Pharmacological activation of serotonergic activity in Atlantic salmon (*Salmo salar*):
A model of chronic stress

By Kia Wee Tan

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Kia Wee Tan

Abstract

Chronic stress is the principal component to many human and animal disorders. As stress responses are remarkably conserved between mammals and teleosts, the use of teleosts in comparative models has been a useful tool in neurobiology. In particular, salmonid fishes have emerged as model organisms for chronic social stress due to their well-characterized social behavior and physiology.

The serotonergic system is involved in a wide variety of functions, among which are the control of the neuroendocrine stress response and the regulation of an array of behaviors such as feeding and aggression. In salmonid models of chronic social stress, serotonergic activity in subordinate individuals has been found to be elevated in many brain regions, in some instances to pathological levels. In this study, the serotonergic system of Atlantic salmon was pharmacologically activated with fluoxetine for 18 days and the behavioral, physiological and neurochemical responses were examined. Fluoxetine induced anorexia, surfacing behavior and an increased tendency for treated fish to position themselves in a head up, tail down position. Physiologically, fluoxetine treated salmon had increased brain stem serotonergic and norepinephrine activity, greatly elevated cortisol levels, larger relative heart sizes, upregulated hypothalamic AVT and 5-$HT_{1A\beta}$ receptor mRNA levels. All of these behavioral and physiological parameters are strikingly similar to those exhibited by subordinate salmonids in chronic social stress models. These results indicate that long term pharmacological activation of the serotonergic system in Atlantic salmon recapitulates many of the typical behavioral and physiological markers found in salmonid models of chronic social stress. The utilization of fluoxetine and other pharmacological tools to manipulate serotonergic signaling may thus be of further use in elucidating the intricacies of the stress response and mechanisms that lead to its dysfunction.

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List of Abbreviations:

ACTH	Adrenocorticotropin
AVP	Arginine Vasopressin
AVT	Arginine Vasotocin
BDNF	Brain Derived Neurotrophic Factor
CART	Cocaine and Amphetamine Regulated Transcript
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
CRH	Corticotropin Releasing Hormone
CSI	Cardiosomatic Index
DHBA	3,4-dihydroxybenzyl amine hydrobromide
EDTA	Ethylene diamine tetraacetic acid
GIRK	G-protein coupled Inwardly Rectifying K^+ channel
GPCR	G-protein Coupled Receptor
GR	Glucocorticoid Receptor
GRE	Glucocorticoid Response Element
HPA	Hypothalamic-Pituitary-Adrenal
HPI	Hypothalamic-Pituitary-Interrenal
HPLC	High Performance Liquid Chromatography
i.p.	intraperitoneal
MAO	Monoamine Oxidase
MHPG	3-methoxy-4-hydroxyphenylglycol
MR	Mineralocorticoid Receptor
NE	Norepinephrine
NFAT	Nuclear Factor of Activated T cells
PCA	Perchloric Acid
PCNA	Proliferating Cell Nuclear Antigen
POMC	Proopiomelanocortin
RIN	RNA Integrity Number
(m)RNA	(messenger) Ribonucleic Acid
SAM	Sympathetico-adrenomedullary
SERT	Serotonin Transporter
SSRI	Selective Serotonin Reuptake Inhibitor
TPH	Tryphophan Hydroxylase
α-MSH	α-melanocyte-stimulating hormone
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytrypamine

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1 Introduction

Organisms that face a perceived threat to their biological functions activate an array of behavioral and physiological mechanisms to fulfill a current or anticipated demand and thus improve their chances of survival. These perceived threats, both internal and external are termed "stressors"[1] and the mechanisms that are activated, such as the sympathetic-adrenal medullary (SAM) or the hypothalamic-pituitary-adrenal (HPA) axis have become widely known as the neuroendocrine stress response. It has however been argued recently, that the activation of mechanisms comprising the stress response, may not only be elicited under stressful situations. In fact, these mechanisms may also be activated under non-aversive or harmful situations, such as increased metabolic demand during behavioral activity[2,3]. Therefore, Koolhaas[4] proposes a more restricted definition of stressors as "*conditions where an environmental demand exceeds the natural regulatory capacity of an organism, in particular situations that include unpredictability and uncontrollability*". Notably, unpredictability and uncontrollability are particularly potent factors in stress-induced pathology[5,6] as well as being important variables in determining the success of behavioral strategies[7]. Hence with these concepts in mind, I have chosen to adhere to Koolhaas's working definition of stress in this thesis.

As stress is a risk factor for a variety of human and animal disorders, such as mood disorders[8] and cardiovascular disease[9], the stress response and its regulation is of considerable research interest. Inadequate, excessive and/or prolonged activation of the stress response can itself be damaging and lead to a reduced ability to cope with stressors[4]. In particular, stress responses sustained over long periods have been shown to cause alterations at various levels of the stress response pathways[10–12]. In addition, the behavioral response to stressors varies between individuals[6] and can change depending on the severity and/or duration of the stressor. For example, severe or prolonged (chronic) stress, may bias individuals from active behavior (proactive) to behavioral inhibition (reactive)[13–15]. In this respect, it has been proposed that a stress-induced switch to reactive behavior is adaptive in unpredictable environments, while proactive behavior is more suited to stable environments[6,7].

One common paradigm utilized in both mammalian and teleost models in order to study the effects of acute and chronic stress is subordination, i.e. defeat stress[16–19]. Under these

conditions, the subordinate may experience unpredictable and unilateral aggression from the dominant over extended periods of interaction[17].

1.1 Fish as models for chronic stress: Social subordination in salmonids

Salmonid fish have been used in neurobiological research as a model for social stress due to their well characterized behavioral, physiological and neuroendocrine responses to social subordination[20–22]. Exploiting the normally territorial nature of salmonids, interaction of a subordinate fish with a dominant leads to behavioral and physiological changes in the stressed subordinate that are similar to those found in mammalian models[16]. For example, subordinate salmonids display a reduced feeding intake, elevated plasma cortisol, serotonergic activity and decreased expression of brain neuroplasticity markers[17,23–26]. Furthermore, in the brain, the increase in serotonergic activity appears to include the hypothalamus, telencephalon and brain stem, and may last over several weeks (so long as the subordinate is kept together with the dominant)[17,20]. Notably, the reduced feeding in subordinates is not due to interference or continued aggression by the dominant conspecific as it persists after the subordinate has been isolated. In fact, the reduced feed intake recovers gradually after separation of the dominant/subordinate pair, during which time, there is also a decline in serotonergic activity back to baseline[24]. Interestingly, Vindas et. al.[27] recently characterized a neuroendocrine phenotype in farmed Atlantic salmon (*Salmo salar*) where individuals which had been subjected to chronic social stress displayed an elevated level of serotonergic activity and an inability of the serotonergic system to respond further to acute stress, despite showing a functional cortisol stress response. This illustrates that serotonergic dysfunction can be found in fish undergoing severe and prolonged stress.

1.2 The Neuroendocrine Stress Response

The neuroendocrine stress response in both mammals and teleosts, is activated and regulated in a similar manner. This response consists of the activation of two main systems:

- The rapid sympathetic-adrenal medullary (SAM) system (sympathetic-chromaffin in fish)

- The slower hypothalamic-pituitary-adrenal (HPA) axis (hypothalamic-pituitary-interrenal (HPI) axis in fish)

The SAM response is initiated by the sympathetic nervous system and its main effect is to promote the release of pre-synthesized epinephrine and norepinephrine into the bloodstream from the adrenal medulla (or chromaffin cells of the head kidney in fish). This results in mobilization of energy stores into the bloodstream, improved capacity of red blood cells to carry oxygen and increased cardiovascular function[28,29].

Activation of the HPA axis begins in the hypothalamus with the release of Corticotropin Releasing Hormone (CRH). CRH stimulates release of Adrenocorticotropic Hormone (ACTH) from the anterior pituitary into the bloodstream. ACTH in turn stimulates cortisol secretion from the adrenal cortex (or interrenal cells in fish). In addition, neuropeptides such as Arginine Vasopressin (AVP) may also contribute directly and/or indirectly, by potentiating the release of ACTH and cortisol. Cortisol in turn exhibits negative feedback on all levels of the HPA/HPI axis[30].

The transcriptional effects of cortisol are classically mediated through corticosteroid receptors, which are ligand-binding nuclear receptors. Cortisol being highly lipophilic, diffuses freely through the plasma membrane into the cytosol. In mammals, a high affinity (10-fold higher) Mineralocorticoid Receptor (MR) and a lower affinity Glucocorticoid Receptor (GR) have been described to bind cortisol. Ligand-bound corticosteroid receptor dimers in the nucleus bind to Glucocorticoid Response Elements (GREs) in the promoter region of target genes and may activate or repress transcription, depending on the GRE. In salmonids, one high affinity MR homologue and two lower affinity GR homologues (GR1 and GR2) have been described, with GR2 showing a higher cortisol affinity[31].

1.3 Serotonergic Transmission and Control of the Stress Response

Serotonin (or 5-hydroxytryptamine, 5-HT) is a major monoamine neurotransmitter and neuromodulator in the central nervous system (CNS). Because the serotonergic system is evolutionarily very ancient, much homology can be found between the molecular, cellular, anatomical, and functional components between mammals and teleosts. For example, many proteins involved in the serotonergic system first identified in mammalian models have been now cloned and characterized in zebrafish (*Danio rerio*) such as the 5-HT transporter (SERT) and the 5-HT synthesis enzyme tryptophan hydroxylase (TPH)[32]. The functionality of serotonergic transmission in mammals and teleosts also bear much similarity to each other[33], although differences have been found. For example, in teleosts there is the presence of

additional clusters of serotonergic cell bodies[34] and differential expression of specific isoforms of SERT[35].

Serotonergic cell bodies are found in multiple clusters mainly distributed throughout the mid and hindbrain, which send projection axons to all other regions of the brain. This in turn, can release 5-HT both at the axon terminals and along the axon[36]. In zebrafish, the best characterized teleost, additional clusters of serotonergic neurons are found in the hypothalamus, pre-optic area and posterior tuberculum[33]. Because 5-HT receptors are also often found extrasynaptically, and 5-HT can act through volume transmission, the wide distribution of serotonergic fibers allows for an extensive serotonergic regulation throughout the brain on specific neurons that express the appropriate receptors. Accordingly, serotonergic transmission plays a role in very diverse functions, which include housekeeping tasks like thermoregulation and food intake[37], more complex physiological regulation such as that of the generalized stress response[38] and also behavioral responses and affective states, such as aggression and anxiety-like behavior[30].

The ratio of the concentration of 5-Hydroxyindoleacetic acid (5-HIAA; which is the main catabolite for 5-HT) over the total concentration of 5-HT, i.e. [5-HIAA]/[5-HT], is commonly taken as a measure of serotonergic activity in a particular brain area[39]. In brief tissue concentrations of 5-HT may not be sufficient to adjudge serotonergic activity, since it is not possible to distinguish between actively signaling 5-HT in the extracellular space, vesicle-bound intracellular and/or cytoplasmic free 5-HT. However, as vesicle-bound 5-HT is protected from monoamine oxidases (MAO) located in the outer mitochondrial membrane[40], the catabolite 5-HIAA is generated only from cytoplasmic 5-HT. Therefore, all other parameters being equal, a higher concentration of actively signaling extracellular 5-HT leads to increased reuptake by the 5-HT transporter located in the plasma membrane, leading to an increased cytoplasmic concentration of 5-HT and thus increased substrate availability for MAO. However, the [5-HIAA]/[5-HT] ratio may be affected not just by normal 5-HT turnover from activity, but also from factors such as MAO activity, synthesis rates and by reuptake blockers such as fluoxetine. Furthermore, MAO[40] and TPH2[41] activity are known to be regulated by glucocorticoids and therefore may also perturb this ratio.

5-HT receptors are a very diverse family of membrane receptors found both in the CNS and other tissues. Presently 14 receptors, classified into 7 families based on their pharmacology, signal transduction and structure, have been found, and with the exception of the 5-HT_3 type,

all of them are G-protein coupled receptors (GPCRs)[42]. The 5-HT_{1A} receptor in the CNS is well described as being involved in affective disorders and is present as both a heteroreceptor on non-serotonergic target neurons (such as hippocampal neurons) and as a somatodendritic autoreceptor on serotonergic neurons[43]. 5-HT_{1A} autoreceptors inhibit firing of serotonergic neurons by hyperpolarization through G-protein-coupled inwardly-rectifying potassium channels (GIRK), and therefore have influence on all serotonergic transmission[43]. Postsynaptically, 5-HT_{1A} and 5-HT_{2C} receptors have been shown in mammals and teleosts to control cortisol release[30], thus illustrating a direct coupling of the serotonergic system to stress-induced activation of the HPA/HPI axis.

1.4 Pharmacological Activation of the Serotonergic System

The time course of serotonergic response in various brain regions to social subordination in salmonids have been thoroughly described by Øverli et al.[23].Furthermore, along with the serotonergic response, other consequences of subordination have been described, such as elevated plasma cortisol and reduced feeding behavior[44]. Pharmacologically, in mammals and fish, the use of 5-HT receptor agonists has been successfully utilized for the study of the link between 5-HT receptors to the HPI axis[44–47] and feeding behavior[34,37,48]. However, as these studies used short-duration selective agonists for specific receptor subtypes, it is thus not possible to compare their effects to the generalized upregulation of serotonergic signaling sustained over several days found in salmonid chronic social stress models[17,20,25]. It is hence of interest to study the long term effects of a more generalized activation of the serotonergic system in salmonid fish as it is the serotonergic correlate of chronic stress and may reveal alteration in serotonergic signaling that precede dysfunction.

Fluoxetine is a selective 5-HT reuptake inhibitor (SSRI) that inhibits the major 5-HT reuptake pump, SERT, in the brain. This causes an increase in the extracellular concentration of actively signaling 5-HT, mimicking the effects of increased serotonergic activity. Notably, in ecotoxicology[49,50] as well as experimental studies[32,51], fluoxetine has been found to be effective as an SSRI in teleosts. Furthermore, fluoxetine also has the longest half-life (2 days in humans) of the SSRIs[52], allowing for a more infrequent administration which makes it practical in experimental settings to diminish the possible stress caused by drug administration. The possible role of fluoxetine as a pharmacological tool to investigate

behavioral and physiological effects of chronic 5-HT activation has however not been explored in the salmonid fish model.

This study was conducted on Atlantic salmon because the relatively stress-sensitive nature of this salmonid species might allow for larger effects to be seen compared to relatively stress-tolerant species such as rainbow trout (*Oncorhynchus mykiss*). In addition, Vindas et. al.[27] recently found serotonergic dysfunction in a chronically stressed subpopulation of farmed Atlantic salmon, thus confirming that chronic stress may result in alterations of serotonergic regulation in salmonids. In the present study I therefore administered fluoxetine to Atlantic salmon for 2 weeks in order to activate serotonergic signaling, mimicking the serotonergic effects of chronic social stress. Data on behavioral, physiological and neurochemical parameters related to serotonergic activity and the neuroendocrine stress response were quantified here and I compared my results with those reported by research on salmonid social stress models.

2 Aims of Study

The aims of this study are:

- To test an oral “stress-free” method of fluoxetine administration on isolated Atlantic salmon over 2 weeks.
- To verify drug effects on serotonergic signaling in Atlantic salmon.
- To quantify the behavioral, physiological and neurochemical response to fluoxetine treatment over 2 weeks and compare neurobiological/behavioral profiles to those reported in salmonid chronic social stress models.

3 Materials and Methods

3.1 Experimental Animals and Location

Atlantic salmon from the Department of Animal and Aquacultural Sciences (University of Life Sciences, Ås, Norway) were transferred on the 12th of June to a 1250L tank at the fish holding facility at the Department of Biosciences, University of Oslo.. The tank was continuously supplied with dechlorinated Oslo tap water (1000L h^{-1}) and aerated with bubbled air. During the holding period and throughout the experiment, the water temperature was between 8.5-10^{0}C, dissolved oxygen content was 85-95% and the light regime was 12/12 light/dark. Fish were hand fed with 3 mm commercial food pellets (Skretting, Norway)

3.2 Pre-experimental Procedure

Eight 250L glass aquaria, covered on 3 sides with opaque black plastic, were divided into 4 compartments each, by PVC walls, forming 32 compartments in total. Each compartment was continuously supplied with dechlorinated Oslo tap water (5L h^{-1}) and aerated with bubbled air. Waterproof video cameras (Colour CCD cameras, IR- YC-25V, with a 3.6 mm lens) controlled by an MSH-video multicam surveillance system (M. Shafro & Co., Riga, Latvia), were placed approximately 70cm in front of each aquaria.

Atlantic salmon were transferred and isolated on the 16th of August from the group holding tank. Fish were hand fed daily 10 dry pellets between 1300 and 1500. Feeding behavior was assessed and scored daily by observing the response of the fish to food items dropped one by one into the tank, according to the following scale (modified from Sørensen et. al.[25]):

- 0 – Fish does not respond to food, even when dropped directly in front of it.
- 1 – Fish only eats pellets that fall directly in front of it.
- 2 – Fish moves less than half body length to eat food, ignores otherwise.
- 3 – Fish moves to eat food throughout the tank, but returns to original position each time.
- 4 – Fish swims actively after food items throughout the tank.

Individuals were acclimated for a period of 5 weeks until full feeding behavior (scored 4 on the feeding scale) was observed (a total of 1 individual was excluded).

Following the acclimatization period, fish were weighed by anesthetizing them lightly in 50mg/L MS-222 (Syndel Laboratories Ltd, BC, Canada). Thereafter, fish were fed with dry

pellets once every second day with a ration corresponding to 0.05% of their body weight. In addition, fish were fed small quantities of dried shrimp hydrated in deionized water over 9 days, in order to acclimatize them to this type of food (which was later used to administer fluoxetine, as explained further below).

3.3 Preparation of Drugs

Fluoxetine solution was prepared by dissolving 4 mg fluoxetine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) per ml of deionized water. In addition, a control solution containing only deionized water was also prepared. Both solutions were placed into identical tubes and were administered blindly (the identity of each tube was marked by a person not involved in handling of the fish). Solutions were stored at -20^{0}C in the dark.

Prior to feeding, approximately 10g of dried shrimp were reconstituted in fluoxetine or control solutions for 2hrs at room temperature. The weight of the shrimp after reconstitution was recorded and used to calculate the ration quantity for each fish that would result in the delivery of 4 ug/g body weight of fluoxetine, following the formula:

Shrimp ration = 0.001 (Shrimp Wet Weight x Fish Weight) / (Shrimp Wet Weight - Shrimp Dry Weight)

For intraperitoneal (*i.p.*) injections, 0.9% w/v sodium chloride (Sigma-Aldrich) was added to the fluoxetine and control solutions and vortex-mixed until completely dissolved.

3.4 Experimental Procedure

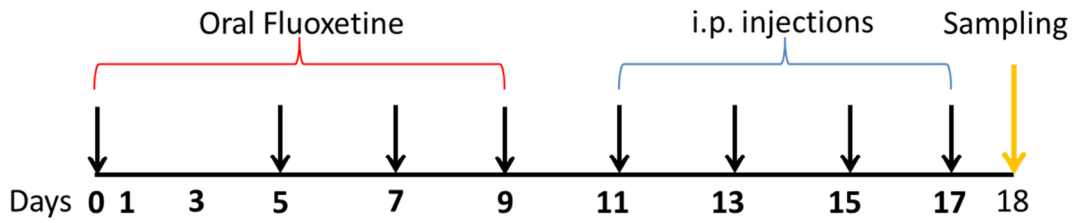

Figure 1: Timeline of Experiment. Feeding responses were assessed on every marked day before sampling.

Tanks were assigned randomly to each of the two treatments. Fish were fed once every 2 days at 0.05% body weight with pelleted food for the first 9 days of the experiment. Fluoxetine-shrimp rations were given on days 0, 5, 7, and 9 as shown on Figure 1. The spacing between the first and second doses (days 0 and 5 of experiment) was due to a logistics issue. Subsequently, all fish were fed 1 pellet every 2 days to assess feeding behavior. The feeding

amount was reduced to minimize discrepancy between normally eating fish and individuals which had developed varying readiness to accept food. On days 11, 13, 15, and 17 of the experiment, fish were lightly anesthetized with 50mg/L MS-222 and a drug solution amount corresponding to 4ug/g body weight was delivered via *i.p.* 2 hours after feeding. All fish were monitored after injection to ensure that they recovered properly from the handling and the anesthetic. The order of feeding and injections were randomized.

3.5 Sampling

On the last day of the experiment, fish were sampled in random order. Following rapid anesthetization in 800mg/L MS-222, fish were weighed and blood was collected from the caudal vein before the fish were sacrificed by decapitation and the brains and hearts dissected. Blood collection was done with 1 ml syringes containing the anticoagulant ethylenediaminetetraacetic acid (EDTA), and kept on ice for no more than 3 minutes before centrifugation at 4^0C and 8000g, for 5 min. Blood plasma was then quickly frozen on dry ice and stored at -80^0C. The brains were quickly excised (within 2 min) and further divided into *brain stem*, which was snap frozen in liquid nitrogen and stored at -80^0C, *hypothalamus* and *pituitary*, which were placed separately on RNA later® (Ambion, Austin TX, USA), and the *telencephalon* which was placed in 4% paraformaldehyde in phosphate buffered saline. Hearts were dissected and ventricles (after atrium and bulbus removal) were placed on previously weighed RNA later® Eppendorf tubes and reweighed. All tissues in RNA later® were kept at 20^0C for 1 day according to manufacturer's instructions and subsequently stored at -20^0C.

3.6 RNA extraction and Quantitative PCR

Total RNA was extracted from the hypothalamus using Trizol® reagent (Invitrogen, Carlsbad, CA, USA) extraction, according to manufacturer`s protocol. In short, samples were thawed, weighed and homogenized with 15µl/mg Trizol®. Extracted total RNA was treated with DNAse using a TURBO DNA-free^{TM} Kit (Ambion, Austin TX, USA) according to manufacturer's instructions to remove DNA contamination. The RNA concentration was then quantified using the Nanodrop ND-2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA work was carried out on ice and stored at -80^0C. 12 out of 27 RNA samples were randomly selected and RNA quality checked using the Bioanalyzer 2100 (Agilent Technologies Inc. The RIN scale assigns a number between 1 and

10 to RNA samples, with 10 indicating intact RNA. All samples scored between 9.9-10 on the RIN scale, which confirmed excellent RNA quality.

1µg of total RNA was reverse-transcribed into cDNA using OligodT_{18-20} primers (Invitrogen) and Superscript® III (Invitrogen) in a total volume of 20ul. The reaction was diluted 20X with nuclease free water and stored at -20^{0}C.

Quantitative PCR was carried out using 1uM of forward and reverse primers in 1 x SYBR® GREEN I Master Mix (Roche Diagnostics, Basel, Switzerland) with 3µl of 1:20 cDNA as the template. Total reaction volume was 10µl. The reactions were run on a Lightcycler® 96 (Roche Diagnostics). Cp values were obtained from the Lightcycler® 96 software (Roche Diagnostics). Primer efficiencies were calculated per primer pair, per reaction plate by using Linreg software (version 2012.1), according to the florescence values recorded during the amplification cycles. All samples were run in triplicates. Primer information is shown in Table 1 below.

Table 1: Primer pairs used in Quantitative PCR

Gene	Primer Sequence 5' → 3'	References
AVT	Fwd GAGGGGATGGGCTGCTATGT Rev CAAACCGCCCATTAAGGCAG	Thörnqvist P-O. & Winberg S. Unpublished
CRH	Fwd AACCAGCTCGACGACTCGATGG Rev GCTATGGGCTTGTTGCTGTAACTG	Nilsen T. O. & Ebbesson, L. O. E. Unpublished
MR	Fwd AGACTCGACCCCCACCAAG Rev CGTTAGTGGGACTGGTGCTC	Kiilerich et al. 2011[53]
GR1	Fwd ACGACGATGGAGCCGAAC Rev ATGGCTTTGAGCAGGGATAG	Kiilerich et al. 2011[53]
GR2	Fwd TGGTGGGCTGCTGGATTTCTGC Rev CTCCCTGTCTCCCTCTGTCA	Kiilerich et al. 2011[53]
SERTa	Fwd ACAAACCACTCCCTCCTCCT Rev CGGCTACATGGCTGAAATGC	Thörnqvist P-O. & Winberg S. Unpublished
5-$HT_{1A\alpha}$	Fwd ATGCTGGTCCTCTACGGGCG Rev CGTGGTTCACCGCGCCGTTT	Thörnqvist P-O. & Winberg S. Unpublished
5-$HT_{1A\beta}$	Fwd TTGATCATGCGTTCCCAGCCGA Rev AAAGGAATGTAGAACGCGCCGA	Thörnqvist P-O. & Winberg S. Unpublished
PCNA (Proliferating Cell Nuclear Antigen)	Fwd TGAGCTCGTCGGGTATCTCT Rev CTCGAAGACTAGGGCGAGTG	Vindas et al. 2014[54]
BDNF (Brain Derived Neurotrophic Factor)	Fwd ATGTCTGGGCAGACCGTTAC Rev GTTGTCCTGCATTGGGAGTT	Vindas et al. 2014[54]
EF1α (Elongation Factor-1α) ***Reference Gene***	Fwd CCCCTCCAGGACGTTTACAAA Rev CACACGGCCCACAGGTACA	Ingerslev et al. 2006[55]

3.7 Brain stem Neurochemistry

Frozen brain stems were homogenized in 4 % (w/v) ice cold perchloric acid (PCA) containing 0.2% EDTA and 3,4-dihydroxybenzyl amine hydrobromide (DHBA, 40 ng/ml) as an internal standard using a Potter–Elvehjem homogenizer. After spinning samples for 10 min at 13000 rpm and 4°C, the supernatant was analyzed by means of high-performance liquid

chromatography (HPLC). The mobile phase was made up of 12 µM EDTA, 86 mM sodium phosphate and 1.4 mM sodium octyl sulphate in deionized water (resistance 18.2 MW), containing 7 % acetonitrile set to pH 3.1 using phosphoric acid. The system contains a solvent delivery system (Shimadzu, LC-10AD), an auto-injector (Famos, Spark), a reverse phase column (4.6 mm´100 mm, Hichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at -40 mV and +320 mV. A conditioning electrode with a potential of +40 mV was used to oxidize possible contaminants before analysis. Brain stem concentrations of 5-HT, the 5-HT catabolite 5-hydroxyindoleacetic acid (5-HIAA), norepinephrine (NE) and the NE catabolite 3-methoxy-4-hydroxyphenylglycol (MHPG) were quantified by comparison with standards and corrected for recovery of the internal standard using HPLC software (CSW, Data Apex Ltd, the Czech Republic).

3.8 Radioimmunoassay for Cortisol

Plasma samples were thawed on ice and 100µl aliquots were mixed with 500µl of ethyl acetate (Merck KGaA, Darmstadt, Germany) and vortexed for 10 seconds, in order to extract steroids into the organic phase, which was then separated by centrifugation for 5 mins at 8000g at 4^0C. Thereafter, 50µl of this extract was used for analysis of cortisol content. 50µl of [1,2,6,7-^{3}H] cortisol (Amersham Pharmacia Biotech, Little Chalfront, UK, 60 Ci mmol-1) diluted in ethyl acetate, was mixed together with the extract and evaporated to dryness under vacuum. Donkey anti-cortisol (AbD Serotec, Kidlington, UK) was prepared 1:600 in a radioimmunoassay buffer containing 0.1% Bovine Serum Albumin (w/v) (Sigma-Aldrich) and phosphate buffered saline (PBS, pH 7.4). 200µl of this antibody solution was added to the assay tubes, which were then placed on a rotary shaker for 15mins in 4^0C. Subsequently, samples were left for 18hrs in the dark at 4^0C. Thereafter, 100µl of a dextran-coated charcoal buffer (0.1% dextran (w/v) (Sigma-Aldrich) and 0.5% activated charcoal (Sigma-Aldrich) in PBS was added to the tubes, vortexed, and left to stand for 5 min in order to bind free steroids. The charcoal was pelleted by centrifugation at 8000g for 10mins at 4^0C and 200µl of the supernatant was transferred into a scintillation tube containing 4ml of Ultima Gold scintillation fluid (Perkin Elmer, Waltham, MA, USA). Samples were then counted on a Packard Tri-Carb A1900 TR liquid scintillation analyzer (Packard Instrument, Meriden, CT, USA).

A 3-parameter hyperbolic function was fitted to the plot of the percentage of ^{3}H-cortisol bound against a standard curve using SigmaPlot 11 (SPSS Science, Systat Software Inc., San Jose, CA, USA). All samples were run in duplicate. Samples were reran, in cases were values fell outside the standard curve, with a more appropriate amount of extract..

3.9 Video Analysis

Video recordings taken during feeding times were used to characterize behavior. However, no data was taken during actual feeding. That is, the quantification of swimming behavior was done when fish were undisturbed. In order to quantify the tendency to stay at the top of the water column, total time spent in this section, during a 5 min period, beginning 5 min after the room door was opened, was analyzed. Many fish species avoid spending too much time at the water surface, since it leaves them more vulnerable to possible predator attacks[56]. Therefore, total time spent near the surface, has been commonly used as a metric in order to assess normal/aberrant behavior[50,57,58]. The head up tail down positioning was defined as a behavior where the fish was angled more than 45^{0} upwards while not engaged in locomotor activity. Even though the camera arrangement did not allow for quantitative measurements of fish body posture, video recordings were sufficient to distinguish the display of this particular behavior. Therefore, each fish was classified as either showing or not showing this head up tail down behavior on the day before terminal sampling (day 17 of the experiment).

3.10 Statistical Analysis

All statistical analysis was performed on Graphpad Prism 6 (Graphpad Software, San Diego, CA, USA). Data on surfacing behavior and body weight was analyzed by Repeated Measures ANOVA and differences between groups on each day were confirmed by a Tukey post-hoc test. Data on head up tail down behavior was analyzed by Fisher's Exact Test. Normality was assessed by means of a D'Agostino-Pearson test and values were either log or arcsine transformed if necessary. Cortisol, heart size, brain stem neurochemistry and quantitative PCR values, were analyzed by means of a Student's t-test or Welch's t-test (non-parametric data). Data sets were subjected to Grubbs test in order to detect possible outliers at $\alpha = 0.01$. Differences were considered significant at $p < 0.05$.

4 Results

4.1 Behavioral Observations

4.1.1 Feeding behavior

Fluoxetine treatment over several days decreased feeding behavior, as depicted in Figure 2. The increasing reluctance of fluoxetine treated fish to seek and ingest pellets was especially evident after 11 days of treatment, at which point oral delivery of fluoxetine via food had to be changed to *i.p.* delivery. Furthermore, from day 15 of treatment until the end of the experiment most of the fluoxetine-treated group did not respond at all to food (neither shrimp nor dry pellets). Note that control fish maintained normal feeding behavior throughout the experiment, even after receiving *i.p.* saline injections.

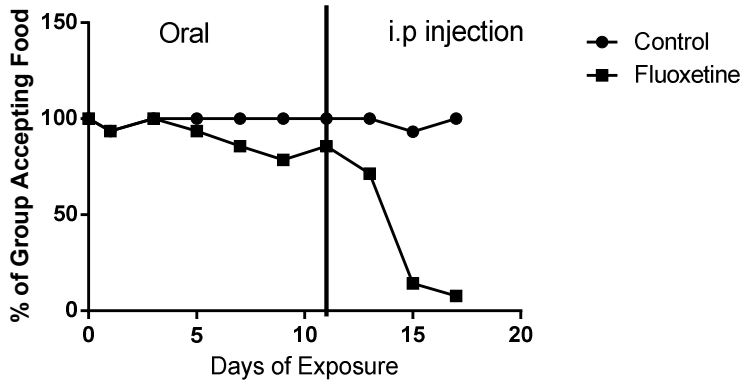

Figure 2: Fluoxetine decreased response to food in Atlantic salmon
Percentage of individuals in each group accepting food. (n = 15 for control, n = 13 for fluoxetine).

4.1.2 Positioning in the water column

After 9 days exposure to fluoxetine, treated fish showed an increased tendency to place themselves in a head up, tail down position. That is, they were inclined with the head higher up in the water column than the tail, as depicted on Figure 3A. Importantly, this behavior was only evident in the fluoxetine-treated group. After 17 days of exposure to fluoxetine, the head up, tail down position was observed in 12 out of 13 fluoxetine treated fish, but none of the 15 control fish ($p < 0.0001$). Fluoxetine treated fish also showed a greatly increased tendency to place themselves in the top half of the tank ($p < 0.0001$) (Figure 3B).

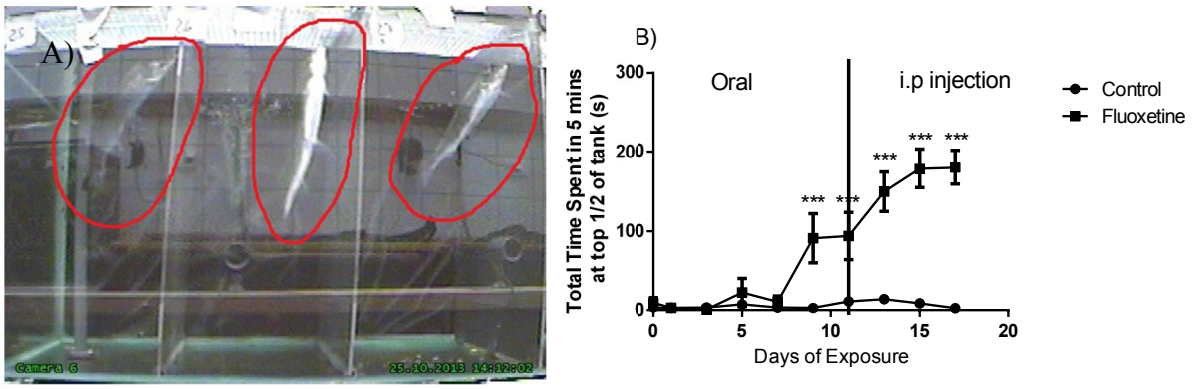

Figure 3: Altered water column and body positioning in Atlantic salmon
A) Video frame showing tendency of fish to place themselves with a head up/tail down position high in the water column. ($p < 0.0001$) B) Total time(s) spent during 5 min at top half of the tank. Presented as means ± S.E.M. (n = 15 controls, n = 13 fluoxetine) , $F_{(9, 234)} = 22.87$, $p < 0.0001$

4.2 Physiological Parameters

4.2.1 Blood plasma cortisol

Treatment with fluoxetine significantly increased cortisol levels compared to controls (Figure 4; $p = 0.0003$).

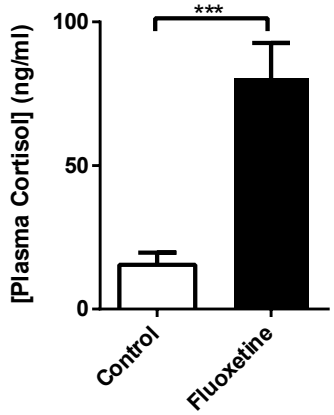

Figure 4: Fluoxetine treatment increased plasma cortisol levels in Atlantic salmon
Mean plasma concentrations of cortisol (± S.E.M.) for control (n = 15) and fluoxetine (n = 11) treated fish. Welch's t-test: $t_{(12.46)} = 5.01$; $p < 0.0001$.

4.2.2 Cardiosomatic index (Ventricle Weight/Body Weight)

Fluoxetine treatment significantly increased the cardiosomatic index (CSI; Figure 5, $p = 0.024$). Importantly, this increase was not caused by a difference in body weight between the

groups, since there were no significant differences between groups neither at the start (p = 0.2144), nor at the end of the experiment (p = 0.9712).

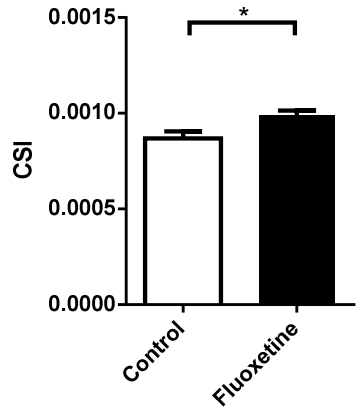

Figure 5: Fluoxetine treatment increased CSI in Atlantic salmon
A) Mean cardiosomatic index (CSI, ± S.E.M) for control (n = 14) and fluoxetine fish (n = 11) at the end of the experiment. Students t test: $t_{(23)} = 2.491$; $p = 0.024$

4.2.3 Brain Stem Neurochemistry

The concentration of 5-HIAA in the fluoxetine-treated group was significantly increased compared to controls (Figure 6A, p = 0.0041). Meanwhile 5-HT levels in the fluoxetine-treated group were significantly lower than controls (Figure 6B, $p < 0.0001$). Consequently, the [5-HIAA]/[5-HT] ratio was also significantly increased in the fluoxetine-treated group compared to controls (Figure 6C, p = 0.0008).

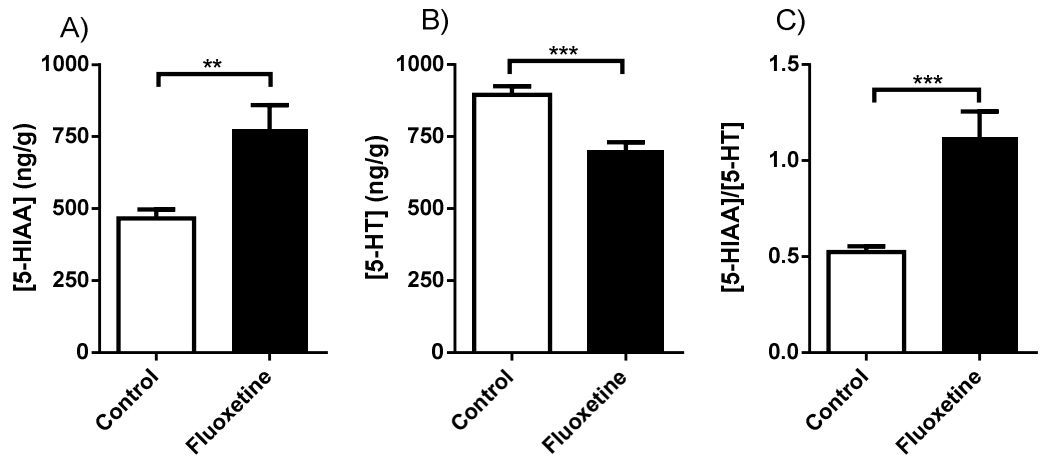

Figure 6: Fluoxetine altered serotonergic signaling in Atlantic salmon after 17 days of exposure.
Mean concentrations (± SEM) in brain stem of 5-HIAA, 5-HT and the [5-HIAA]/[5-HT] ratio for control (n = 15) and fluoxetine (n = 12) fish. Students t test statistics: A) $t_{(13.83)} = 3.428$; $p = 0.0041$. B) $t_{(25)} = 4.546$; $p < 0.0001$. C) $t_{(12.16)} = 4.408$; $p = 0.0008$

Treatment with fluoxetine also significantly increased the concentration of MHPG in the brain stem as compared to controls (Figure 7A, $p = 0.0056$). However, NE levels were found to be similar between groups (Figure 7B, $p = 0.6744$). Meanwhile, the [MHPG]/[NE] ratio was significantly increased in the fluoxetine-treated group compared to controls (Figure 7C, $p = 0.0086$).

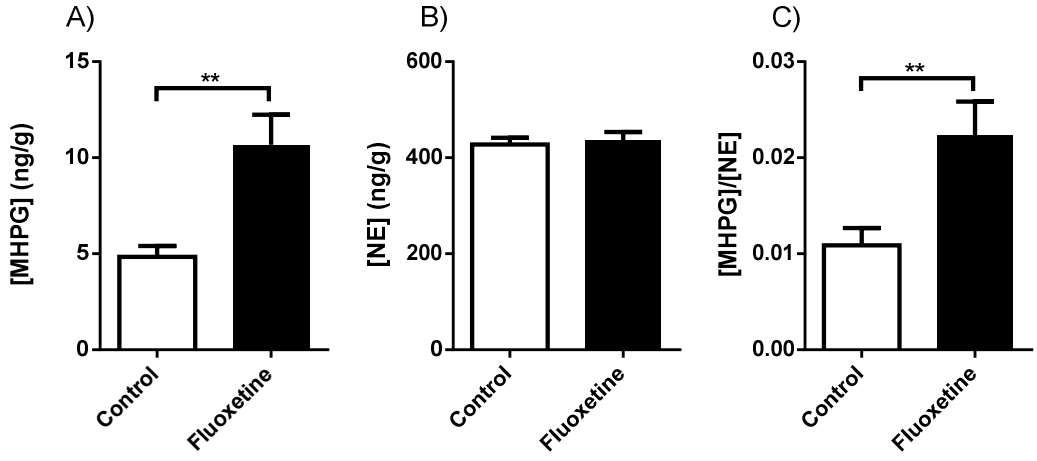

Figure 7: Fluoxetine increased norepinephrine signaling in Atlantic salmon
Mean concentrations (± SEM) in brain stem of MHPG, NE and the [MHPG]/[NE] ratio for control and fluoxetine fish. Students t test statistics: A) $t_{(10.21)} = 3.491$; $p = 0.0056$; $n_{control} = 13$, $n_{fluoxetine} = 9$. B) $t_{(24)} = 4.254$; $p = 0.6744$; $n_{control} = 15$, $n_{fluoxetine} = 11$. C) $t_{(13)} = 3.019$; $p = 0.0086$; $n_{control} = 13$, $n_{fluoxetine} = 9$.

4.2.4 Hypothalamic relative gene expression

Fluoxetine treatment significantly increased hypothalamic AVT (Figure 8A, $p = 0.0402$) and 5-$HT_{1A\beta}$ (Figure 8B, $p = 0.0384$) mRNA levels. Note that significance of 5-$HT_{1A\beta}$ was contingent on the removal of one outlier (Grubbs, $\alpha = 0.01$) from the control group.

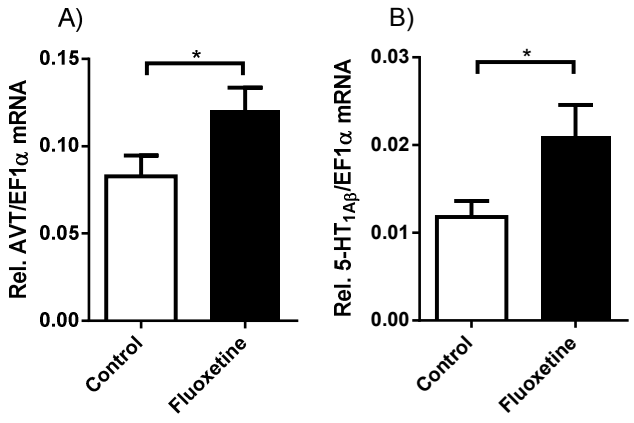

Figure 8: AVT and 5-HT$_{1A\beta}$ hypothalamic mRNA levels are increased by fluoxetine treatment
Average (± SEM) mRNA levels of AVT and 5-HT$_{1A\beta}$ relative to the reference gene EF1α in hypothalamus of control (n = 15) and fluoxetine (n = 12) fish. Students t statistics: A) $t_{(25)} = 2.164$; $p = 0.0402$. B) $t_{(16.39)} = 2.251$; $p = 0.0384$.

No significant differences were found between treatment groups on hypothalamic relative mRNA levels of CRH, MR, GR1, GR2, SERT, 5-HT$_{1A\alpha}$, PCNA or BDNF (Table 2).

Table 2: Average (± SEM) mRNA levels of Corticotropin Releasing Hormone (CRH), Mineralocorticoid Receptor (MR), Glucocorticoid Receptor 1 (GR1), Glucocorticoid Receptor 2 (GR2). Serotonin Transporter (SERT) and 5-HT$_{1A\alpha}$ relative to the reference gene Elongation Factor 1α (EF1α) in hypothalamus of control (n = 15) and fluoxetine (n = 12) fish.

Gene	Control	Fluoxetine	t-statistic	*p*-value
CRH	0.027 (±0.00348)	0.027 (±0.00178)	$t_{(20.47)} = 0.00797$	0.99
MR	0.0001103 (±0.000022)	0.00012 (±0.000023)	$t_{(25)} = 0.2691$	0.79
GR1	0.00037 (±0.000044)	0.00047 (±0.000043)	$t_{(25)} = 1.622$	0.1174
GR2	0.007469 (±0.000822)	0.008835 (±0.001585)	$t_{(25)} = 0.8102$	0.4254
SERT	0.00233 (±0.00042)	0.0031 (±0.00073)	$t_{(25)} = 0.9553$	0.3486
5-HT$_{1A\alpha}$	0.01166 (±0.001564)	0.01383 (±0.00196)	$t_{(25)} = 0.8798$	0.3874
PCNA	0.1178 (±0.01166)	0.1028 (±0.00607)	$t_{(20.68)} = 1.142$	0.2664
BDNF	0.05212 (±0.006523)	0.05117 (±0.004589)	$t_{(25)} = 0.1136$	0.9105

5 Discussion

In the present experiment I found that fluoxetine treatment affected not only the behavior of Atlantic salmon, but also a range of physiological parameters that could be linked to behavioral and endocrine stress responses. In brief, it was observed that fluoxetine decreased receptiveness to food, increased time spent high up in the water column and their tendency to be positioned with their heads elevated. Physiologically, plasma cortisol and the relative heart size were found to be significantly increased. Furthermore, as adjudged from neurochemical analysis, brain stem serotonergic and noradrenergic activity was increased in fluoxetine treated fish. Finally, in the hypothalamus, relative mRNA expression levels of the neuropeptide AVT and the serotonin receptor 5-$HT_{1A\beta}$ were also significantly increased.

5.1 Activity of the HPI axis

Administration of fluoxetine would appear to have induced an activation of the HPI axis in Atlantic salmon, as indicated by plasma cortisol levels. Plasma cortisol concentrations were found to be elevated by a factor of 5 (approximately) in fluoxetine-treated fish, which is comparable to cortisol responses obtained after acute stress in salmonid fishes[23,59]. Considering the effect of fluoxetine on the serotonergic system (discussed further below), the fluoxetine-increased cortisol levels are in agreement with previous findings regarding a stimulatory effect of 5-HT activation on HPI axis activity in comparative models. For example, in the Gulf toadfish (*Opsanus beta*)[45] and Arctic charr (*Salvilinus alpinus*)[44], administration of an 5-HT_{1A} receptor agonist induces a cortisol response. Furthermore, plasma cortisol responsiveness to stress has been reported to be positively associated with heart size in rainbow and brown trout[60]. In previous experiments in this lab on rainbow trout, 21 and 45 days of oral cortisol administration induced a significant CSI increase of 20% and 24% respectively, while shorter treatments of up to 7 days appeared not to have an effect on the CSI[61]. In the present experiment, we found that fluoxetine-treated fish had a 13.8% larger CSI compared to controls, suggesting that the HPI axis activation could have been sustained over many days. Both central and peripheral mechanisms could have caused the simultaneous increase of cortisol and CSI, as fluoxetine treatment is also known to increase plasma serotonin levels[62], which were not measured in this experiment. For example, a peripheral mechanism may act through the 5-HT_{2A} receptor activity, which are found to be expressed in cardiomyocytes and mediate cardiac hypertrophy in murine cardiac disease models[60,63,64]

through the calcineurin/NFAT pathway. Similarly, peripheral 5-HT_{1A} receptors in the head kidney of goldfish (*Carassius auratus*) capable of stimulating cortisol production have been reported[65], but the receptor abundance varies greatly between teleost species[45]. Disregarding the specific mechanism at hand, fluoxetine treatment increases not only cortisol levels but also heart size in salmon after 2 weeks treatment. Furthermore, both these results indicate an increased activity of the HPI axis, possibly over several days.

CRH and AVT (the teleost homolog of AVP) are neuropeptides that play a central activating role in the HPI axis.. As mentioned in the introduction, CRH and AVT induce pituitary release of ACTH which triggers cortisol production from the interrenal cells[66,67]. Fluoxetine treated salmon showed similar transcript levels of CRH in the hypothalamus compared to controls. Although speculative, the measured elevated cortisol levels may have suppressed CRH mRNA levels at the timepoint of sampling, as CRH transcription is known to be under negative feedback control via the glucocorticoid receptor acting on GREs in teleost fish[68] and mammals[30]. mRNA transcript levels of both teleost paralogs of the glucocorticoid receptor and the putative mineralocorticoid receptor in the hypothalamus were similar between treatment groups, hence glucocorticoid-receptor signaling was not affected by fluoxetine. However, I did not find any relationship between cortisol levels and CRH expression in the hypothalamus (results not shown). Notably, it has been reported that glucocorticoid-induced suppression of CRH transcription is region-specific and that hypothalamic CRH is not necessarily downregulated by cortisol[69]. If the assumption is made that transcript levels of CRH in the hypothalamus are reflective of signaling levels of CRH down the HPI axis, then it is possible that in the current experiment CRH was not the cause of the elevated cortisol levels in fluoxetine-treated salmon. This is in contrast to the findings of Mennigen et. al[70] where fluoxetine treatment in goldfish, at a similar averaged daily dose every 3 days for 2 weeks, increased hypothalamic CRH mRNA. The discrepancy might be attributed to the different dose timings used (i.e. every 2 days in the current experiment vs. 3 days in theirs).

On the other hand, mRNA levels of AVT in the hypothalamus were found to be significantly elevated in fluoxetine-treated fish. AVT, apart from independently stimulating ACTH release[71] and negatively regulating appetite in fish[72] and mammals[30], also has a potentiating effect on CRH function in salmonids[71]. In mammals, CRH and AVP neurons in the paraventricular nucleus of the hypothalamus are under direct control by serotonergic signaling through the 5-$HT_{2A/C}$ and 5-HT_{1A} receptors[38,73]. Therefore, it is possible that AVT could have

been the mechanism of HPI axis activation in fluoxetine-treated fish, induced by an increased 5-HT signaling to fluoxetine administration. In teleosts, central administration of AVT in isolated rainbow trout induced sizable increases in mean plasma cortisol concentrations[72,74]. In addition, Gesto et al.[72] found increased serotonergic activity in the hypothalamus and the telencephalon in response to central administration of AVT, which the authors cite as evidence of bidirectional crosstalk between AVT signaling and serotonergic activity. In contrast to the results found in the present experiment, blue head wrasse (*Thalassoma bifasciatum*), administered fluoxetine daily for 2 weeks, had a decreased expression of AVT in the anterior hypothalamus[75]. This discrepancy may be the product of dose-dependent effects, as the averaged daily dose administered by Semsar et. al.[75] was three-fold higher than in my experiment. It is possible that different doses of fluoxetine have different effects, as the presynaptic 5-HT receptors appear to have greater sensitivity to 5-HT signaling[46]. In addition, the relatively more stressful regime of daily injections used by Semsar et. al.[75] may have caused a compensatory downregulation of AVT mRNA similar to that found in rainbow trout subjected to repeated stress[76].

5.2 Feeding Behavior

An anorectic effect of both acute and chronic fluoxetine administration is documented in humans[37], murine models[37,77,78] and in teleosts[58,70]. In the present experiment, the response to food in Atlantic salmon was decreased after exposure to fluoxetine. This decline was particularly marked following the switch from oral to *i.p.* administration, perhaps due to increased bioavailability of fluoxetine. Importantly, I do not believe that the decrease in food consumption was due to the stressfulness of *i.p.* injections, since this did not seem to affect the appetite of vehicle-injected controls (see below for the implications of the switch in mode of administration). Furthermore, the switch from oral to *i.p.* administration was made because of an increasing reluctance of fluoxetine-treated salmon to accept food, which is further evidence that the anorectic effect was not specific to the mode of administration. Also, the fluoxetine treated salmon refused both untreated food pellets and treated shrimp, demonstrating that anorectic behavior was not due to aversive taste conditioning. Feeding behavior in mammals and teleosts is under reciprocal control of a feeding and a satiety center, located in the hypothalamus[48] and innervated by serotonergic fibers[37]. A diverse array of orexigenic and anorectic CNS and peripheral neuropeptides modulate feed intake[79]. In teleosts, a common consequence of stress is a disruption in feeding behavior, an elevation of

serotonergic activity and an activation of the HPI axis[66]. Interestingly, CRH and AVT have been shown to modulate both the HPI axis and feeding behavior by independent mechanisms[80,81].

The mechanisms mediating the anorectic response in the present experiment to fluoxetine are still unclear. Direct serotonergic signaling to the hypothalamus feeding centers mediated through 5-HT_{1B} and 5-HT_{2C} receptors is one possible mechanism for anorexia which has been described in murine models, along with the action of 5-HT on peripheral receptors[37]. Central effects of serotonergic signaling on appetite in teleosts have also been demonstrated, but 5-HT_{1A} and 5-HT_{2C} appear to be involved, while 5-HT_{1B} is not[34]. AVT signaling triggers transcription of 2 anorectic neuropeptides, CART and α-MSH (derived from POMC)[72]. Anorexia as a result of fluoxetine administration in goldfish has also been previously reported[70], probably induced via CRH.

In addition, the neuropeptides CRH and AVT are also potent activators of the HPI axis as discussed above. As elevated cortisol levels were seen in the present experiment in the anorexic group, it would be relevant to consider the possible contribution of elevated cortisol to this anorexic effect observed in fluoxetine-treated fish. In mammals, moderately elevated plasma cortisol levels have orexigenic effects[82]. Similarly, stress-induced cortisol elevation has been linked to a reduction in feed intake in many teleost studies. These studies are complicated by the fact that the stressors used, such as social or confinement stress can regulate via serotonin and/or neuropeptides (e.g. CRH) feeding behavior independent of cortisol[80]. In two rainbow trout studies, exogenously administered cortisol induced a loss of appetite[83,84]. The interpretation of one of these studies has however been questioned, as there was significant aggression found among the cortisol-treated cohort which might have been the cause of anorexia, rather than cortisol[80]. On the other hand, Øverli et. al.[85] report no such reduction in feed intake during oral administration of cortisol to rainbow trout. However, published and unpublished results from our lab indicate that direct oral administration of cortisol to isolated rainbow trout have found no reduction over short periods of time (e.g. 2-4 days), but significant reductions in food intake over longer periods (7-21 days) at plasma cortisol concentrations comparable to levels found under chronic social subordination[86]. Furthermore, Nørstrud et. al.[61] reports that the observed reduction in food intake was associated with a "spitting" behavior, whereby the fish would attempt to eat the food, but were apparently unable to swallow. This was distinct from the present experiment, where no

attempts to eat food were seen at all in long term fluoxetine-treated fish. While the possibility cannot be excluded that the fluoxetine-induced anorexia in the present experiment was a cortisol-induced effect, the dramatic reductions of feeding behavior seen in contrast to cortisol administration studies suggest that 5-HT mediated AVT signaling could be a more likely mechanism.

5.3 Monoamine signaling and behavior

In addition to decreased feeding behavior, there were two other, possibly closely related behavioral responses which emerged in the fluoxetine-treated salmon group. The first was a dramatic increase in time spent at the top half of the water column (described in the literature as a surfacing behavior[51] reduced geotaxis[87] or top-dwelling[51]) while the second was a tendency to be positioned "head up, tail down" at a near vertical angle. These two behavioral phenotypes have been observed in other studies involving the serotonergic system, either separately or together. Surfacing behavior after fluoxetine treatment has been reported in mosquitofish (*Gambusia affinis*)[50], hybrid striped bass (*Morone saxatilis* x *M. chrysops*)[58] and several zebrafish[87] studies. This is however inconsistent as other studies have also reported contrary results[57]. Furthermore, the "head up, tail down" behavior has also been observed in the mentioned hybrid striped bass experiment after fluoxetine treatment[58].

Even though the mechanisms that underlie these behavioral responses to fluoxetine treatment, are still unknown, it might be possible that these behaviors are evidence of 5-HT toxicity elicited by massive drug-induced overdoses that do not occur physiologically. Stewart et. al.[51] supports this argument, and considers these behaviors as teleost correlates of the 5-HT syndrome. The 5-HT syndrome has been described in mammals under similar circumstances, such as during an SSRI overdose or when two or more serotonin-perturbing treatments are administered together. Herculano et. al.[87] points out, however, that even though 5-HT toxicity cannot be excluded, in some cases smaller doses were more effective at eliciting surfacing behavior than much larger doses.

Interestingly, subordinate farmed Atlantic salmon subjected to chronic social stress (inferred from physiological parameters) display a surfacing, head up tail down and anorectic behavioral phenotype very similar to that observed in the present experiment[2]. In addition, cortisol and brain 5-HT parameters in these fish were found to be elevated compared to normally behaving control fish under basal conditions[27]. Surfacing and head up tail down

behavior has also been seen in subordinate rainbow trout and Arctic charr after a single dyadic contest, commonly displayed after receiving high levels of unilateral aggression from the dominant (Riise & Øverli, unpublished observations). Subordination in a dyadic contest in rainbow trout and Arctic charr is known to increase serotonin turnover in the brainstem (and other regions)[23,24]. Extrapolating these findings (which are similar to those found in rats[88]) to zebrafish and/or Atlantic salmon, we can speculate that physiologically relevant levels of serotonergic activity may be capable of producing the surfacing, head up tail down behavioral profile exhibited in the current experiment.

In the present experiment, indicators of serotonergic and norepinephrine activity were increased by fluoxetine treatment. Increased 5-HT and NE activity has been associated with generally stressful situations[20] such as social subordination[23] and nonsocial stressors[89]. Therefore, it appears that the physiological and behavioral profile of a fish undergoing chronic social stress strongly resembles the physiological (i.e. increased cortisol, relative heart size, 5-HT and NE activity) and behavioral (increase in surfacing, head up tail down and anorectic behavior) profile exhibited by the fluoxetine-treated salmon in this experiment.

Even though the neurochemical profile of the salmon in the current experiment bear strong similarity to that of chronically subordinate fish, the possibility cannot be dismissed that 5-HT_{1A} autoreceptors might also have been strongly activated on serotonergic neurons of the hypothalamus. In the hypothalamus, the serotonin receptor 5-$HT_{1A\beta}$ was upregulated while 5-$HT_{1A\alpha}$ was not. This upregulation could suggest that neurons expressing 5-$HT_{1A\beta}$ were preferentially active compared to those expressing 5-$HT_{1A\alpha}$. In mammals, the expression of 5-HT_{1A} in neuronal cells is negatively regulated by Freud1 and Freud2 repression factors[43]. Freud1 and Freud2 are in turn negatively regulated by intracellular calcium[90,91], which could come from voltage-gated calcium channels or intracellular calcium stores during activity[92,93]. A presynaptic upregulation of the autoreceptor decreases serotonergic tone, while a postsynaptic upregulation increases serotonergic signaling. From these data, it is however not possible to speculate on the identity (presynaptic serotonergic or postsynaptic, non-serotonergic, possibly neuropeptidergic) or outgoing transmission of the 5-$HT1_{A\beta}$ expressing neurons. It is interesting to note that a genotype indicative of 5-HT vulnerability to depression (C(-1019)G(rs6295)Htr1a), is predicted based on murine models to result in increased 5-HT_{1A} autoreceptor expression on serotonergic raphe cells[43]. Thus, the 5-$HT_{1A\beta}$ upregulation in the current experiment may be a precursor to pathology.

The role of fluoxetine treatment on SERT mRNA levels in the raphe nuclei reported in the mammalian literature describe mixed effects, either upregulation, downregulation, or no changes[94]. Furthermore, in teleosts, the transcriptional response of SERT to fluoxetine has not yet been characterized. Therefore the interpretation of the results obtained in the present experiment (no change in SERT mRNA levels in the hypothalamus), remains to be elucidated.

5.4 Markers of Neurogenesis and Neuroplasticity

The modulation of neurogenesis and neuroplastic markers in teleosts in response to environmental stimuli is thought to follow a biphasic U-shaped curve, where mild and/or acute stress are stimulatory and severe and prolonged stress are inhibitory[95]. For example in rainbow trout subjected to chronic social stress, cell proliferation in the telencephalon is decreased[25]. One of the proposed mechanisms modulating neurogenesis in mammals[96] as well as in teleosts[86] is cortisol. While cortisol levels of fluoxetine treated Atlantic salmon in the current experiment were significantly elevated to levels comparable to chronic stress[54], no effects on mRNA levels of the cell proliferation marker PCNA or the neurotrophic peptide BDNF were found in the hypothalamus. As only a single time point was sampled, it is not possible to establish if the lack of change was maintained throughout the experiment or if it had crossed the biphasic curve described in Sørensen et. al.[95].

5.5 Experimental Considerations

In the administration of fluoxetine to salmon in the current experiment, there are two considerations that may have affected the results, or the interpretation thereof. The first is an interruption of treatment early on in the experiment. Due to logistical issues, the time between the first and the second dose of the drug was 5 days, instead of 2 days as was maintained for the remainder of the experiment. The half-life of fluoxetine is 2 days in humans[52] and 15hrs in rats[97] at the start of treatment. In hybrid striped bass, 6 days of recovery after 6 days of immersion treatment (100µg/L) is sufficient for 5-HIAA concentrations in the brain to recover to pre-exposure levels[58]. While it is difficult to compare immersion doses with *i.p.* administration doses, it would be prudent to consider the primary time course of treatment as a 12 day treatment, starting on Day 5 of the experiment. If the pharmacokinetics of elimination in Atlantic salmon are similar to mammals, it is also of note that fluoxetine inhibits its own metabolism, causing the half-life in humans to rise from 2 to 6 days after

multiple treatments[52]. It is unlikely but cannot be excluded that the initial dose affected metabolism of the subsequent treatments as a small amount of fluoxetine might still be present after 5 days, thus accelerating the appearance of the behavioral phenotypes observed.

The second consideration is the switch from oral to *i.p.* administration at Day 11 of the experiment, necessitated by the increasing reluctance to accept food. Orally administered fluoxetine is subject to extensive first-pass metabolism by the liver, with only 38% reaching general circulation in humans[52]. To our knowledge, no studies on the oral bioavailability of fluoxetine in teleosts have been done, as ecotoxicological studies are focused on the gills as the most likely mode of entry. On the assumption that first-pass metabolism in the liver by Atlantic salmon removes a significant, but unknown proportion of orally administered fluoxetine, the behavioral data could be considered a preliminary evaluation of dose-dependent effects, with higher effective doses producing stronger effects. Notably, all behavioral effects reported were also observed to a lesser degree prior to the switch in administration. Thus, the behavioral effects described here appear not to be specific to the mode of administration.

6 Conclusions and Future Perspectives

Delivery of the drug fluoxetine in food to Atlantic salmon using rehydrated shrimp was partially successful, as long term treatment induced loss of appetite. Oral administration of exogenous substances incorporated in food is a relatively stress-free mode of administration, which is important when attempting to study the stress response, as habituation or sensitization to a repeated procedural stressor may alter or mask treatment effects. In future work, oral delivery of any substance raises the consideration of first-pass metabolism by the liver. These effects cannot be dismissed by current behavioral data, especially since stronger effects were seen after the switch to *i.p*. administration, even though the nominal dose delivered was unchanged. A possibility that there was an interaction between drug and injection procedure cannot be excluded.

In the current experiment, Atlantic salmon treated with fluoxetine for 2 weeks show an array of physiological and behavioral responses that seem to parallel those observed in conditions of chronic stress. The hypothalamic neurogenic markers however, appear not to be affected by fluoxetine treatment. This experiment was done in social isolation, with no external stressor or social interaction applied. A likely interpretation then is that the serotonergic activity seen during social stress may be the primary driver of many physiological and behavioral responses to social subordination. Notably, a 5-$HT_{1A\beta}$ upregulation was found in the hypothalamus. Given the central integrating role of the hypothalamus in the regulation of the stress response, it is of particular interest to elucidate whether this upregulation is specific to autoreceptors in serotonergic neurons (which reduces serotonergic tone), or on target neurons which mediate downstream effects of serotonergic signaling. It is also striking that social interactions are not required to elicit behavioral responses that are remarkably similar to those elicited under social stress. This suggests that the head up tail down and surfacing behaviors reported by Vindas et. al.[27] in Atlantic salmon and Riise & Øverli (Riise & Øverli, unpublished) in subordinate Arctic charr and rainbow trout, may not be conditioned responses to species-typical aggression, but rather a more generalized response to severe stress. However, the stress response is highly interconnected and it is thus difficult to disentangle the causative roles of each component under conditions with an external stressor. Therefore, manipulation of specific components of the same, in the absence of a stressor, may allow dissociation of each component under experimental conditions.

As the resolution of the current experiment is relatively broad, a more detailed examination of these specific results should be attempted. For example, investigation of the specific localization of the 5-HT receptor upregulation could yield information on specific neuronal populations that are affected. Also, suitable in-vivo molecular and genetic methods, such as calcium imaging and transgenic lines, may be of use in a finer-grained examination of neural circuitry involved in the behavioral responses. In addition to exploring the current results in greater detail, another venue of inquiry could focus on elucidating more of the similarities and differences in behavior and physiology between pharmacological and stressor-induced serotonergic activity in the brain. A third line of questioning could seek to understand the mechanisms behind the heart size increase in fluoxetine treated animals, as it is not clear if this is caused by central or peripheral mechanisms.

In conclusion, this study shows that fluoxetine treatment of Atlantic salmon for 18 days induces heightened serotonergic and noradrenergic activity in the brain stem, causes behavior that is similar to that observed in highly stressed salmonids, increases heart size, activates the HPI axis and upregulates a 5-HT receptor that is closely associated with affective disorders in the hypothalamus. This illustrates how comparative animal models may be used to discern the mechanisms involved in pharmacological manipulation of brain systems.

7 References

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