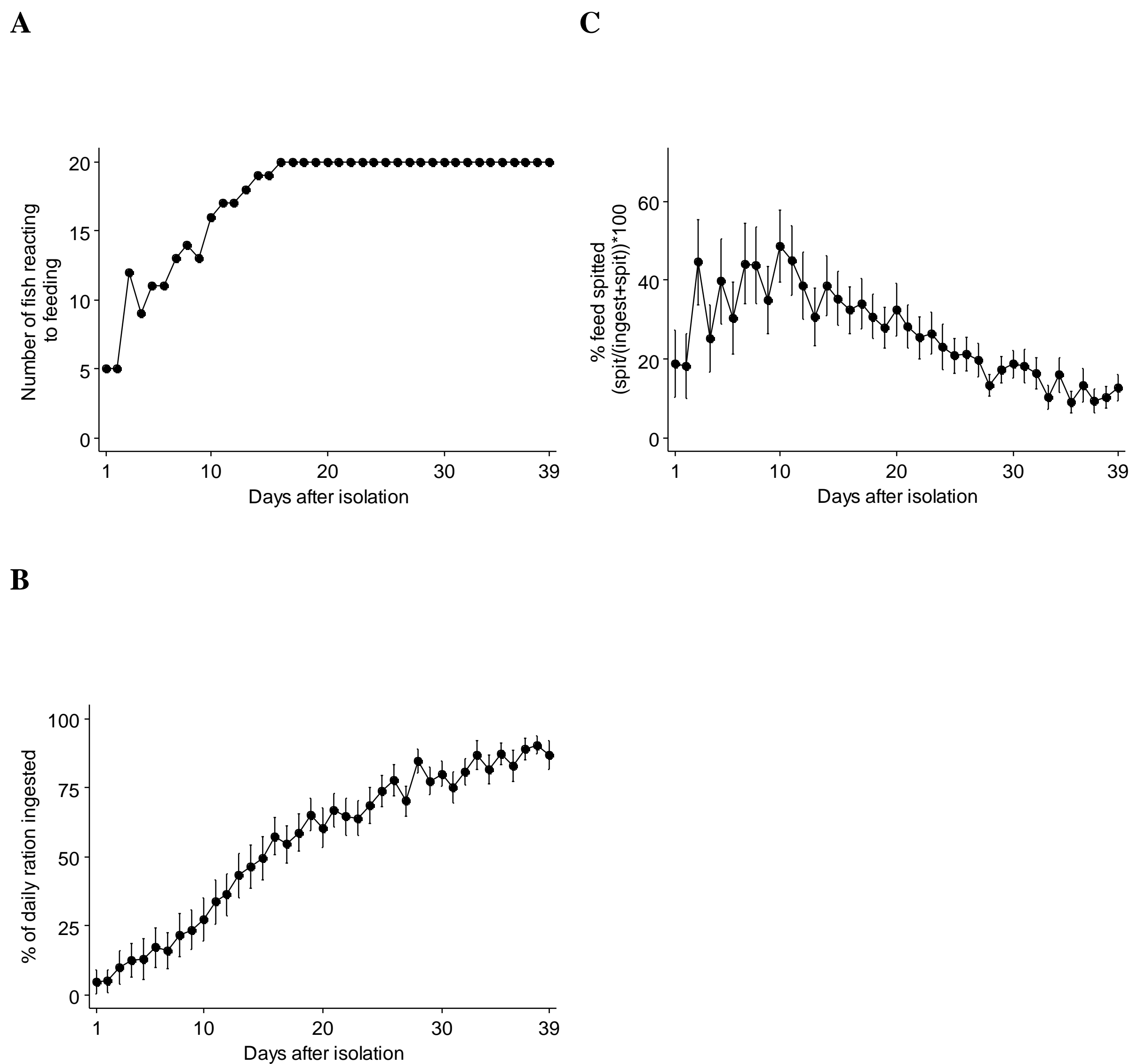

Figure 4 Over time more fish would attempt to eat, ingest more feed and spit less
The number of fish reacting to feeding increased with time after transfer to rearing in isolation (**A**). Feed intake increased as more of the fish reacted and successfully ingested feed (**B**). Spitting behavior (as number of pellets spitted out of all pellets reacted upon, in percent) reached a peak after 10 days before declining (**C**). N = 20. Data in **A** presented as number of fish reacting to feed for any given day and as mean ± s.e.m in **B** and **C**.

3.2 Study 2: Effect on chronic cortisol administration on molecular appetite markers

To examine whether cortisol influenced known appetite markers, cortisol was given in the feed for 2 or 7 days, while control groups received untreated feed in the same period. This approach aimed at providing information not only about the effect of cortisol treatment, but also about changes over time.

3.2.1 Feed intake and growth in respect to cortisol treatment

In the following sections, the data is divided into groups depending on duration of cortisol treatment (i.e. 2 days and 7 days). For feed intake two repeated measure ANOVAs (RM ANOVA) were performed using the feed intake for a given day as response, and treatment and day as factors (Figure 5). This subdivision into two separate analysis was made since 50% of the fish were sampled after 2 days of treatment (see final n in Figure 5 caption). Due to a logistical issue, some feeding data for day 7 were missing, resulting in this day being omitted from the analysis, it is however depicted in the graph. The RM ANOVA for feed intake in the first 12 days (excluding day 7) revealed a significant effect of time (RM ANOVA; $F_{(10,329)} = 27.61$, $p<0.001$), with GG correction for sphericity which provided a p-value of <0.001. There was no significant effect of later treatment grouping (RM ANOVA; $F_{(1,329)} = 0.62$, $p=0.43$) nor any interaction of grouping and days (RM ANOVA; $F_{(10,329)} = 0.43$, $p=0.93$). Feed intake for the last 5 days revealed significant effects of treatment (RM ANOVA; $F_{(1,79)} = 10.46$, $p<0.01$) and days (RM ANOVA; $F_{(4,79)} = 2.83$, $p<0.05$), however when correcting for sphericity the adjusted p-value for time was $p=0.07$. In addition, no interactions were found (RM ANOVA; $F_{(4,79)} = 1.73$, $p=0.15$). From visual observation, reduced feed intake in cortisol treated fish appeared to result, at least in part, from the same spitting behavior as noted in study #1. This spitting behavior was, however, not assessed in this study.

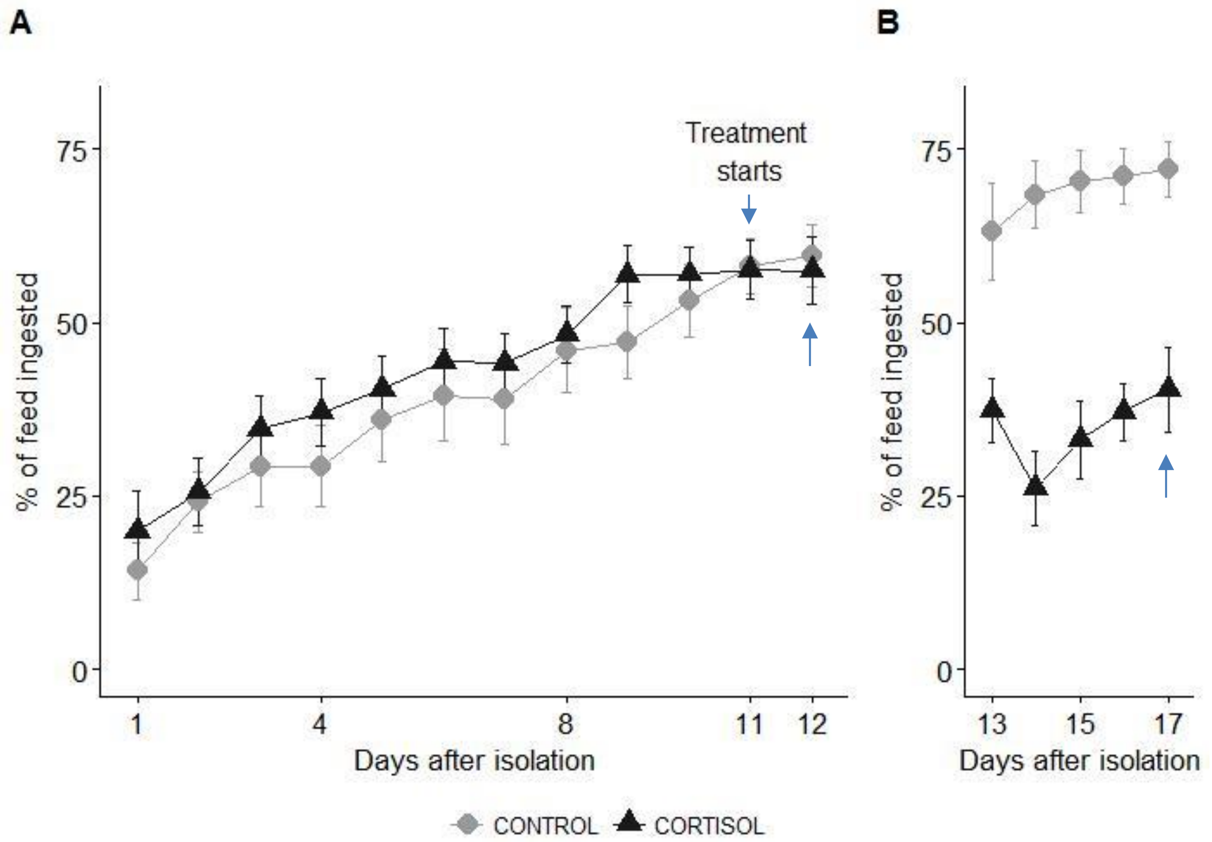

Figure 5 Cortisol treatment reduced feed intake in fish fed cortisol for 7 days
Percent of daily ration eaten per day, with n=14 in control and n=16 in cortisol group in **A**, and n=7 in control and n=8 in cortisol group in **B**. Upward pointing arrows indicate sampling time points of 7 control and 8 cortisol treated fish in both A and B. The sampling was subsequent to feeding on the indicated days. Data presented as mean ± s.e.m.

The cortisol data was compared by two-way ANOVA with log transformed cortisol levels as response variable, and treatment and time as factors where the analysis showed a significant effect of treatment (ANOVA; $F_{(1,29)} = 79.19$, $p<0.001$) and interaction of treatment and time (ANOVA; $F_{(1,29)} = 5.71$, $p<0.05$). A Tukey HSD test revealed that plasma cortisol was significantly different between cortisol treated fish compared to controls following both 2 (Tukey; $p<0.001$) and 7 (Tukey; $p<0.001$) days of treatment (Figure 6A). There was no significant effect of treatment (ANOVA; $F_{(1,29)} = 1.17$, $p=0.28$), treatment period (ANOVA; $F_{(1,29)} = 0.14$, $p=0.72$) nor any interaction effect (ANOVA; $F_{(1,29)} = 1.03$, $p=0.32$) on SGR, Figure 6B.

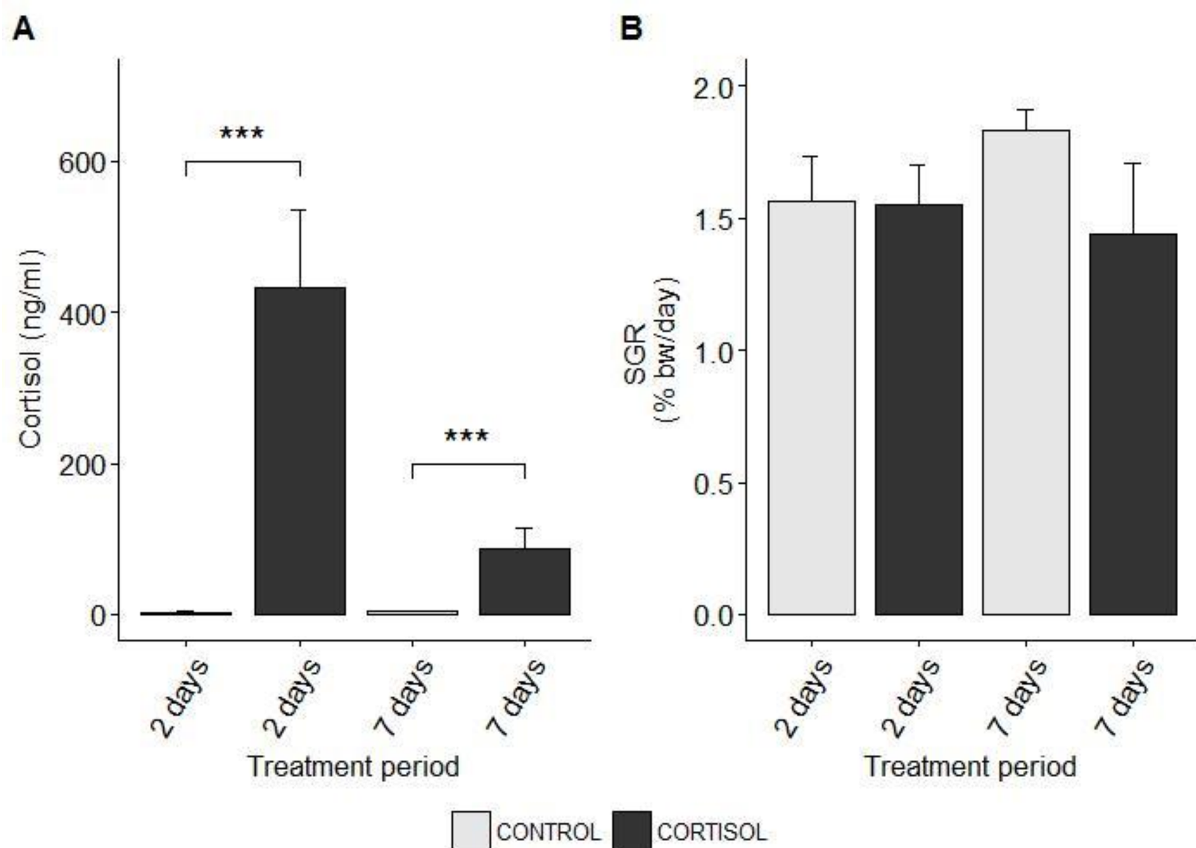

Figure 6 Cortisol treatment increased plasma cortisol levels but had no significant effect on specific growth rate (SGR)

Cortisol levels were higher in the cortisol treated fish compared to control groups (**A**). Specific growth rate (percent growth per day) did not change significantly during the treatment period (**B**). All control groups n=7 and all cortisol groups n=8. Data presented as mean ± s.e.m. Tukey HSD post-hoc test: *** = p<0.001 versus treatment control

3.2.2 Signaling peptides in plasma

Protein levels of two different appetite markers were analyzed in plasma of the fish treated with cortisol for 2 and 7 days. Two-way ANOVA revealed significant effects of treatment (ANOVA; $F_{(1,29)} = 5.25$, $p<0.05$), borderline significance of treatment period (ANOVA; $F_{(1,29)} = 4.08$, $p=0.05$) but no significant interactions (ANOVA; $F_{(1,29)} = 2.15$, $p=0.15$) on plasma leptin levels (Figure 7A). Plasma vasotocin (Figure 7B) showed no significant change in treatment nor interaction (ANOVA; $F_{(1,29)} = 1.75$, $p=0.20$ for treatment and $F_{(1,29)} = 0.62$, $p=0.44$ for interaction), however there was a significant effect of treatment period (ANOVA; $F_{(1,29)} = 5.10$, $p<0.05$).

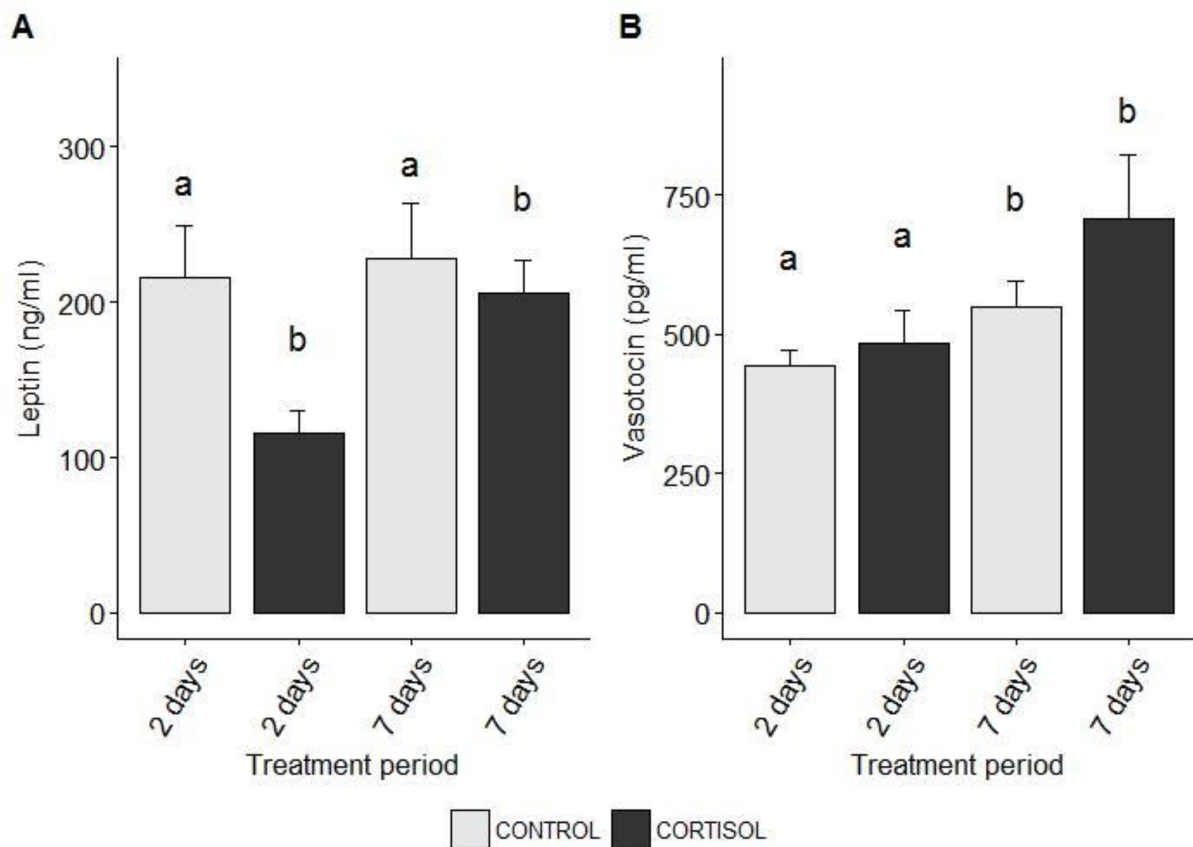

Figure 7 Cortisol treatment significantly affected levels of leptin, while treatment period affected levels of vasotocin
Plasma leptin was significantly lowered of cortisol treatment compared to the control groups, $p<0.05$ (**A**). Plasma vasotocin significantly increased over time, $p<0.05$ (**B**). Bars for a given parameter that do not share a common letter are significantly different from one another. All control groups n=7 and all cortisol groups n=8. Data presented as mean ± s.e.m.

3.2.3 Effects of cortisol treatment on mRNA levels of hypothalamic appetite markers

mRNA levels of three appetite markers were measured in the hypothalamus of controls and fish treated with cortisol for 2 and 7 days. There was a significant effect of cortisol treatment, but not of treatment period on expression of CRF (ANOVA; $F_{(1,29)} = 4.45$, $p<0.05$). There was no significant interaction effect between cortisol treatment and treatment period, indicating that the effect of cortisol did not depend on treatment duration ($F_{(1,29)} = 0.64$, $p=0.43$ for period and $F_{(1,29)} = 0.06$, $p=0.81$ for interaction) (Figure 8A). No significant effects of cortisol treatment were found for NPY mRNA expression ($F_{(1,29)} = 1.36$, $p=0.25$ for treatment, $F_{(1,29)} = 0.68$, $p=0.42$ for treatment period, $F_{(1,29)} = 0.56$, $p=0.46$ for interaction) (Figure 8B). No significant effect of treatment was found for CART mRNA expression ($F_{(1,29)} = 3.92$, $p=0.06$). Nor was there was a significant effect of treatment period or interaction ($F_{(1,29)} = 1.10$, $p=0.30$ and $F_{(1,29)} = 0.05$, $p=0.82$ for period and interaction, respectively) (Figure 8C).

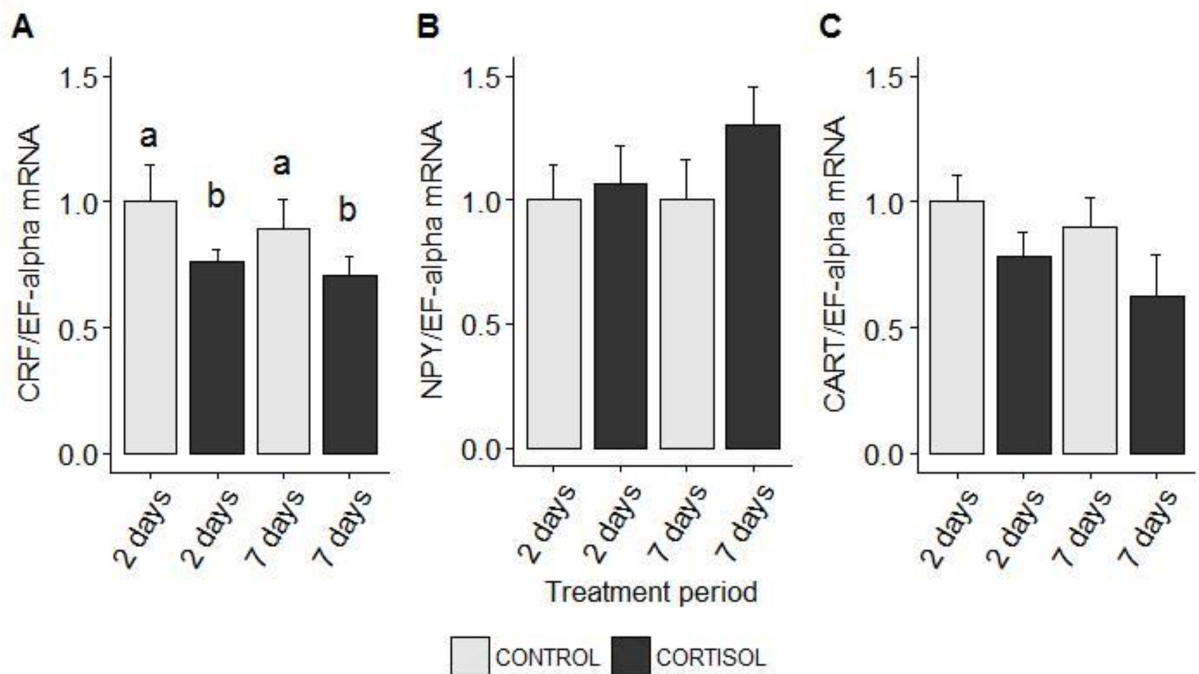

Figure 8 Cortisol treatment significantly decreased expression of corticotropin-releasing factor (CRF) Corticotropin-releasing factor (CRF) was significantly decrease after cortisol treatment, $p<0.05$ (**A**). Neither neuropeptide Y (NPY) (**B**) nor cocaine- and amphetamine-regulated transcript (CART) (**C**) expression was significantly altered by cortisol treatment. Bars for a given parameter that do not share a common letter are significantly different from one another. All control groups n=7 and all cortisol groups n=8. Data presented as mean ± s.e.m.

3.3 Study 3: Gut volume after exogenous cortisol exposure

To examine if cortisol affected the attributes of the upper GI of fish, cortisol was given as an intraperitoneal (IP) injected implant, and feed intake noted over a period of 7 days followed by measurement of stomach volumes post mortem. The fish were sampled when the cortisol treated group started to struggle to ingest feed i.e. showing spitting behavior, but before they ingested less feed than the control fish.

3.3.1 Feed intake during acclimation and treatment

Feed intake for each fish per day was noted as percent of daily ration ingested, where 100% would entail 0.8% of the specific individual's body weight (Figure 9A). There were no significant differences between the control group and the cortisol group in feed intake during the 7 day treatment period (T-test; $t_{(22)} = 0.26$, p=0.80) (Figure 9B).

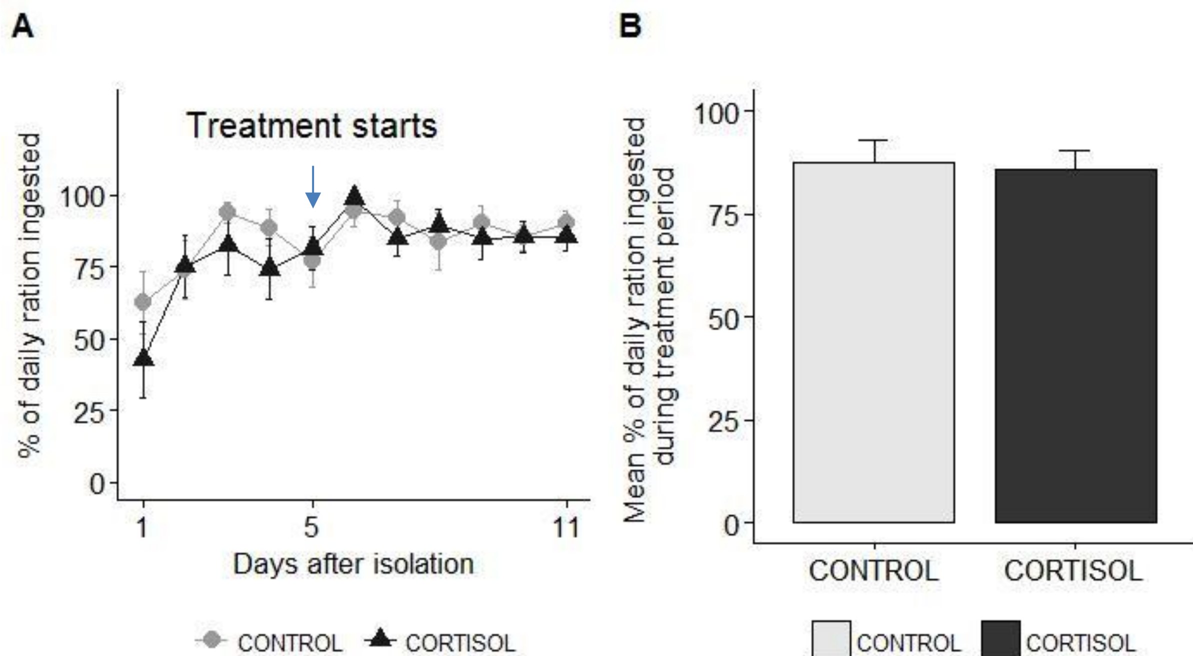

Figure 9 Cortisol treatment had no significant effect on feed intake
Percent of daily ration ingested per day (**A**), and mean percent feed ingested per day during treatment period (**B**). Control group (n=12), cortisol group (n=12). Data presented as mean ±s.e.m.

3.3.2 Cortisol and spitting behavior

To confirm that the plasma cortisol levels were increased by the cortisol implants, cortisol levels were analyzed by T-test, which revealed significantly higher levels of plasma cortisol in the cortisol group (T-test; $t_{(14)} = -4.08$, $p<0.01$) (Figure 10A). Spitting behavior was also noted in this experiment, as percent of daily ration spitted during the 7 day treatment period as seen in Figure 10B. There were no significant differences between treatment groups in spitting behavior during treatment period (T-test; $t_{(22)} = -0.98$, $p=0.34$).

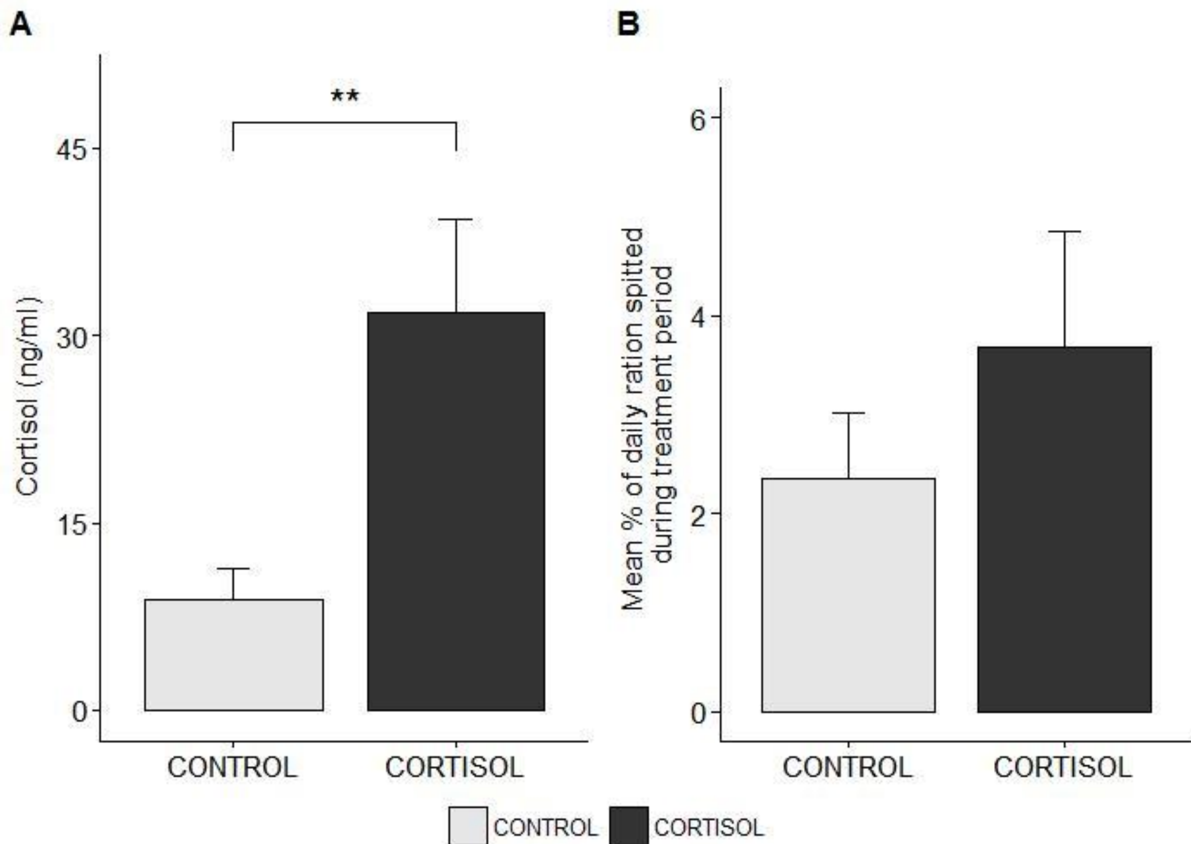

Figure 10 Cortisol treatment increased plasma cortisol levels, but had no significant effect on spitting behavior
Cortisol treated fish had significantly higher levels of plasma cortisol compared to the treatment control, p<0.01 (**A**). Spitting behavior showed as mean percent of daily ration spitted during treatment period (**B**). Control group (n=8), cortisol group (n=8) in **A**, and control group (n=12), cortisol group (n=12) in **B**. Data presented as mean ±s.e.m. T-test: ** = p<0.01

3.3.3 Growth and plasma leptin

Specific growth rate (SGR, $\frac{(BW_1 - BW_0)}{t}$) was significantly reduced by the cortisol treatment (T-test; $t_{(22)} = 4.02$, $p<0.001$) as showed in Figure 11A. The change in condition factor (CF_1 - CF_0) was also significantly lowered by the cortisol treatment (T-test; $t_{(22)} = 2.35$, $p<0.05$) (Figure 11B). There were no significant differences in plasma leptin between the treatment groups (T-test; $t_{(14)} = -0.17$, $p=0.87$) (Figure 11C).

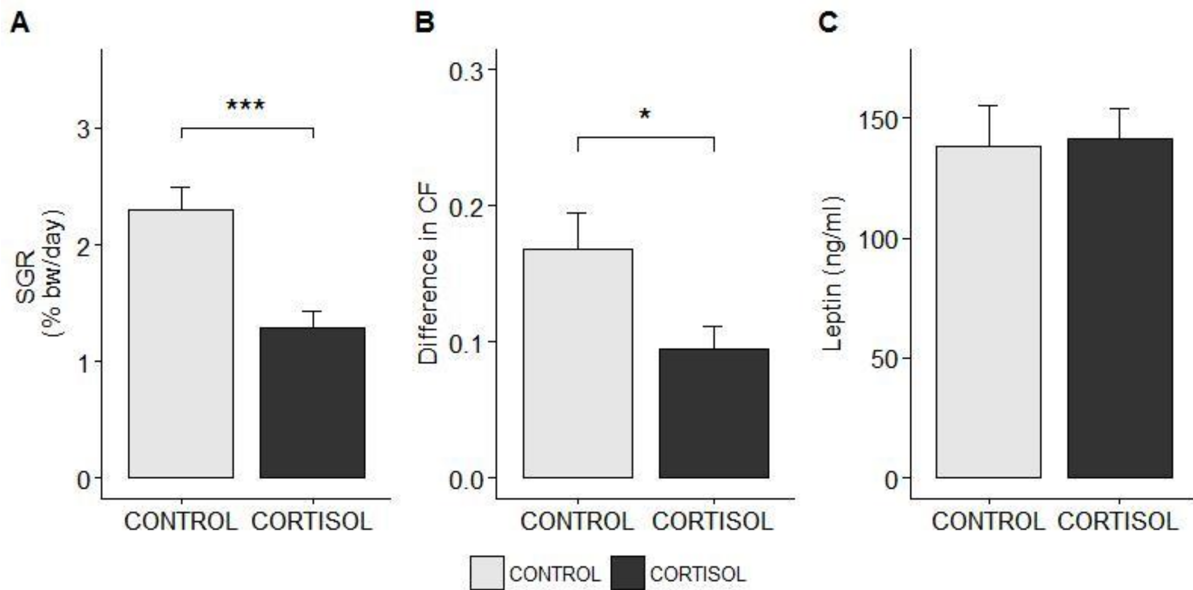

Figure 11 Growth rate was significantly reduced by 7 days of exogenous treatment
There was a significant reduction in specific growth rate (SGR) in cortisol treatment group, p<0.001 (**A**). There was also a significance in difference in condition factor (CF) between start of treatment and end of treatment, p<0.05 (**B**). Plasma leptin levels were not altered by treatment (**C**). Control group (n=12) and cortisol group (n=12) for graph **A** and **B**, and control group (n=8) and cortisol group (n=8) for graph **C**. Data presented as mean ±s.e.m. T-test: * = p<0.05, *** = p<0.001

3.3.4 Stomach volume

To establish the relationship between stomach volume and body weight, a linear model was formed, using body weight as predictor and stomach volume as response. There was a significant correlation (Pearson's; $r^2 = 0.41$, p=0.001) between body weight and stomach volume. While the control group retained its significance when the correlation was separated into groups (Pearson's; $r^2 = 0.47$, $p<0.05$), the significance disappeared from the cortisol group (Pearson's; $r^2 = 0.29$, p=0.09) (Figure 12A).

To investigate whether cortisol affected the stomach volume, relative stomach volumes were calculated as $(\frac{stomach\ volume}{BW_1}) \times 100$. Stomach volumes were significantly reduced by the cortisol treatment (T-test; $t_{(20)} = 2.14$, $p<0.05$), but note that statistical significance was contingent on the removal of one outlier from the cortisol treated group (Bonferroni outlier test; adjusted $p<0.05$) (Figure 12B).

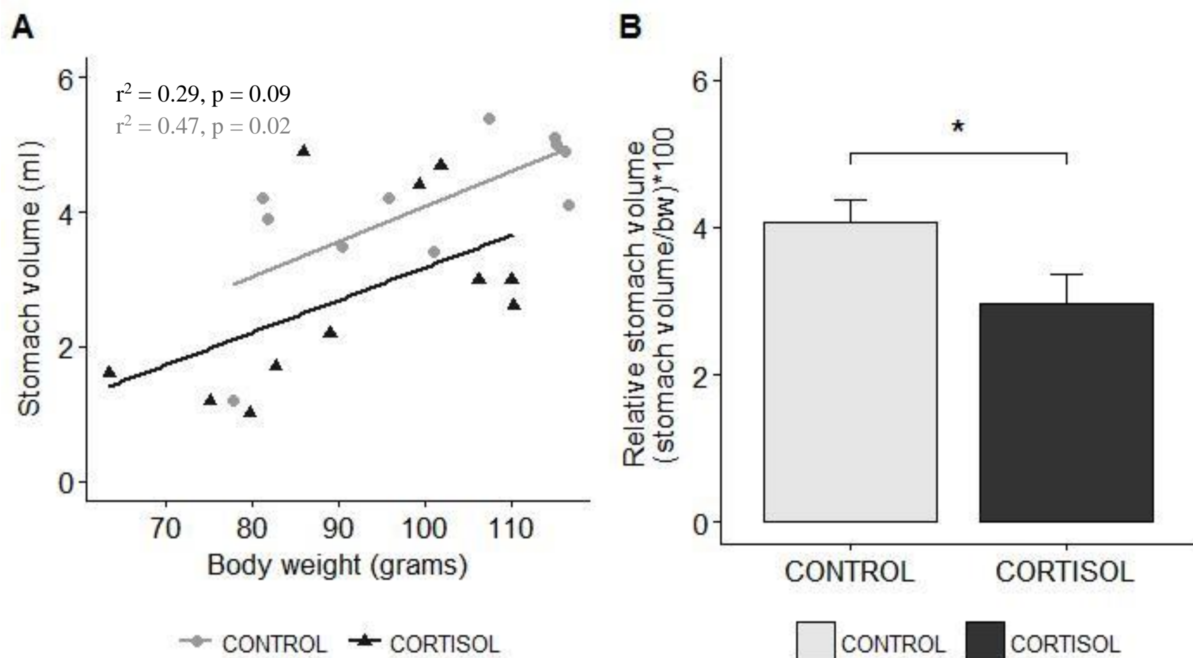

Figure 12 Stomach volume was significantly lower in the cortisol treatment group
There was a significant correlation between stomach volume and body weight, Pearson's p=0.001 (not shown, see text), but when separated into groups, the correlation was no longer significant for the cortisol group (Pearson's p=0.09) (**A).** Stomach volume was significantly lower in the cortisol treated group (**B**). Control group (n=11) and cortisol group (n=11). r^2 signifies the Pearson correlation coefficient and p signifies the significance level. Data presented as one dot/triangle per individual with regression line in **A**, and as mean ± s.e.m in **B**. T-test: * = p<0.05

4 Discussion

In this study I sought to test the hypothesis that stressed salmonid fish will attempt to feed but not be able to successfully ingest food and that this behavior is transient. I also wanted to investigate if appetite markers were affected by acute cortisol treatment. Additionally, I aspired to investigate if cortisol implantation affected the physiology of the upper GI tract by measuring stomach volume.

To study my hypothesis, I examined feed intake in response to transfer to a novel environment, measured how protein and mRNA expression levels of selected neuroendocrine appetite markers were affected by cortisol treatment, and how stomach volumes changed in response to cortisol implantation. In addition to the expected general increase in feed intake during acclimation to a novel environment, a primary observation is a high incidence of attempted feeding subsequent to transfer, a behavior that decreased over time. This transition in behavior from complete anorexia via a period of attempted, but unsuccessful feed intake, to normal feed intake suggests that stress inhibits the desire to eat only temporarily, while there is an extended period when fish indeed has appetite but are physically unable to ingest food.

4.1 Feeding behavior can be separated into three phases

I will in the following paragraphs elaborate in more detail on the somewhat peculiar phenomenon observed in the long-term behavioral study (study #1), that fishes go through a phase of actively taking available feed pellets into the mouth cavity, but apparently without being physically able to swallow. It would appear that feeding behavior can be divided into three phases: *Passive/anorexic*: where the fish is completely inactive and does not respond to feeding in any manner. Feed can land on its nose, yet the fish remains immobile. *Active but unable*: where the fish actively seeks the feed, meaning the fish look at it, swim towards it and engulf it. However, the food is spitted/coughed back out. *Active and able*: where the fish actively seek the feed and successfully ingest it without spitting. Spitting behavior is most prominent subsequent to transfer or when exposed to high enough exogenous cortisol levels. This finding suggests that stressed fish has an appetite, but there is a temporary impediment somewhere along the esophagus and/or upper digestive tract hindering the fish to ingest feed in the period where the stress response is at the descending arm of the peak. Only a very

limited number of fish (4 of 20) skip the passive/anorectic phase and go straight to the active but unable phase (1 of 20 fish even went straight to ingesting 94% of daily ration on the first day of feeding), as seen in Figure 3A and C. What is revealed from these studies, are that the individual differences are enormous. The fish that feed first exhibits a proactive coping mechanism to stress, whereas those that starts later display a reactive coping mechanism [77]. This is the most likely explanation as to why some fish eat the day after transfer while others might wait as long as a month (or longer) to eat the first pellet.

The effect of stress on appetite and feed intake has generally been studied in grouped fish, rather than in isolation. This method is adequate when studying stress response in relation to or together with social hierarchy, but unsuitable when social rank is not one of the study parameters. Additionally, it is challenging to assess the feeding behavior for the individual fish, and may result in an extrapolated conclusion that the fish has lost their appetite when more feed is left uneaten after feeding. However, when fish are isolated, the stress effects resulting from subordination is abolished, and feeding behavior can be examined more closely and correctly.

4.2 None of the studied appetite markers indicated inhibition of appetite

Fish fed with cortisol showed significantly decreased CRF mRNA expression. This may be a result of the negative feedback loop excised of cortisol on the hypothalamus, but nevertheless given the known anorectic effects of CRF the observation is not in line with an appetite suppressive effect of cortisol. However, other studies have found CRF transcription highly upregulated, e.g. in response to high stocking density, expression of CRF mRNA increased to levels 4 times higher than control after 6 hours [66], while subjection to cortisol treatment by osmotic pumps increased expression approximately 2.5 times control levels after 14 days [40]. Whether it is due to the methods of achieved/administered cortisol, the doses, or even the duration of the experiments, the reasons for the marked reduction is yet to be unraveled.

There was no significant alteration in the other two studied hypothalamic appetite markers, NPY and CART. This finding is somewhat contradictory to previous studies where gene expression of NPY and CART was significantly altered as immediate as 6 hours and also after 14 and 28 days following subjection to stress or cortisol treatment [40, 66]. NPY mRNA

expression in both the mentioned studies was significantly increased, although in this study, NPY mRNA was barely elevated (p=0.25). This discrepancy could indicate that the time frames of this current study lie somewhere in-between the other two resulting in a decrease both before and after an increase, or that the triggering of the stress response was performed differently, i.e. osmotic cortisol pump and high stocking density versus cortisol lased feed. CART mRNA was also significantly lowered in response to high stocking density for 6 hours [66], however not significantly decreased in response to cortisol delivered by osmotic pumps for 14 days [40], which could indicate that the effect on CART is mainly acute and is approximately rescued after 7 days, as shown here (Figure 8C). However, CART is a satiety hormone [59], and decreased levels should therefore not be associated with decreased feed intake. These findings indicate that CART is not a possible culprit in relation to the observed reduction in feed intake. NPY however, is an orexigen, meaning increased levels should result in increased feed intake. Yet, this is not the case when fish were treated with cortisol for 14 days by osmotic pumps. Even though NPY mRNA was significantly increased, the fish ate significantly less [40]. This, together with slightly elevated NPY levels in this current study (Figure 8B), indicates that the orexigenic effects of NPY is inhibited by other anorexigenic mechanisms.

Moreover, a decrease in plasma leptin was observed in fish receiving exogenous cortisol through the feed. Treatment but not time appeared as a significant factor, however one should not entirely ignore that leptin levels were notably lower after 2 days compared to 7 days of treatment (Figure 6A). Cortisol has previously been shown to increase the expression of leptin mRNA in hepatocytes of rainbow trout *in vitro* after 24 hours of cortisol treatment [40]. Such an increase was however, not evident in fish treated with cortisol-feed for 2 days. On the contrary, these fishes had the lowest levels of plasma leptin coinciding with prominent levels of plasma cortisol. Leptin was also measured after IP cortisol admission (study #3) where control and cortisol treated fish had similar levels, revealing that acute cortisol treatment did not affect plasma leptin. Furthermore, even though the fish who received IP injection of cortisol ate as much as the control group, the cortisol treated fish had significantly lower growth rates compared to control treated fish, as seen in Figure 10A and B. This implies that the nutrients in the ingested feed was not fully utilized, thus corresponding well with previous findings, that cortisol alters function of the GI tract [78]. However, fish fed cortisol for 7 days did not have significant reduction in growth rates even though these fish ate significantly less than the control fish. This could be a result of different cortisol doses given in the two studies.

Fish who received cortisol intraperitoneally had 31.86 ng/ml ± 6.07 ng/ml plasma cortisol compared to fish who received cortisol through the feed, where levels were 86.79 ng/ml ± 28.18 ng/ml (data as mean ± s.e.m). This difference could be due to the fact that cortisol levels raised much quicker in fish fed cortisol as it was dependent on how much feed the fish ate. Thus, by day 7 of treatment with cortisol-feed the fish had already experienced cortisol levels in accordance with what is seen for 2 days of treatment in Figure 5A. Hence, the reduction in growth rates due to cortisol treatment could be a result of more steady increase in the IP injected study versus the sharp peak in the cortisol fed study.

4.3 Reduction of stomach volumes

I also found that fish with cortisol implants had smaller stomach volumes compared to controls ($p<0.05$). This finding could very well be a possible explanation to as why stressed aquaculture fish suffer a reduction in feed intake, and why migrating salmonids are found without food in their digestive system. Fasting is known to reduce stomach length and mass [79], but in this case, the duration of the experiment in combination with a relatively moderate dose of exogenous cortisol, was specifically intended to end before any reduction in feed intake commenced. A decrease of stomach volume occurring due to stress or stress hormones has not previously been documented in fishes that still take food. Indeed, stress is known to alter the function of the GI tract in both mammals and fish [15, 36-38, 80], and has also been reported to affect the upper esophageal sphincter (UES) tone in humans [81]. The UES in fish, however, is mainly known to be involved in the process of drinking while in seawater, while swallowing reflexes in response to food, appears not to be extensively studied. The swallowing reflex in teleost fishes is at least partially under the control of the glossopharyngeal-vagal motor complex (GVC) located in the medulla oblongata, where the vagal X_1 branch from GVC constricts the UES through acetylcholine neurotransmitters to the muscular end- plate. Thus, UES striated muscles are constricted directly by activation from vagal stimuli through nicotinic acetylcholine receptors (nAChR) [82]. The activity of the GVC neurons is under control of the area posterma in the medulla oblongata and is stimulated and inhibited by angiotensin II and atrial natriuretic peptide, respectively. GVC activity can also be inhibited by adrenaline, noradrenaline and dopamine in a dose-dependent manner, suggesting the presence of adrenergic and dopamine receptors in the GVC, which when inhibited results in a more relaxed sphincter and thus increased drinking [83].

Although mechanisms regulating constriction of the esophagus or innovation of the swallowing reflex are poorly described in fish, particularly in response to stress, it could be noted that Farrar and Rodnick (2004) found that cortisol doses as low as 36 ng/ml in males and 3.6 ng/ml in females induced more forceful contractions in rainbow trout heart strips, while blocking the GR with the GR antagonist mifepristone abolished this effect [84]. The fact that cortisol has this contractile effect in the striated muscles of the heart, could imply that something similar might happen in the striated muscles in the esophagus/UES and the smooth muscles of the gut. When plasma cortisol levels as low as 30 ng/ml increases contractility in the heart, it is not entirely unlikely that cortisol levels 20 times that influence the muscles in the gut. Given that cortisol levels are highly elevated in migrating salmonids [24, 25] as well as in stressed aquaculture fish [7-14] such an effect of cortisol could possibly contribute to reduced feed intake under both these circumstances.

4.4 Methodical considerations

Ideally, all of these three studies should have been merged to one study. In that respect that feed intake and feeding behavior after transfer to novel environment would be noted followed by cortisol treatment when feed intake had normalized. Thereafter sampling of the fish when the cortisol treated group would express spitting behavior where appetite markers in plasma and hypothalamus were measured and volume of the stomach assessed. However, due to the time frame of this project, it could simply not be done. As mentioned in section 2.2 (materials and methods for study #2), the attempt to achieve exogenous cortisol levels by IP injection failed, and the time limit prohibited another pursuit.

In addition, one cannot exclude the possibility that protein degradation in plasma could have occurred after four years in a -20 °C freezer. Hypothalamus RNA integrity seemed well enough (Table 2B), but no integrity testing was done for plasma.

One could argue that the observed spitting behavior seen after a few days of ingesting cortisol treated feed is due to different taste of the pellets compared to untreated feed. This argument however, is flawed. The same behavior is evident after transfer to novel environment and after IP injection of cortisol, thereby concluding that spitting behavior is directly connected to the stress response.

Moreover, isolation of experimental fish such as rainbow trout can act as a stressor, seeing as these fish are social animals and form hierarchies. However, isolation is unavoidable in this case due to the difficulties of observing individual feeding behavior when in communal rearing tanks.

Another key thing to remember, is that MS-222 is known to increase cortisol levels, and may aggravate the stress response when used as a tranquilizer, however, studies in salmon has shown that cortisol release peaks 30 minutes after exposure followed by a drop to control levels four hours later [85], implying no long term effects.

4.5 Future perspectives

To further assess the impact on the upper esophageal sphincter and gut physiology, a histological examination could be useful. Furthermore, one could prepare stomach and esophagus strips and follow the protocol from Farrar and Rodnick (2004) [84] to investigate if the phenomenon is mediated through the same, or similar, mechanisms. One could also explore if serotonin plays a crucial role, as this molecule is a known regulator of the HPI axis in fish and abundant in the gastrointestinal tract [86, 87]. Another possible experiment could be to evaluate spitting behavior after blocking the receptors for glucocorticoids (intracellular and/or membrane bound), catecholamines, nicotinic acetylcholine, atrial natriuretic peptide, all together, in different combinations or separately. One could also examine if the inability to ingest feed is mediated trough nitric oxide and other non-adrenergic non-cholinergic neurotransmitters in the gut. One could also investigate the effects of catecholamines and hypothalamic-sympathetic-chromaffin cell axis, as the activation of the HPI axis increases both the storage and release of catecholamines during a stress response [88]. Further, one could try to determine where along the pathway from the brain to the muscle cells in the esophagus the inability to ingest food originates. Moreover, one could in addition investigate if some of the known enteric neuropeptides, like calcitonin gene related peptide, NPY, tyrosine hydroxylase, substance P, vasoactive intestinal polypeptide and galanin, plays a role in constricting UES during stress in salmonids [89, 90]. As this finding was unscrutinized until now, much is left to explore about this phenomenon.

4.6 Summary and conclusion

These studies revealed that rainbow trout will exhibit a spitting behavior in an intermediate period after transfer to novel environment, between the passive/anorectic stage and actively feeding. I also found that none of the investigated appetite markers indicated reduced appetite as a cause to the reduced feed intake. Furthermore, I found that fish implanted with cortisol had smaller stomachs even though there was no reduction in feed intake. Therefore, if cortisol and stress truly reduced appetite, one would expect that attempts to feed would decrease in stressed fish. One would also expect an expression profile of appetite markers indicative of reduced appetite. On the contrary, I found small or no effects of cortisol on the levels of appetite markers. This supports our hypothesis that feed intake is not reduced due to a lack of appetite, but rather the physical ability to ingest feed. This conclusion applies to the period of post-stress acclimation when active feeding attempts can be seen, but engulfed feed items are regurgitated instead of consumed.

The question of why salmonids has evolved such a mechanism, remains unanswered. It could be a way of conserving energy during times of migration, seeing as keeping a digestive system functioning while there is little to no food available is unfavorable. Simultaneously, it could be due to the fact that there is high activity in the sympathetic nervous system during acute stress, and having food in the GI tract could be adverse when not digested properly.

From these findings, I further hypothesize that cortisol might induce a contraction of the muscles in the GI tract walls, alternatively generate changes making the passages narrower. A reduced stomach volume might imply a direct effect of cortisol on the GI tract walls, however this must be investigated further. Revealing this mechanism could have huge impacts on the aquaculture industry in relation to moderating the reduced growth in anorectic fish and to minimizing leaked nutrients from uneaten and undigested feed [91].

5 Appendices

5.1 List of abbreviations

ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
AVT	Arginine vasotocin
bp	Base pairs
BW	Body weight
CART	Cocaine- and amphetamine-regulated transcript
cDNA	Complementary DNA
CF	Condition factor
CRF	Corticotropin-releasing factor
CRFR	Corticotropin-releasing factor receptor
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DR	Dissociation reagent
EF 1-α	Elongation factor 1-α
ELISA	Enzyme linked immunosorbent assay
FL	Fork length
GG	Greenhouse-Geisser
GI	Gastrointestinal
GR	Glucocorticoid receptor
HPA	Hypothalamus-pituitary-adrenal
HPI	Hypothalamus-pituitary-interrenal
ICV	Intracerebroventricular
IP	Intraperitoneal
JAK	Janus kinase
mRNA	Messenger RNA
NPY	Neuropeptide Y
OD	Optical density
PC	Prohormone convertase
PCR	Polymerase chain reaction

PI3K	Phosphatidylinositol-3-kinase
POMC	Proopiomelanocortin
qPCR	Quantitative polymerase chain reaction
RIN	RNA integrity number
RM ANOVA	Repeated measure ANOVA
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Room temperature
SD	Standard deviation
s.e.m	Standard error of mean
SGR	Specific growth rate
STAT	Signal transducers and activators of transcription

5.2 Functions and packages in R

The functions and packages used to compute the statistics and produce the graphs for this thesis included the functions “aov”, “anova”, “cor.test”, “cooks.distance”, “lm”, “t.test” in the R stats package, the functions “annotate”, “geom_vline”, “scale_fill_grey”, “scale_y_discrete”, “scale_x_discrete”, and “theme” in the ggplot2 package [92], the function “geom_signif” in the ggsignif package [93], the functions “ggarrange”, “ggbarplot”, “ggdotplot”, “ggline”, “ggpaired”, “ggpar”, “ggscatter”, “stat_compare_means”, “stat_cor” and “theme_pubr” in the ggpubr package [94], the function “dunn.test” in the dunn.test package [95], the function “outlierTest” in the car package [96], the functions “as.data.table”, “melt” and “merge” in the data.table package [97], the function “homog.test” in onewaytests package [98], the function ezANOVA in the ez package [99], and the function “%>%” in the magrittr package [100].

5.3 Supplementary tables

Number of fish	20
Body weight start (grams)	217.8 ± 12.65
Body weight end (grams)	307.8 ± 17.83

Table 5 Physiological information about the fish in study #1
Data presented as mean ± s.e.m.

	CONT2	CORT2	CONT7	CORT7
Number of fish	7	8	7	8
Mean feed intake in treatment period (% of daily ration)	55.82 ± 5.53	64.37 ± 6.79	67.00 ± 4.60	39.35 ± 3.89
Body weight treatment start (grams)	157.36 ± 9.66	168.44 ± 10.23	165.29 ± 9.16	155.38 ± 7.63
Body weight end (grams)	176.07 ± 10.16	187 ± 11.1	196.43 ± 9.11	179.81 ± 8.53
Plasma cortisol (ng/ml)	2.46 ± 1.32	433.29 ± 101.51	4.03 ± 1.28	86.79 ± 28.18
Leptin (ng/ml)	215.49 ± 33.58	115.5 ± 14.84	227.45 ± 35.41	205.51 ± 21.15
Vasotocin (pg/ml)	442.97 ± 29.14	483.19 ± 61.45	549.03 ± 46.24	707.44 ± 116.18

Table 6 Physiological information about the fish in study #2
CONT2 = control treatment 2 days, CORT2 = cortisol treatment 2 days, CONT7 = control treatment 7 days and CORT7 = cortisol treatment 7 days. Data presented as mean ± s.e.m.

	Control	**Cortisol**
Number of fish	12 (8)	12 (8)
Mean feed intake in treatment period	87.50 ± 5.41	85.68 ± 4.57
Body weight treatment start (gram)	82.27 ± 3.77	82.97 ± 3.53
Body weight end (grams)	98.33 ± 4.48	92.01 ± 4.33
Specific growth rate	2.29 ± 0.20	1.29 ± 0.14
Stomach volume (ml)*	4.08 ± 0.33	2.75 ± 0.42
Plasma cortisol (ng/ml)	(8.79 ± 2.12)	(31.86 ± 6.07)
Plasma leptin (ng/ml)	(137.83 ± 13.92)	(141.41 ± 10.32)

Table 7 Physiological information about the fish in study #3
Parenthesis represent data from 8 individuals in each group. * represents data from 11 individuals from each group. Data presented as mean ± s.e.m.

6 References

1. Epstein, F.H. and J.A. Epstein, *A Perspective on the Value of Aquatic Models in Biomedical Research.* Experimental Biology and Medicine, 2005. **230**(1): p. 1-7.
2. Steenbergen, P.J., M.K. Richardson, and D.L. Champagne, *The Use of the Zebrafish Model in Stress Research.* Progress in Neuro-Psychopharmacology and Biological Psychiatry, 2011. **35**(6): p. 1432-1451.
3. Thorgaard, G.H., et al., *Status and Opportunities for Genomics Research with Rainbow Trout.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2002. **133**(4): p. 609-646.
4. Sørensen, C., I.B. Johansen, and Ø. Øverli, *Neural Plasticity and Stress Coping in Teleost Fishes.* General and Comparative Endocrinology, 2013. **181**: p. 25-34.
5. Bostock, J., et al., *Aquaculture: Global Status and Trends.* Philosophical Transactions of the Royal Society B: Biological Sciences, 2010. **365**(1554): p. 2897-2912.
6. Merino, G., et al., *Can Marine Fisheries and Aquaculture Meet Fish Demand from a Growing Human Population in a Changing Climate?* Global Environmental Change, 2012. **22**(4): p. 795-806.
7. Pickering, A.D. and A. Stewart, *Acclimation of the Interrenal Tissue of the Brown Trout, Salmo trutta L, to Chronic Crowding Stress.* Journal of Fish Biology, 1984. **24**(6): p. 731-740.
8. Wedemeyer, G.A., *Physiological Response of Juvenile Coho Salmon (Oncorhynchus kisutch) and Rainbow Trout (Salmo gairdneri) to Handling and Crowding Stress in Intensive Fish Culture.* Canadian Journal of Fisheries and Aquatic Sciences, 1976. **33**: p. 2699-2702.
9. Fagerlund, U.H.M., J.R. McBride, and E.T. Stone, *Stress-related Effects of Hatchery Rearing Density on Coho Salmon.* Transactions of the American Fisheries Society, 1981. **110**: p. 644-649.
10. Sørum, U. and B. Damsgard, *Effects of Anaesthetisation and Vaccination on Feed Intake and Growth in Atlantic salmon (Salmo salar L.).* Aquaculture, 2004. **232**(1-4): p. 333-341.
11. Peters, G. and R. Schwarzer, *Changes in Hematopoietic Tissue of Rainbow Trout under Influence of Stress.* Diseases of Aquatic Organisms, 1985. **1**(1): p. 1-10.
12. McCormick, S.D., et al., *Repeated Acute Stress Reduces Growth Rate of Atlantic Salmon Parr and Alters Plasma Levels of Growth Hormone, Insulin-like Growth Factor I and Cortisol.* Aquaculture, 1998. **168**(1-4): p. 221-235.
13. Barton, B.A., *Salmonid Fishes Differ in Their Cortisol and Glucose Responses to Handling and Transport Stress.* North American Journal of Aquaculture, 2000. **62**(1): p. 12-18.
14. Iversen, M., B. Finstad, and K.J. Nilssen, *Recovery from Loading and Transport Stress in Atlantic Salmon (Salmo salar L.) Smolts.* Aquaculture, 1998. **168**(1-4): p. 387-394.
15. Barton, B.A., C.B. Schreck, and L.D. Barton, *Effects of Chronic Cortisol Administration and Daily Acute Stress on Growth, Physiological Conditions, and Stress Responses in Juvenile Rainbow Trout.* Diseases of Aquatic Organisms, 1987. **2**: p. 173-185.

16. Greogry, T.R. and C.M. Wood, *The Effects of Chronic Plasma Cortisol Elevation on the Feeding Behaviour, Growth, Competitive Ability, and Swimming Performance of Juvenile Rainbow Trout.* Physiological and Biochemical Zoology, 1999. **72**(3): p. 286-295.
17. Davis, K.B., et al., *Growth, Body Composition and Hepatic Tyrosine Aminotransferase Activity in Cortisol-fed Channel Catfish, Ictalurus punctatus Rafinesque.* Journal of Fish Biology, 1985. **27**(2): p. 177-184.
18. Merriam-Webster, *Appetite*, in *Merriam-Webster*. 2017.
19. Tataranni, P.A., et al., *Effects of Glucocorticoids on Energy Metabolism and Food Intake in Humans.* American Journal of Physiology - Endocrinology and Metabolism, 1996. **271**(2): p. E317-E325.
20. Björntorp, P. and R. Rosmond, *Obesity and Cortisol.* Nutrition, 2000. **16**(10): p. 924-936.
21. Wendelaar Bonga, S.E., *The Stress Response in Fish.* Physiological Reviews, 1997. **77**(3): p. 591-625.
22. Dittman, A. and T. Quinn, *Homing in Pacific Salmon: Mechanisms and Ecological Basis.* Journal of Experimental Biology, 1996. **199**: p. 83-91.
23. Stabell, O.B., *Homing and Olfaction in Salmonids: A Critical Review with Special Reference to the Atlantic Salmon.* Biological Reviews, 1984. **59**(3): p. 333-388.
24. Carruth, L.L., R.E. Jones, and D.O. Norris, *Cortisol and Pacific Salmon: A New Look at the Role of Stress Hormones in Olfaction and Home-stream Migration.* Integrative & Comparative Biology, 2002. **42**(3): p. 574-581.
25. Carruth, L.L., et al., *Elevation of Plasma Cortisol During the Spawning Migration of Landlocked Kokanee Salmon (Oncorhynchus nerka kennerlyi).* Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 2000. **127**(2): p. 123-131.
26. Høgåsen, H.R., *Physiological Changes Associated with the Diadromous Migration of Salmonids*. Canadian Special Publication of Fisheries and Aquatic Sciences, ed. R.H. Haynes. Vol. 127. 1998: NRC Research Press. 128.
27. Kadri, S., et al., *What Controls the Onset of Anorexia in Maturing Adult Female Atlantic Salmon?* Functional Ecology, 1995. **9**(5): p. 790-797.
28. Calderwood, W.L., *The Life of the Salmon*. 1907, London: Edward Arnold Publishers Ltd.
29. Pérez, M.H.-A., et al., *A Membrane Glucocorticoid Receptor Mediates the Rapid/Non-Genomic Actions of Glucocorticoids in Mammalian Skeletal Muscle Fibres.* The Journal of Physiology, 2013. **591**(20): p. 5171-5185.
30. Espinoza, M.B., et al., *Cortisol Induces Reactive Oxygen Species Through a Membrane Glucocorticoid Receptor in Rainbow Trout Myotubes.* Journal of Cellular Biochemistry, 2017. **118**(4): p. 718-725.
31. Groeneweg, F.L., et al., *Rapid Non-Genomic Effects of Corticosteroids and Their Role in the Central Stress Response.* Journal of Endocrinology, 2011. **209**(2): p. 153-167.
32. Huang, T.-S., P. Ruoff, and P.G. Fjelldal, *Diurnal Expression of Clock Genes in Pineal Gland and Brain and Plasma Levels of Melatonin and Cortisol in Atlantic Salmon Parr and Smolts.* Chronobiology International, 2010. **27**: p. 1697-1714.
33. Virtanen, E. and L. Forsman, *Physiological Responses to Continuous Swimming in Wild Salmon (Salmo salar L.) Parr and Smolt.* Fish Physiology and Biochemistry, 1987. **4**(3): p. 157-163.
34. Takahashi, H., et al., *Expression of Glucocorticoid Receptor in the Intestine of a Euryhaline Teleost, the Mozambique Tilapia (Oreochromis mossambicus): Effect of*

Seawater Exposure and Cortisol Treatment. Life Sciences, 2006. **78**(11): p. 2329-2335.
35. Selye, H., *Confusion and Controversy in the Stress Field.* Journal of Human Stress, 1975. **1**(2): p. 37-44.
36. Bhatia, V. and R.K. Tandon, *Stress and the Gastrointestinal Tract.* Journal of Gastroenterology and Hepatology, 2005. **20**(3): p. 332-339.
37. Rosengren, M., et al., *The Brain-Gut Axis of Fish: Rainbow Trout with Low and High Cortisol Response Show Innate Differences in Intestinal Integrity and Brain Gene Expression.* General and Comparative Endocrinology, 2018. **257**: p. 235-235.
38. Olsen, R.E., et al., *Acute Stress Alters Intestinal Function of Rainbow Trout, Oncorhynchus mykiss (Walbaum).* Aquaculture, 2005. **250**(1-2): p. 480-495.
39. Ulrich-Lai, Y.M., et al., *Stress Exposure, Food Intake and Emotional State.* Stress, 2015. **18**(4): p. 381-399.
40. Madison, B.N., et al., *Chronic Cortisol and the Regulation of Food Intake and the Endocrine Growth Axis in Rainbow Trout.* Journal of Endocrinology, 2015.
41. Ahima, R.S. and D.A. Antwi, *Brain Regulation of Appetite and Satiety.* Endocrinology and Metabolism Clinics of North America, 2008. **37**(4): p. 811-823.
42. VanGuilder, H.D., K. Vrana, and W.M. Freeman, *Twenty-Five Years of Quantitative PCR for Gene Expression Analysis.* BioTechniques, 2008. **44**(5): p. 619-626.
43. Murashita, K., et al., *Production of Recombinant Leptin and its Effects on Food Intake in Rainbow Trout (Oncorhynchus mykiss).* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2008. **150**(4): p. 377-384.
44. Tartaglia, L.A., *The Leptin Receptor.* Journal of Biological Chemistry, 1997. **272**(10): p. 6093-6096.
45. Aguilar, A.J., et al., *Central Leptin Treatment Modulates Brain Glucosensing Function and Peripheral Energy Metabolism of Rainbow Trout.* Peptides, 2010. **31**(6): p. 1044-1054.
46. Kristensen, P., et al., *Hypothalamic CART is a New Anorectic Peptide Regulated by Leptin.* Nature, 1998. **393**: p. 72-76.
47. Dagogo-Jack, S., G. Tykodi, and I. Umamaheswaran, *Inhibition of Cortisol Biosynthesis Decreases Circulating Leptin Levels in Obese Humans.* The Journal of Clinical Endocrinology and Metabolism, 2005. **90**(9): p. 5333-5335.
48. Balment, R.J., et al., *Arginine Vasotocin a Key Hormone in Fish Physiology and Behaviour: A Review with Insights From Mammalian Models.* General and Comparative Endocrinology, 2006. **147**(1): p. 9-16.
49. Backström, T. and S. Winberg, *Arginine–Vasotocin Influence on Aggressive Behavior and Dominance in Rainbow Trout.* Physiology & Behavior, 2009. **96**(3): p. 470-475.
50. Gesto, M., et al., *Arginine Vasotocin Treatment Induces a Stress Response and Exerts a Potent Anorexigenic Effect in Rainbow Trout, Oncorhynchus mykiss.* Journal of Neuroendocrinology, 2014. **26**: p. 89-99.
51. Aguilera, G., *Regulation of Pituitary ACTH Secretion during Chronic Stress.* Frontiers in Neuroendocrinology, 1994. **15**(4): p. 321-350.
52. Yehuda, R., S. Brand, and R.-K. Yang, *Plasma Neuropeptide Y Concentrations in Combat Exposed Veterans: Relationship to Trauma Exposure, Recovery from PTSD, and Coping.* Biological Psychiatry, 2006. **59**(7): p. 660-663.
53. Doyon, C., et al., *Corticotropin-Releasing Factor and Neuropeptide Y mRNA Levels are Modified by Glucocorticoids in Rainbow Trout, Oncorhynchus mykiss.* General and Comparative Endocrinology, 2005. **146**(2): p. 126-135.

54. Dryden, S., et al., *Increased Neuropeptide Y Secretion in the Hypothalamic Paraventricular Nucleus of Obese (fa/fa) Zucker Rats.* Brain Research, 1995. **690**(2): p. 185-188.
55. Kalra, S.P., et al., *Neuropeptide Y Secretion Increases in the Paraventricular Nucleus in Association with Increased Appetite for Food.* Proceedings of the National Academy of Sciences, 1991. **88**(23): p. 10931-10935.
56. Labrie, F., et al., *Interactions Between CRF, Epinephrine, Vasopressin and Glucocorticoids in the Control of ACTH Secretion.* Journal of Steroid Biochemistry, 1984. **20**(1): p. 153-160.
57. Bernier, N.J., *The Corticotropin-Releasing Factor System as a Mediator of the Appetite-Suppressing Effects of Stress in Fish.* General and Comparative Endocrinology, 2006. **146**: p. 45-55.
58. Carpenter, R.E., et al., *Corticotropin Releasing Factor Induces Anxiogenic Locomotion in Trout and Alters Serotonergic and Dopaminergic Activity.* Hormones and Behavior, 2007. **52**(5): p. 600-611.
59. Murphy, K.G., *Dissecting the Role of Cocaine- and Amphetamine-Regulated Transcript (CART) in the Control of Appetite.* Briefings in Functional Genomics & Proteomics, 2005. **4**(2): p. 95-111.
60. Arunangsu Dey, et al., *Biological Processing of the Cocaine and Amphetamine-regulated Transcript Precursors by Prohormone Convertases, PC2 and PC1/3.* Journal of Biological Chemistry, 2002. **278**: p. 15007-15014.
61. Dhillo, W.S., et al., *Hypothalamic Interactions Between Neuropeptide Y, Agouti-Related Protein, Cocaine- and Amphetamine-Regulated Transcript and Alpha-Melanocyte-Stimulating Hormone in vitro in Male Rats.* Journal of Neuroendocrinology, 2002. **14**(9): p. 725-730.
62. Okumura, T., et al., *Cocaine-Amphetamine-Regulated Transcript (CART) Acts in the Central Nervous System to Inhibit Gastric Acid Secretion via Brain Corticotropin-Releasing Factor System.* Endocrinology, 2000. **141**(8): p. 2854-2860.
63. Stanley, S.A., et al., *Regulation of Rat Pituitary Cocaine- and Amphetamine-Regulated Transcript (CART) by CRH and Glucocorticoids.* American Journal of Physiology - Endocrinology and Metabolism, 2004. **287**(3): p. E583-E590.
64. Backström, T., et al., *Stress Effects on AVT and CRF Systems in Two Strains of Rainbow Trout (Oncorhynchus mykiss) Divergent in Stress Responsiveness.* Hormones and Behavior, 2011. **59**(1): p. 180-186.
65. Cortés, R., et al., *Effects of Acute Handling Stress on Short-Term Central Expression of Orexigenic/Anorexigenic Genes in Zebrafish.* Fish Physiology and Biochemistry, 2018. **44**(1): p. 257-272.
66. Conde-Sieira, M., et al., *Effect of Different Glycaemic Conditions on Gene Expression of Neuropeptides Involved in Control of Food Intake in Rainbow Trout; Interaction with Stress.* Journal of Experimental Biology, 2010. **213**: p. 3858-3865.
67. Bernier, N.J. and R.E. Peter, *The Hypothalamic-Pituitary-Interrenal Axis and the Control of Food Intake in Teleost Fish.* Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology, 2001. **129**(2-3): p. 639-644.
68. Schreck, C.B., B.L. Olla, and M.W. Davis, *Behavioural Response to Stress*, in *Fish Stress and Health in Aquaculture*, G.K. Iwama, et al., Editors. 1997, Cambridge University Press: Cambridge. p. 145-170.
69. Beitinger, T.L., *Behavioral Reactions for the Assessment of Stress in Fishes.* Journal of Great Lakes Research, 1990. **16**(4): p. 495-528.
70. Castanheira, M.F., et al., *Coping Styles in Farmed Fish: Consequences for Aquaculture.* Reviews in Aquaculture, 2017. **9**(1): p. 23-41.

71. Martins , C.I.M., et al., *Linking Fearfulness and Coping Styles in Fish.* PLoS ONE, 2011. **6**(11): p. e28084.
72. Øverli, Ø., et al., *Stress Coping Style Predicts Aggression and Social Dominance in Rainbow Trout.* Hormones and Behavior, 2004. **45**(4): p. 235-241.
73. Nørstrud, K.S., *Cortisol-Induced Cardiac Remodeling in Rainbow Trout (Oncorhynchus mykiss): Time-Course and Receptor-Specific Effects*. 2013, University of Oslo: Oslo, Norway.
74. Pottinger, T.G. and T.R. Carrick, *Stress Responsiveness Affects Dominant–Subordinate Relationships in Rainbow Trout.* Hormones and Behavior, 2001. **40**(3): p. 419-427.
75. Øverli, Ø., C.A. Harris, and S. Winberg, *Short-Term Effects of Fights for Social Dominance and the Establishment of Dominant-Subordinate Relationships on Brain Monoamines and Cortisol in Rainbow Trout.* Brain, Behavior and Evolution, 1999. **54**(5): p. 263-275.
76. R Core Team, *R: A Language and Environment for Statistical Computing*. 2017: R Foundation for Statistical Computing, Vienna, Austria.
77. Koolhaas, J.M., et al., *Coping Styles in Animals: Current Status in Behavior and Stress-Physiology.* Neuroscience and Biobehavioral Reviews, 1999. **23**: p. 925-935.
78. Mommsen, T.P., M.M. Vijayan, and T.W. Moon, *Cortisol in Teleosts: Dynamics, Mechanisms of Action, and Metabolic Regulation.* Reviews in Fish Biology and Fisheries, 1999. **9**(3): p. 211-268.
79. Krogdahl, Å. and A.M. Bakke-McKellep, *Fasting and Refeeding Cause Rapid Changes in Intestinal Tissue Mass and Digestive Enzyme Capacities of Atlantic salmon (Salmo salar L.).* Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 2005. **141**(4): p. 450-460.
80. Konturek, P.C., T. Brzozowski, and S.J. Konturek, *Stress and the Gut: Pathophysiology, Clinical Consequences, Diagnostic Approach and Treatment Options.* Journal of Physiology and Pharmacology, 2011. **62**(6): p. 591-599.
81. Cook, I.J., J. Dent, and S.M. Collins, *Upper Esophageal Sphincter Tone and Reactivity to Stress in Patients with a History of Globus Sensation.* Digestive Diseases and Science, 1989. **34**(5): p. 672-676.
82. Ando, M., M. Ogawa, and M. Fukuda, *A Vagal Nerve Branch Controls Swallowing Directly in the Seawater Eel.* Journal of Comparative Physiology B, 2013. **183**(8): p. 1015-1021.
83. Ito, S., T. Mukuda, and M. Ando, *Catecholamines Inhibit Neuronal Activity in the Glossopharyngeal-Vagal Motor Complex of the Japanese Eel: Significance for Controlling Swallowing Water.* Journal of Experimental Biology, 2006. **305**(6): p. 499-506.
84. Farrar, R.S. and K.J. Rodnick, *Sex-Dependent Effects of Gonadal Steroids and Cortisol on Cardiac Contractility in Rainbow Trout.* Journal of Experimental Biology, 2004. **207**: p. 2083-2093.
85. Zahl, I.H., et al., *Anesthesia Induces Stress in Atlantic Salmon (Salmo salar), Atlantic Cod (Gadus morhua) and Atlantic Halibut (Hippoglossus hippoglossus).* Fish Physiology and Biochemistry, 2010. **36**(3): p. 719-730.
86. Winberg, S., et al., *Serotonin as a Regulator of Hypothalamic-Pituitary-Interrenal Activity in Teleost Fish.* Neuroscience Letters, 1997. **230**: p. 113-116.
87. Caamaño-Tubío, R.I., et al., *Peripheral Serotonin Dynamics in the Rainbow Trout (Oncorhynchus mykiss).* Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 2007. **145**(2): p. 245-255.

88. Reid, S.G., M.M. Vijayan, and S.F. Perry, *Modulation of Catecholamine Storage and Release by the Pituitary-Interrenal Axis in the Rainbow Trout, Oncorhynchus mykiss.* Journal of Comparative Physiology B, 1996. **165**(8): p. 665-676.
89. Lang, I.M. and R. Shaker, *Anatomy and Physiology of the Upper Esophageal Sphincter.* The American Journal of Medicine, 1997. **103**(5): p. 50S-55S.
90. Olsson, C., *Autonomic Innervation of the Fish Gut.* Acta Histochemica, 2009. **111**(3): p. 185-195.
91. Selvik, J.R. and T. Høgåsen, *Kildefordelte Tilførsler av Nitrogen og Fosfor til Norske Kystområder i 2015 - Tabeller, Figurer og Kart*. 2017, Norsk institutt for vannforskning (NIVA). p. 13-14.
92. Wickham, H., *ggplot2: Elegant Graphics for Data Analysis*. 2009: Springer-Verlag, New York, USA.
93. Ahlmann-Eltze, C., *ggsignif: Significance Brackets for 'ggplot2'*. 2017.
94. Kassambara, A., *ggpubr: 'ggplot2' Based Publication Ready Plots*. 2017.
95. Dinno, A., *dunn.test: Dunn's Test of Multiple Comparisons Using Rank Sums*. 2017.
96. Fox, J. and S. Weisberg, *An {R} Companion to Applied Regression*. 2011.
97. Dowle, M. and A. Srinivasan, *data.table: Extension of `data.frame`*. 2017.
98. Dag, O., A. Dolgun, and N.M. Konar, *onewaytests: One-Way Tests in Independent Groups Designs*. 2017.
99. Lawrence, M.A., *Ez: Easy Analysis and Visualization of Factorial Experiments*. 2006.
100. Bache, S.M. and H. Wickham, *magrittr: A Forward-Pipe Operator for R*. 2014.