Norwegian University
of Life Sciences

Master's Thesis 2020 60 ECTS
Faculty of Environmental Sciences and Natural Resource Management

Characterizing and Reversing the Salmonid Depression-like State Profile

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Acknowledgements

Firstly, I would like to give my sincerest thanks to my main supervisor, Dr. Marco Vindas, for providing me with the opportunity to do this project and guiding me through completing it in spite of every possible challenge that emerged. I learned so much valuable information throughout the process of running the experiment, analyzing the data, and writing up the results that has left me equipped with a whole new wealth of knowledge and skills moving forward. Additionally, I would like to thank my co-supervisor Dr. Øyvind Øverli for all of the guidance I received turning this thesis into a polished and effective final product. Also, without the continued help of Tone Vågseth, Ole Folkedal, and Ivar Helge throughout my time at Matre, the buspirone and novel environment experiments would not have been possible, thank you again for everything.

The path to completion was a tough one, and sometimes felt as if every unlucky thing that could have happened did, but I could not be happier with this completed thesis and the overall experience that led to writing it. To my friends both near and far, thank you for offering both help and much needed laughs and distractions when I felt stuck, I'm sure you're all relieved that I'm done talking about salmon for a while at least. Finally, I would like to thank my family for their unending patience as I stayed up to the early hours of the morning working, stressed over every minute detail, and explained every thrilling detail of my writing process more than once. Specifically, I would like to thank my father Shahram Shapouri for stepping in every time I mixed up my document formatting beyond recognition or came close to losing months of work due to technical issues; this thesis would not have been remotely as polished looking and functional without the continuous technical help.

Abstract

Depressive or anxious symptoms are typically connected to chronic and unpredictable high-stress situations or otherwise adverse experiences. In aquaculture, hierarchical disputes and confrontations between individuals in dense populations are difficult to avoid or defuse as a result of the confined environment, meaning that generally submissive and reactive fish are typically chronically stressed and continually exposed to environmental stressors. Prolonged exposure to these and other stressors can result in a sustained depression-like state (DLS) that inhibits growth and social behaviors, while also reducing immune capability and greatly increasing the risk of mortality.

Chemically treating the serotonergic system of DLS fish and/or changing their social environment is theorized to stimulate interactive behaviors as well as increase interest in food, beginning the reversal of the growth stunted state. In this study, buspirone, a serotonin agonist, was used to decrease anxiety symptoms in young Atlantic salmon (*Salmo salar*). In addition, the environment of potential DLS fish was manipulated to try and reverse the DLS profile. The aims were to determine the exact effect of buspirone on control fish via bath treatment, manipulate the serotonergic system to reverse the DLS profile, and determine if reversal of this profile can also be achieved purely through altering their environment. To determine dosages and the appropriate method of administering buspirone to fish, a pilot study was performed on a series of healthy juvenile salmon that were experiencing temporary stress from a novel environment. This experiment showed buspirone having a clear effect on reducing stress, resulting in less socially inhibited behavior and significantly increased movement and usage of the entire water column. However, when DLS fish were treated with this same dosage, there was no change in behavior, with all fish continuing to show stress behaviors such as tight grouping and less independent movement.

While no behavioral changes were noted, buspirone treated fish had apparent higher plasma cortisol levels when sampled after acute stress confinement, as well as showing a more heterogeneous response compared to untreated fish. Meanwhile, basal (unstressed) levels remained the same for both treatments. This reversed response from DLS fish versus healthy controls could suggest an alteration in the serotonergic system in these fish affecting the reaction to serotonin agonists, possibly as a result of prolonged stress. Finally, altering the social dynamics of fish showing the DLS profile by removing larger competition and eventually transitioning them to saltwater tanks showed growth and survival rates that rivaled their non-DLS counterparts, showing that some variations of this DLS profile can be reversed.

1. Introduction

Humans have been domesticating the animals around us for the past 11,000 years, adapting wild species for labor, food, and companionship. Mammals, birds, fish, and even reptiles have been domesticated over the years to meet the demands of human food production (Zeder, 2012). Recently, aquaculture has emerged as a formidable industry to supplement the globally rising demand for nutrition in the human population. The most large-scale industrial farming processes typically involve salmonids such as rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). Atlantic salmon farming especially is now a driving economical force in European nations such as Norway and Scotland, and is expanding worldwide. With this rapid growth comes questions and concerns regarding animal welfare, not only in the context of the conditions the animals are kept in but the limits of adaptation for these animals after only a few generations in captivity. Inside this artificial, limiting environment, the natural diversity of behaviors and strategies these fish employ in the wild is stifled. In captivity and the highly competitive environment it fosters, certain subsets of the population simply cannot adapt and inevitably fall behind.

1.1 Study Species

Salmonid fishes have been steadily increasing in interest as the focus of a myriad of scientific studies in recent years. This is partly due to the fact that these species have always been economically important for food and their recent introduction to intensive farming (*i.e.* aquaculture) systems (Liu et al., 2011). Salmonids are also the focus of many ecological and biomedical studies. Rainbow trout (*Onchorhynchus mykiss*), for example, have been used as models in the fields of genetics, cancer research, and toxicology (Thorgaard et al., 2002). Ecologically speaking, Atlantic salmon (*Salmo salar)* have a complicated life cycle which includes fresh- and saltwater stages (Figure 1) which also makes them subjects of great interest in behavioral and evolutionary ecology. That is, salmon are anadromous fish, which means that they spawn in freshwater, spend their adult lives at sea and return to their natal freshwater bodies to spawn (Hoar, 1988; Stefansson et al., 2008). Anadromy is a life history strategy typically fraught with hazards and requires a period of metamorphosis in which individuals transition from one stage to the next, typically initiated by seasonal hormonal changes. Anadromy is a strategy chosen on the individual level based on overall fitness, growth, and the general value of taking such a dangerous risk for the benefit of increased resources out at sea (Railsback et al., 2014). With access to greater food resources, individuals that have migrated out to sea grow larger, improving their reproductive fitness and social position amongst the group upon their return to their natal rivers and streams (McDowall, 2001).

In the wild, Atlantic salmon spawn in shallow, fast flowing freshwater rivers or streams. The eggs, once fertilized, lie buried under a layer of gravel for up to several months. Newly hatched salmon, called alevins, remain beneath the gravel layer on the stream bed until their egg yolk sacs are fully absorbed. Once grown into free swimming fry, they emerge from the stream bed and into the water column to grow into the next life stage for a young salmon, known as the parr stage (Keenleyside & Yamamoto, 1962). During this stage, parr feed intensively on aquatic invertebrates and insects by establishing territories, which they aggressively defend from intruders, in high-velocity water flow areas, chosen to maximize feeding opportunities (Orlov et al., 2006, Keenleyside & Yamamoto, 1962).

With this territorial and aggressive behavior, social hierarchies characterize salmon populations. In this context, environments in nature with a steady, reliable food supply have been shown to favor the growth of socially dominant individuals, and therefore result in an uneven distribution of resources, while subordinates tend to fall behind in growth due to lack

of access to food resources and territories (Harwood et al., 2003).While socially dominant individuals are typically first to attain adequate size for sea migration, their absence allows subordinates to take advantage of food resources and eventually grow enough to migrate, though in a much longer time-frame than their dominant counterparts. Studies on juvenile Arctic charr (*Salvelinus alpinus*) have shown that while socially subordinate individuals experience inhibited food intake in the presence of larger dominants, the absence of these dominant individuals reverses the inhibition and causes the smaller fish to eat normally (Øverli et al.,1998). This parr stage lasts on average about two to four years (Hansen & Quinn, 1998).

Prompted by seasonal changes in both photoperiod and temperature, as well as by body fat resources, the parr undergo a process known as smoltification, which entails a series of physiological, morphological and behavioral changes that prepare them for migration and life out at sea. Adaptations suited for life in freshwater are transformed to then sustain life in seawater, maintaining osmoregulation as well as effectively concealing themselves from predators. While parr have a dark coloration, which helps them blend with the substrate (since they swim close to the stream bottom against the current), smolt have a light silver tone on the body which helps them blend with the water since they swim often at the top of the water column in the sea. Coloration and pattern changes from parr to smolt are regulated by pigment compounds called purines, specifically guanine and hypoxanthine, resulting in the smolts' silver coloration (Hoar, 1988). In addition, smolts forgo territorial behavior in favor of protective schooling and move together downstream towards brackish estuaries and eventually into the open ocean (Stefansson et al., 2008; Orlov et al., 2006). One of the key sites of this smolt transformation is the gill, which is the main site for hydromineral balance and osmoregulation in fish (Breves et al., 2017). In short, the salmon gill changes from retaining salts in a freshwater rich environment (*i.e.* rivers and lakes) to retaining water in a salt rich environment (the sea). Without this important adaptation, salmon would not be able to migrate into the sea (Fjelldal et al., 2018). Smolts are also characterized by a lean body, which is the result of increased oxidative metabolism, with a rate of oxygen utilization to make energy from carbohydrates around 30% higher than parr, which leads to reduced body weight and loss of fat reserves (Hoar, 1988).

After the smoltification process, salmon living in the ocean are known as post-smolts and display far less territorial and aggressive behaviors towards each other, favoring schooling behavior in the open water. When out at sea, early post-smolts typically select a depth where they do a majority of their swimming, typically one to three meters from the surface, based on factors such as avian predation risk, temperature, salinity, and food availability (Thorstad et al., 2012). As previously explained, the principal benefit of anadromy is the increased access to abundant food opportunities, which allows salmon to increase their body mass of up to 1000-fold (Rikardsen & Dempson, 2010). Salmon out at sea are highly opportunistic feeders, feeding on various different species of fish and invertebrates to support this rapid growth spurt needed to return upstream in their natal rivers to spawn (Thorstad et al., 2012).

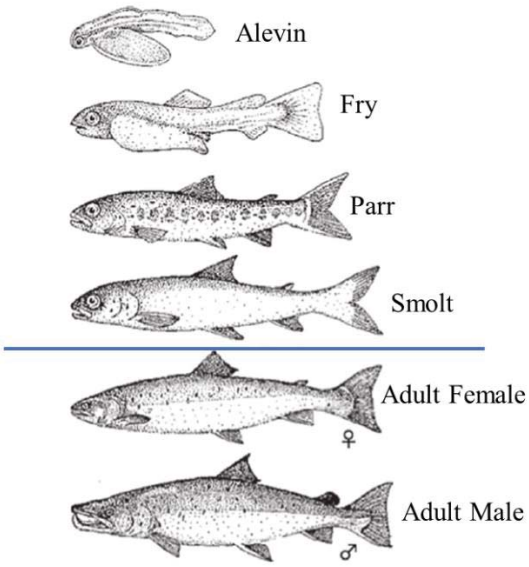

Figure 1. Salmon life cycle, showing freshwater life stages above the blue line and saltwater stages below. Illustration has been modified from Mcmenamin & Parichy, 2013.

1.2 Domestication and Individual Variation

In an aquaculture environment, certain traits are artificially selected for in order to maximize profitability, such as rapid growth. Other traits meanwhile, such as tendencies for aggression and dominance, are involuntarily selected for in conjunction with the intentional ones. For example, rapid growth has been associated with more dominant and aggressive individuals, which are not really suited for keeping large numbers of fish in close proximity. This is an unnatural situation since in aquaculture systems the selection for traits that promote a more consistent access to food in a dense environment benefits exclusively socially aggressive individuals, while in the wild, multiple different approaches to food acquisition can be equally successful and not necessarily particularly favored (Cubitt et al., 2008). In this context, farmed Atlantic salmon raised for generations in aquaculture systems are more aggressive than their wild counterparts and readily beat them in dyadic dominance contests (Adams & Huntingford, 2005). Since high aggression is counterproductive in farm environments, breeding programs should ideally select for traits that generally fare better in crowded, dense environments with consistently available food, though achieving this ideal is generally unrealistic (Adams & Huntingford, 2005).

Regarding the seawater phase in sea cages, salmon cannot engage in typical behaviors found in the wild, such as migratory, spawning or feeding behaviors, since they are limited to a relatively small contained area of water densely populated with other salmon (Juell, 1995; Fernö et al., 2011). In this setting, in which a natural diversity of behaviors and strategies are essentially impossible, fish are forced into more inflexible conditions which some individuals may experience as highly stressful, in addition to also being exposed to a series of unnatural stressors, such as handling and delousing (Pickering & Pottinger, 1989). While a natural stream or river environment provides a varied selection of situations and conditions that each favor different life history strategies, aquaculture settings contain crowded populations of fish all in an identical situation. This manufactured environment essentially now favors more aggressive and bold individuals due to the nature of fish farms:

dense populations taking advantage of a consistent food source without the threat of predators (Fernö et al., 2011).

Commonly found in aquaculture environments is a large size and growth disparity between fish, as some succeed in coping with this artificial environment, while other individuals do not and therefore grow slowly or not at all. This inability for some individuals to succeed in farm conditions has been often linked to socially dominant individuals consuming more food overall compared to submissive fish, and creating a growth differential that only widens over time (Cubitt et al., 2008). While growth disparities in nature often lead to larger fish migrating earlier downriver towards the ocean, smaller fish remain and grow for an additional amount of time, and therefore, subordinate individuals may have a chance also at acquiring resources (Harwood et al., 2003). However, the confined nature of aquaculture environments keeps big and small fish together, drawing from the same food and territorial resources which promotes and maintains growth disparities. Furthermore, additional features of the aquaculture industry are also contributing factors to the growth disparity found in these populations. That is, the aquaculture environment may be experienced as unusually chaotic for some fish, with regular movement through sorting, vaccination, changing containers, and increased susceptibility to illness and predators, such as birds, in a confined space (Fernö et al., 2011). These compounding stressors also contribute to repressing natural behaviors and limit growth. In salmonids, stress often leads to behavioral inhibition, including anorexia (Øverli et al., 2004; Vindas et al., 2019; Øverli et al., 1998). Responses to stressful stimuli are discussed further in section 1.4.

Coping styles is a term used to describe the set of behavioral and physiological responses to stressors on the individual level that remain consistent over time (Koolhaas, 2008; Øverli et al., 2007). Fish who are typically more socially dominant tend to have more of a proactive coping style, counteracting stressors by actively avoiding them or responding with aggression (Øverli et al., 2007). Conversely, timid and submissive fish tend to evade conflict entirely by remaining on the bottom or close to the sides of their environments. This response to stress is known as a reactive coping style. In dominance contests, for example, a socially subordinate individual responds with immobility and reduced levels of aggression and interactions (Øverli et al., 2007). Remaining submissive and taking avoidant measures to not encounter conflict in an aquaculture setting results in drastically lessened access to food and reduced growth overall (Metcalfe et al., 2003). Because aquaculture environments (and hence associated genetic selection programs) generally favor individuals with proactive coping styles, allowing them to take advantage of the available resources and grow more successfully, reactive individuals fall progressively lower on the social ladder and are more likely to experience chronic stress in aquaculture environments. Coping styles and personalities are linked to both neurochemistry and an individual's endocrine system, meaning that chronic, inescapable stress can potentially have long-term adverse effects on physical fitness and the normal exhibition of behaviors (Øverli et al., 2007; Vindas et al., 2017).

1.3 Depression-like States

As explained above, chronic stress can occur for some individuals as the result of living in aquaculture environments with, amongst others, forced social dynamics, handling and restricted access to food. For example, subordinate individuals do not have the same opportunities for escape, avoidance, and defusing conflict situations as they would in the wild, where space confinements in rivers, streams and the ocean are less restrictive and often allow for targeted fish to simply move away from dominants and other stressors (Keenleyside & Yamamoto, 1962; Adams & Huntingford, 2005). In addition, regardless of dominance status, reactive individuals may be unable to adequately cope or respond to

stressors common in aquaculture, which may result in maladaptive behavior. In this context, it is common that in aquaculture systems, a subset of the salmon population is often found to be lagging behind in growth, uninterested in food, rapidly losing weight to the point of emaciation, and showing very little movement or behavior towards others (Vindas et al., 2016; Stien et al., 2013). This condition is similar to what is described in mammals as learned helplessness and is referred to as a depression-like state (DLS), likened to major depressive disorder in humans (Vindas et al., 2019). These fish are also generally thought to not tolerate saltwater and the transition into saline environments, and are typically not moved into sea cages in aquaculture settings while normal conspecifics are.

As described previously, while some individuals would approach a challenging situation or threat in a more aggressive and direct manner, it has been shown that others take a more conservative approach in terms of resource usage and energy expenditure, as well as reduce their risk of injury (Hsu et al., 2008; Briffa & Elwood, 2009). Essentially, it is hypothesized that the DLS phenotype could be an adaptative strategy for vulnerable individuals to avoid adverse interactions with other more socially dominant and aggressive conspecifics, particularly in risky environments, where social threats cannot be escaped or avoided (Nesse, 2000; Vindas et al., 2019). However, this strategy is not without its own risks, since inadequate nutrition results in lower weight, suppressed immune responses, and heightened risk of death (Larson et al., 2006).

In addition to reduced growth, a depressive state causes impaired cognitive functioning, lower overall physical health, and increased risk of mortality (Nettle, 2004; Larson et al., 2006). Additionally, elevated stress puts fish at risk by lowering the efficacy of their immune system response, heightening the potential of fatal bacterial or fungal infections (Pickering & Pottinger, 1989; Tort, 2011). It is hypothesized that the adaptiveness of a DLS is dependent on the environment. That is, while in the wild a DLS could be an effective strategy for temporarily disengaging from a risky or possibly fatal interaction that may be reversed once the risk has subsided, an artificial environment, such as aquaculture conditions, prolong these adverse interactions indefinitely, effectively extending the period of a DLS leading to pathology (Vindas et al.; 2016; Nesse, 2000; Vindas et al., 2019). Reversing this profile successfully in the freshwater stage would considerably reduce mortalities caused by chronically stressed, potentially immunocompromised fish entering saltwater environments, currently a commonplace issue in aquaculture.

1.4 The Stress Response

Stress is generally defined as a condition in which effectively maintaining homeostasis and essential life functions is disturbed or threatened by stimuli known as "*stressors*" (Wendelaar-Bonga, 1997; Korte et al., 2005). This disturbance elicits a series of physiological and behavioral processes known as the stress response. DLS fish show a constant stress response as a key symptom of this state, meaning their bodies are continuously experiencing the repercussions of chronic stress (Vindas et al., 2016). In teleost fish, the function of both the brain-sympathetic-chromaffin (BSC) and the hypothalamic-pituitary interrenal (HPI) axes govern the most effective way an individual can address or cope with a stressor (Wendelaar-Bonga, 1997). Activation of the HPI axis results in the release of cortisol into the blood, which triggers metabolic processes such as glycolysis in order to maximize efficiency of energy reserves and provide enough energy to escape or otherwise respond to a stressor (Sadoul & Geffroy, 2019). The BSC axis facilitates the secretion of epinephrine and norepinephrine into the circulatory system in order to improve the efficiency of oxygen transport through the blood (Wendelaar-Bonga, 1997).

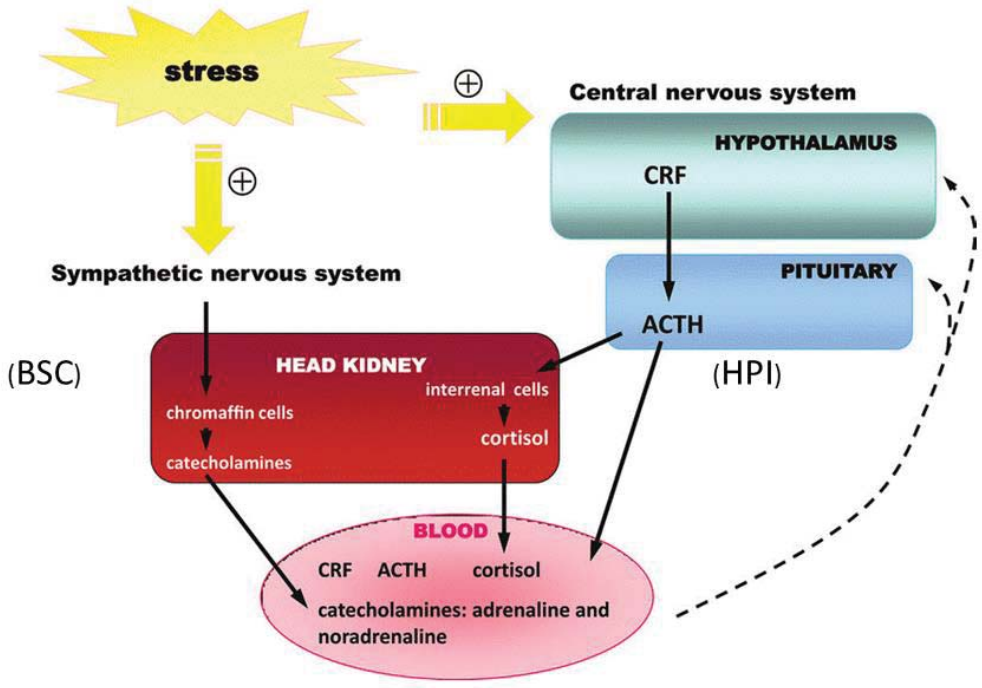

Figure 2: An illustration showing the hypothalamic-pituitary interrenal (HPI) and brain-sympathetic-chromaffin (BSC) axes in fish responding to stress. Illustration has been modified from Kalamarz-Kubiak, 2018.

1.4.1 Cortisol

Corticosteroids are steroid hormones responsible for regulating stress and immune responses in vertebrates, including teleost fish. Cortisol is a naturally occurring glucocorticoid steroid hormone, which, in fish, is synthesized mostly in the interrenal tissue (Milla et al., 2009; Mommsen et al., 1999). Secretion of cortisol is controlled by the hypothalamic-pituitary interrenal (HPI) axis in fish, where the hypothalamus upon receiving a stressful stimulus, releases corticotropic releasing factor (CRF) which promotes the release of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which in turn promotes the secretion of cortisol from the interrenal tissue (Mommsen et al., 1999). The interrenal cells produce cortisol and secrete it into the blood (Sadoul & Geffroy, 2019). The release of cortisol into the body triggers metabolic processes meant to manage the stressful situation

that initiated the release. Glycolysis and gluconeogenesis prompted by the secretion of cortisol provide energy to effectively enact confrontations, escape, or other responses to stressors (Sadoul & Geffroy, 2019). In addition, cortisol affects amino acid metabolism, output of ammonia, and lipolysis, directing bodily resources and energy reserves towards responding to stressors (Kalamarz-Kubiak, 2018). All of these processes when prolonged under extended periods of stress can reduce overall fitness and cause behavioral inhibition, through increased likelihood of illness or loss of body mass from reduced fat stores as a result of increased cortisol levels, and also can induce pathology (Sadoul & Geffroy, 2019; Mommsen et al., 1999).

Normally, brief exposures to stressful stimuli will cause an increase in blood cortisol which then returns to its basal levels after a varied period, ranging from days to weeks. However, prolonged chronic stress will cause elevated blood cortisol for periods of up to four weeks before levels return to normal, varying in duration depending on individual fish and the nature of the stressor with some individuals never recovering at all (Pickering & Pottinger, 1989). With cortisol's known immunosuppressive properties, prolonged heightened blood cortisol will reduce overall fitness and increase mortality rates (Barton et al., 1987). Lowered immune defenses subsequently leave an individual highly susceptible to fungal or bacterial infections, a potentially life-threatening disadvantage in the particular setting of a salmon farm, where crowded conditions can facilitate the spread of parasites or disease (Pickering & Pottinger, 1989; Juell, 1995).

1.4.2 Serotonin

The brain serotonergic system is highly evolutionarily conserved across vertebrates, meaning that serotonin (5-hydroxytryptamine, 5-HT) generally modulates similar functions (Winberg & Thörnqvist, 2016). Serotonin is involved in a myriad of physiological processes, such as respiration, circadian rhythm, aggression and mood control. Notably, 5-HT has a crucial role in the regulation of the stress response by modulating physiological and behavioral outputs in response to stress (Winberg & Thörnqvist, 2016; Larson et al., 2006). Mammalian models have shown that adverse experiences or stressors can affect neuronal development and in turn impair behavior in a way that mirrors depressive symptoms in humans (Kraus et al., 2017). While non-human mammals do not experience depression-like states in the same way humans do, the presence of general behaviors such as anhedonia and loss of appetite, as well as neurophysiological elements such as impaired neurotransmitters characterize an animal model for depression. Teleost fish brains have been found to contain functionally equivalent brain areas to mammals, such as the HPI axis (homologous to the mammalian hypothalamic-pituitary adrenal axis) which, as explained above, coordinates the physiological stress response in fish (Larson et al., 2006). In this context, the distribution of the serotonergic system appears also to be conserved amongst vertebrates, with serotonergic cell bodies located mainly in the raphe nucleus in the hindbrain, although teleost fish also contain additional 5-HT nuclei in the hypothalamus (Winberg & Thörnqvist, 2016).

In subordinate individuals, chronically elevated serotonergic activity levels have been strongly associated with eliciting behavioral inhibition, such as reduced feeding and lowered immune strength, which commonly characterizes subordinate/reactive fish. Conversely, socially dominant individuals are characterized by low serotonergic activity and a more active behavioral profile (Øverli et al., 1999; Winberg & Nilsson, 1993). Other stressors are also present in aquaculture, with factors such as water quality and frequent handling and movement causing stress to fish in a way that could limit growth (Iversen et al., 2005; Fivelstad et al., 2005). In the context of this study, these chronically elevated serotonergic activity levels could possibly be influencing the DLS profile and resultant behavioral inhibition

of DLS fish. Manipulating the serotonergic system in these fish and targeting the potential cause of this behavioral inhibition could be instrumental in reversing this profile.

1.5 Pharmacological Manipulation of the 5-HT System

Pharmacological chemicals affect and alter biochemical functions, and are known specifically as pharmaceuticals when their effects have medicinal value. Pharmaceuticals can target different systems of the body and manipulate the processes in each of these systems differently, such as drugs targeting the immune or endocrine systems. Pharmaceutical chemicals alter behavioral and neurobiological systems to address psychological issues such as anxiety (Ritter, 2008). Medications utilized to treat symptoms of anxiety are generally split into two categories: benzodiazepines and non-benzodiazepines, of which buspirone is the latter. Benzodiazepines work by enhancing the function of the γ-aminobutyric acid$_A$ ($GABA_A$) receptor, located in the central nervous system (Mohler, 2002). They are classified as short-, intermediary-, or long-acting, with different varieties being prescribed to target different issues. Long-acting benzodiazepines are typically prescribed for anxiety, while others are prescribed to control insomnia, as a muscle relaxant or as an anticonvulsant (Mohler, 2002). Non-benzodiazepines work by acting as a partial agonist for serotonin receptors, meaning that they bind to and activate these receptors in the body, which may increase or decrease signaling depending on their target receptors (Gebauer et al., 2011).

Serotonin receptors are found in the peripheral and central nervous systems and regulate the release of various neurotransmitters, which in turn influence both behavioral and physiological responses such as anxiety, hunger, and aggression (Nichols & Nichols, 2008). Buspirone is a drug typically prescribed for anxiolytic purposes, though not as commonly used as selective serotonin reuptake inhibitors (SSRIs), it is still one of the most commonly used drugs (Loane & Politis, 2012). Buspirone targets the 5-HT1A receptor, one subtype of serotonin receptors. It is a partial agonist of post-synaptic 5-HT1A receptors, and a full agonist of pre-synaptic 5-HT1A receptors (Loane & Politis, 2012). Pre-synaptic 5-HT1A receptors are autoreceptors, meaning that they are located in 5-HT neurons and are only sensitive to 5-HT released by their own 5-HT neuron. Meanwhile, post-synaptic 5-HT receptors are found on several types of non-5-HT neurons and may activate or inhibit their neuron once they ligate 5-HT. Targeting these receptors in their different respective locations can therefore elicit a decrease or increase in serotonergic activity depending on the specific target (Loane & Politis, 2012).

Because the systems targeted by buspirone and other pharmaceuticals are highly evolutionarily conserved, experimental trials with these pharmaceuticals on fish have been found to have similar anxiolytic effects. For example, buspirone treated zebrafish (*Danio rerio*) showed greater willingness to explore novel social situations and locations instead of displaying more socially inhibited behaviors (Escobedo & Gould, 2012). Buspirone inhibits anxiety and promotes exploration and movement when administered to fish, meaning that it is decreasing serotonergic activity by activating autoreceptors that are cycling serotonin back to the original pre-synaptic cell (Gebauer et al., 2011; Escobedo & Gould, 2012). Animal trials have also described buspirone as having anti-aggressive effects and causing a reduction of conflict activity after treatment (Jann, 1988).

The purpose of this study is to test the hypothesis that **it is possible to reverse the DLS profile through either manipulation of the serotonergic system with buspirone, or a total change in environment.** To test this, a series of aims were developed for each experimental portion of this study:

- Determine how buspirone affects the behavioral phenotype of salmon in terms of concentration, dosage, and the method of administration.
- Assess if it's possible to reverse a potential DLS profile for fish in freshwater by repeated treatment with buspirone.
- Test if it is possible to reverse a potential DLS profile by changing the social and physical environment.

2. Materials and Methods

2.1 Experimental Facilities and Study Structure

A pilot experiment was performed at the Norwegian University of Life Sciences veterinary faculty facilities in Oslo, Norway, and was conducted in June of 2019 over a period of 10 days. This experiment was conducted in order to determine how fish would respond to buspirone treatment administered through a bath and how dosages would be adjusted.

The remaining experiments were conducted at the Institute of Marine Research (IMR) facility in Matre, Norway. Sample analysis was conducted at The Norwegian university of Life Sciences, Veterinary faculty in Oslo, Norway. These experiments utilized the information gained from the pilot about stress responses and mitigating them, using repeated treatments of buspirone, changes in environmental and social dynamics, to assess the possibility of reversing the DLS profile in the freshwater phase.

2.2 Experimental Fish

All fish for the pilot experiment were obtained from the salmon fish facilities at the Norwegian University of Life Sciences, in Ås, Norway. The fish were reared at this fish facility in indoor experimental tanks (Ø = 3 m, volume = 7 m^3) on a 24-hour light regime, with ambient water temperatures (59.6663° N, 10.7679° E) and *ad libitum* food, following established routines by the university.

The fish used in the buspirone and growth experiments were AquaGen Atlantic QTL-innOva SHIELD. The fish were hatched at the Matre facilities and started feeding on 14/05/2019. The fish were kept in 24 h light conditions from 14/05/2019 to 01/08/2019 and then a 12:12 light/dark regime from 01/08/2019 until the start of the experiments. Feeding followed growth tables and recommendations by Skretting and the feed was provided by automatic feeders during the light hours.

On 02/09/2019 fish were sorted by size before vaccination and 120 fish (smaller than 30 cm which were to be discarded) were selected for the buspirone experiment. During the time of the buspirone experiment (19 days) the fish were kept at a photoperiod of 10:14 light/dark regime at ambient temperatures (60.8760° N, 5.5867° E, 9.4°C on average). Oxygen was maintained at a ≥80 %saturation throughout the experiment. Fish were hand fed 1.2mm pellets twice a day (10:00 and 15:00). A total of six tanks (1 x 1 m) containing 400 L of Matre freshwater were used.

The fish bigger than 30 cm were vaccinated and the fish between 30 and 50 cm were kept in a large indoor tank (6m deep and 8m in diameter), until the start of the growth and survival experiment on 24/10/2019. The fish were kept at ambient natural light prior and throughout the experiment (35 days). The average (± SD) temperatures were 4.7 ± 1.2°C and 8.9 ± 0.1°C for the freshwater and saltwater experimental tanks, respectively. Saltwater was collected from a depth of 90 m with a salinity of 34-35 ppt. Fish were fed Skretting feed through automatic feeders during the day based on information from growth tables used to calculate food intake.

2.3 Experimental Design

2.3.1 Pilot Experiment

The pilot experiment was performed in order to adequately judge dosing of buspirone, as well as the method for administering it to groups of juvenile Atlantic salmon kept in freshwater.

A total of 12 juvenile salmon with an approximate weight of 130 g were brought to the laboratory facilities at the veterinary school for this experiment. Transport by car from the original location at the Norwegian University of Life Sciences in Ås to the veterinary school facilities in Oslo caused some initial stress, and the transferring of these fish to transparent tanks, a novel environment, contributed to these fish experiencing fearful and behaviorally inhibited states. The 12 fish were separated into groups of four in three tanks (100 × 50 × 50 cm) set up in a row with wireless CCTV cameras (Foscam FI9851P, Egnir Invest, Son, Norway) directed horizontally at the tanks, with the video feed controlled remotely by a computer in a different room so as to minimize disruption.

2.3.1.1 Pilot Experiment Part 1: Effect of Different Buspirone Dosages on Behavior

Fish were kept in these tanks with no external constant flow of water (*i.e.* static water conditions) with air stones connected to air pumps to maintain oxygenation, which remained between 85-95% total saturation throughout the experiment. The water temperature was between 13-15°C. All tanks were filled with 250 L dechlorinated Oslo tap water (pH 7.2-7.5). Fish were fed 1.5% of their body weight daily in 3 mm dry food pellets (Skretting, Norway), left undisturbed with the food for a 10 min period, before uneaten food pellets and debris were siphoned out. Video recordings were taken 10 min before, during and 10 min after feeding. Fresh dechlorinated tap water was added after each siphoning to maintain the desired water levels after tank cleanings. In addition, a 25% to 50% water change was performed approximately every 2 days in order to maintain high water quality.

The tanks were assigned to 3 different treatments: control (tank 1), a 3 mg/L “low” buspirone dose (tank 2) and a 5 mg/L “high” buspirone dose (tank 3). The buspirone doses were initially diluted in 5 ml Oslo tap water and the doses were determined by previously reported effects of buspirone on fish (Bencan et al., 2009). The previously diluted buspirone doses were directly added into each tank in order to avoid handling as much as possible. To control for the disturbance of dose treatment, control tanks were also disturbed by adding tap water from a flask into the tank at the same time as the other tanks were treated with buspirone. Video recordings started 10 min before treatment and continued for 2 h. The fish were then left undisturbed for approximately 24 h. At this point, it was observed that all treated fish showed aberrant behavior due to the constant exposure to the buspirone bath and fish were therefore immediately euthanized with an overdose of metacaine (MS-222 at a concentration of 2 g/L, buffered to a pH of 7.2) (Finquel®, Argent Chemical Laboratories, Redmond, WA, USA). This concluded the first part of the pilot experiment: testing buspirone doses and dosage bath time.

2.3.1.2 Pilot Experiment Part 2: Effect of Buspirone Over Time

The observations conducted in the first part of the pilot experiment lead us to conclude that the most appropriate buspirone dose to continue experimenting with was the lower dose (3 mg/L). We therefore proceeded with the second part of the pilot experiment which consisted of treating the remaining 4 fish with repeated 1 h baths of buspirone over the following 5 days. Similar to the first part of the pilot experiment, the buspirone dose was added directly into the home tank. However, the fish were quickly netted and transferred to the adjacent tank (with no buspirone) after 1 hour of treatment. Additional 1-hour baths were given at two-day intervals, resulting in three total baths for this group (Figure 3). Fish were hand fed 3 mm dry pellets (Skretting,Norway), corresponding to an equivalent of 1.5% of their body weight, every day between 10:00 and 11:00. Video recordings were taken starting 10 min before, during, and continuing for 10 min after feeding. On bath days, filming started 10 minutes before treatment and continued throughout the hour-long bath period. After the bath, the fish were netted and moved into the adjacent tank, where filming continued for another 30 minutes as the fish acclimatized, after which feeding and filming scheduling was followed as normal. Movement in the room the fish were being kept was limited during these intervals to reduce as much disturbance as possible. Illustrated below is a figure showing the camera and tank setup for the pilot experiment (Figure 4).

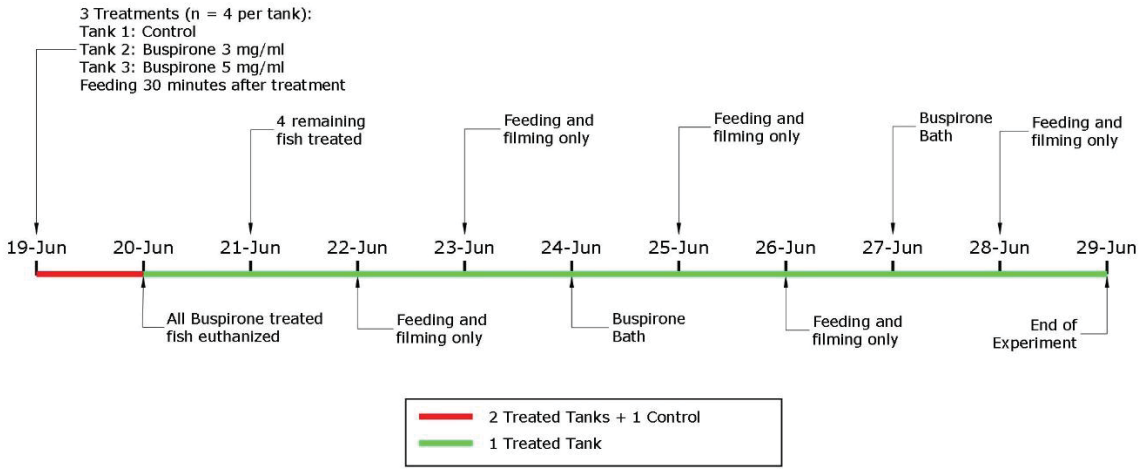

Figure 3: A timeline of events for the pilot experiment (parts 1 and 2), from the first to last experimental day

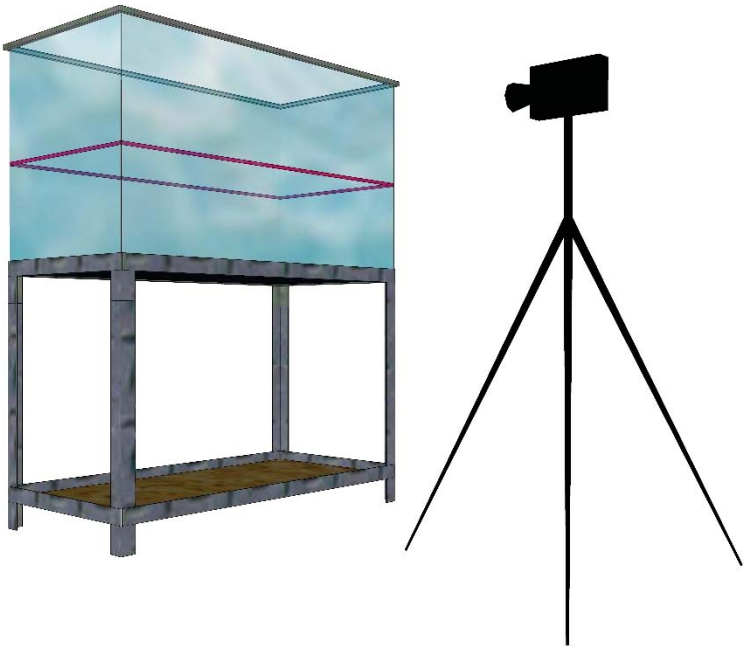

Figure 4: A mockup of the tank and camera setup used in the pilot experiment to film behavior during feeding and treatment with buspirone doses. The line depicted in the middle of the tank was used as a proxy to determine time spent in the bottom and top halves of the aquaria as part of the behavioral analysis (see subsection 2.3 for further below for details).

2.3.2 DLS Reversal by Buspirone

After determining dosages and the method of administering buspirone to Atlantic salmon in freshwater, the main buspirone experiment was conducted on a larger scale with growth-stunted fish. As part of the normal routines established at Matre, small fish (< 30 cm) are selected out of the main population before vaccination since these fish are considered to be too small for vaccination. These undersized fish were sorted out into a holding container from which we collected 120 fish.

The Fish were divided into two groups of 60 in 1x1m opaque containers with air stones and pumps to maintain oxygenation of the water. In these large containers, one group was treated with 3mg/L of buspirone for one hour, while the other group served as non-treated/sham control.

After this initial bath, each group of 60 fish was divided into 3 groups of 20 individuals, they were grouped weighed and placed into 6 adjacent opaque tanks (which minimized disruption from outside sources as well as limiting potential interactions between groups of fish). These tanks were assigned two different treatments: odd numbered tanks (1,3 and 5) were buspirone-treated groups and even numbered tanks (2,4 and 6) served as control groups. The water flow was standardized between all tanks at 10 L/min. All tanks were filmed from above with an automated video recording system, with cameras attached to a single long plank running down a central beam above all the tanks. Each camera was centered as much as possible to the tank below it (Figure 5). Video recording was continuous throughout the day, with clips being manually selected for behavioral analysis. The video feed was displayed on a nearby screen connected to a NoVus multistandard AHD recorder (NHDR-5116AHD, NoVus CCTV, AAT Holding S.A., Warsaw, Poland) that saved all video segments, with all tanks visible at once so the overall behavior of the fish could be observed without disturbing the fish. Due to logistical issues regarding storing video in the

AHD recorder, the first 10 days of video footage was lost. All video analysis was performed on video selected from the remaining days.

Figure 5: A mockup illustrating the 6-tank setup (starting with Tank 1 on the far right, going subsequently to Tank 6 on the left) for the buspirone experiment, including the overhead cameras as described in subsection 2.4.

Throughout the duration of the experiment, the fish were fed with as little disturbance as possible, 1.2 mm food pellets (Skretting NutraOlympic) twice a day (10:00 and 15:00). The photoperiod was maintained at 10:14 light/dark and the average (± SD) temperature was 9.4°C ± 0.9°C. Oxygen was measured twice a week, and levels were maintained above 80% throughout the experimental period. Tanks were cleaned daily to remove excess food and waste material after the second feeding bout. Note that during bath days, the tanks were cleaned more thoroughly while the fish were being treated in the buckets in order to avoid disturbances post-treatment.

On bath days (*i.e.* buspirone treatment days), the fish were netted from their home tanks and placed into 50L buckets in the same groups of 20 individuals. The buckets were either treated with a vial of dissolved buspirone (diluted in the same manner as explained above) or a vial of plain water for the control tanks to create an equal disturbance to the groups being treated with vials of buspirone. After one hour in the treated water all fish were quickly netted and returned to their original tanks. For the first two treatment baths (2/09/19, 6/09/19) a buspirone concentration of 3mg/L was used, while a higher concentration of 5mg/L was used for the final two (17/09/19, 21/09/19) treatments baths. For a complete overview of bath days please refer to Figure 3.

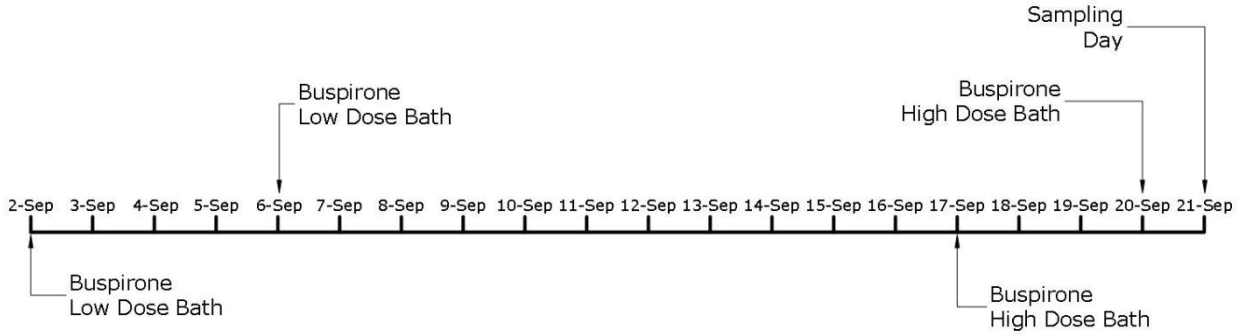

Figure 6: A timeline of events for the main experiment, showing all bath days with low (3 mg/L) and high (5 mg/L) buspirone doses, and the total runtime of the experiment

In total, this experiment ran for 18 days, and included a total of four buspirone baths. Mortalities were minimal, with 3 buspirone and 2 control fish dying from jumping out of their tanks throughout the experimental period.

2.3.3 Sampling

On the sampling day at the end of the experiment, a total of 50 fish were sampled at either basal or post-stress conditions, while the remaining fish were euthanized once sampling had finished. For basal condition (n = 11 buspirone and n = 10 control) fish were netted from their home tanks and immediately euthanized. For post-stress conditions (n = 14 buspirone and n = 15 control), fish were netted from their home tanks and subjected to a 30-min confinement stress test (following methodology by Vindas et. al 2016). The confined test consisted of placing individual fish in a 10L bucket filled with 3L of water from their home tank. The buckets had air stones and pumps to maintain proper oxygen levels for the duration of the stress test. All fish were euthanized with a lethal dose of buffered MS-222 at a concentration of 2 g/L until completely unresponsive and motionless (within approximately 30 s). Fish were rapidly weighed, fork length measured and a blood sample was taken from the caudal vessels with 23G, 1 ml syringes containing the anticoagulant ethylene diamine tetra acetic acid (EDTA). Following centrifugation for 10 min at 9.289 rcf and 4°C, plasma samples were frozen and stored at −80°C for later analysis. Fish were then decapitated and the jaw and gills were trimmed away. The tissue was then sealed in a plastic bag, snap-frozen on dry ice and stored at −80°C for further processing (the processing and analysis of brain samples are not part of this thesis and is ongoing).

2.3.4 Change of Environment Experiment

During the same vaccination event from which the buspirone experiment fish were selected, experimental fish for the novel environment experiment were also selected. While the buspirone experiment fish were all under 30 cm and too small to be vaccinated, the medium-sized fish were in the 30-50cm range and therefore received vaccination. However, these fish were still too small to be considered ready for smoltification and saltwater and are therefore typically destroyed.

After receiving vaccinations, all 30-50 cm fish were placed into a large (8m diameter) indoor tank from 02/09/2019 (vaccination day) to 24/10/2019 (start of experiment). On the 24th a subsample of fish (n = 22) were euthanized in a lethal bath of buffered MS222 weighted, measured and a blood sample was taken from the caudal vessels with 23G, 1 ml syringes containing EDTA. Blood samples were centrifugated for 10 min at 9.289 rcf and 4°C, before being frozen and stored at −80°C for ion analysis. Measuring the amount of

chloride in the blood plasma is used as a proxy to determine saltwater tolerance (Urke et al., 2009; Urke et al., 2013).

At the start of the experimental period for the novel environment experiment, a total of 400 small fish were selected, mildly sedated with MS222, weighed, and measured. The fish were then divided into four 1.5 x 1.5 m tanks, two of which were kept with freshwater and the other two with saltwater. These fish were then kept in these tanks for 35 days before being weighed and measured. In addition, a subsample (n = 16 for freshwater and n = 17 for saltwater), where sampled as described above for blood plasma in order to measure chloride levels.

2.4 Video Analysis

2.4.1 Pilot Experiment

Video recordings were analyzed manually by using a stopwatch, to establish activity levels (by measuring locomotion in seconds when a fish moved further than one body length, the amount of time (s) that fish stayed at the bottom and top half of the tank, and the number of crossings between bottom and top halves were also quantified. In addition, cohesion (*i.e.* how close together fish swim to each other) was calculated by measuring the average distance between each fish to every other fish in the frame (for a detailed explanation of cohesion calculations, please refer to subsection 2.4.3). The quantification of these parameters was used as a proxy for anxiety-like behavior (Blaser et al., 2009; Bencan et al., 2009). All parameters were quantified at three different timepoints: 10 min before, 10 min after they were exposed to the buspirone/sham bath and the last 10 min after 1 h in the bath.

2.4.2 DLS Reversal by Buspirone

Due to logistical reasons and technical issues, video recordings of the first 8 days of the experiment (except for one day, 03/09/2019) were lost. Therefore, plans for video analysis were adjusted to make use of the available days.

2.4.3 Cohesion

The cohesion of the fish groups was measured by selecting a series of screenshots at specific intervals and times throughout the day. In order to examine a varied selection of time points throughout the day and the experimental period, we chose early mornings, feeding times, immediately after buspirone baths and evenings post-treatment baths (before the lights were turned off) on selected days (Table 1). Because cohesion showed such little change between treatments in the pilot experiment, we chose to study it across varied times of day throughout the course of this experiment to provide a comparison between immediate and long-term effects.

Table 1: Detailed explanation for images chosen at different times and dates for the cohesion analysis during different daily events (i.e. feeding, early morning undisturbed behavior, and bath days). T 1-6 indicates tanks 1 through 6.

Day	Time start	Time end	Still images	Activity
03.09.19	10:18	10:21	6 (every 30s for 3min)	5 min before feeding
	10:23	10:26	6 (every 30s for 3min)	5 min directly after feeding
	10:28	10:31	6 (every 30s for 3min)	5 min after end of feeding
10.09.19	10:15	10:18	6 (every 30s for 3min)	5 min before feeding
	10:20	10:23	6 (every 30s for 3min)	5 min directly after feeding
	10:25	10:28	6(every 30s for 3min)	5 min after end of feeding
18.09.19	10:17	10:20	6 (every 30s for 3min)	5 min before feeding
	10:23	10:26	6 (every 30s for 3min)	5 min directly after feeding
	10:28	10:31	6 (every 30s for 3min)	5 min after end of feeding
21.09.19	10:27	10:30	6 (every 30s for 3min)	5 min before feeding
	10:32	10:35	6 (every 30s for 3min)	5 min directly after feeding
	10:41	10:44	6 (every 30s for 3min)	5 min after end of feeding
10.09.19	06:19	06:29	5 (every 2min for 10min)	Undisturbed behavior
15.09.19	06:35	06:45	5 (every 2min for 10min)	Undisturbed behavior
17.09.19	06:29	06:39	5 (every 2min for 10min)	Undisturbed behavior
18.09.19	06:38	06:48	5 (every 2min for 10min)	Undisturbed behavior
19.09.19	06:44	06:54	5 (every 2min for 10min)	Undisturbed behavior
20.09.19	06:37	06:47	5 (every 2min for 10min)	Undisturbed behavior
21.09.19	06:40	06:50	5 (every 2min for 10min)	Undisturbed behavior
17.09.19	T1: 12:58 T2: 12:59 T3: 13:00 T4: 13:01 T5: 13:02 T6: 13:04	T1: 13:20 T2: 13:19 T3: 13:20 T4: 13:21 T5: 13:22 T6: 13:24	One image every 2 mins for 20 mins	5 mins after last fish goes back into home tank after bath
17.09.19	20:00	20:10	One image every 2 mins for 10 mins	Evening at or around 20:00 of bath day
20.09.19	T1: 13:12 T2: 13:11 T3: 13:10 T4: 13:17 T5: 13:13 T6: 13:07	T1: 13:32 T2: 13:31 T3: 13:30 T4: 13:34 T5: 13:33 T6: 13:37	One image every 2 mins for 20 mins	5 mins after last fish goes back into home tank after bath
20.09.19	20:01	20:11	One image every 2 mins for 10 mins	Evening at or around 20:00 of bath day

Once these screenshot images were collected, they were saved as TIFF files and analyzed using the Fiji platform (Schindelin et al., 2012) in ImageJ2 (Rueden et al., 2017). The distance between each fish within the visible tank area were measured in order to establish their overall cohesion, following procedures by Spagnoli et al. 2017. This was done by using the MultiPoint tool, to measure from a point on the nose of the focal fish to a point on the nose of every other fish. These measurements were taken for every single fish within the visible area of the tank (Figure 7). Measurements were by default calculated in pixels by Image J, so they were later converted into cm by comparing to a known standard length within the tank (the grate covering the outflow tube, 21 cm, Figure 8)

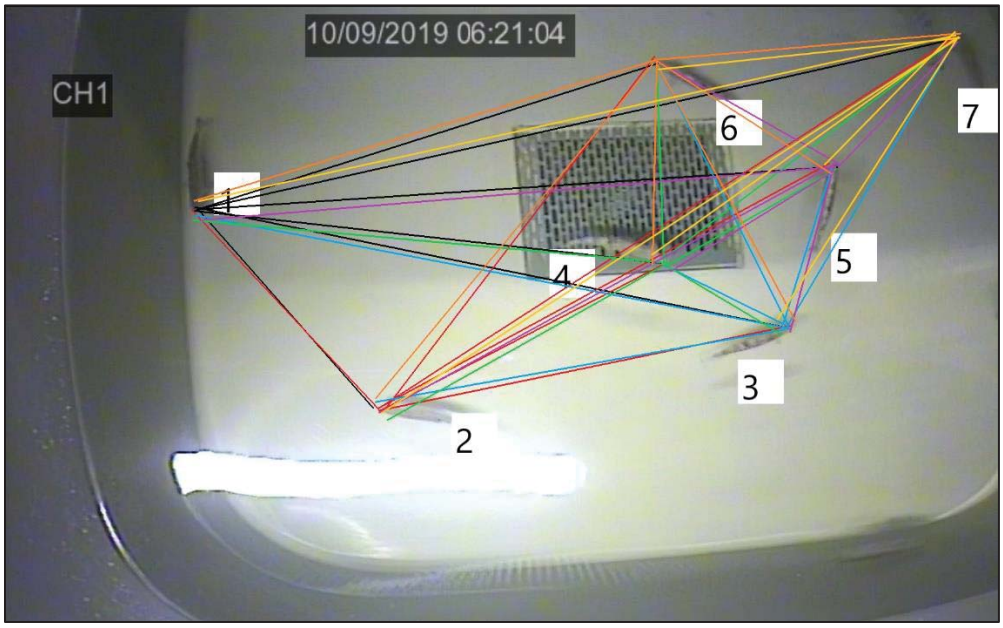

Figure 7: A sample screenshot showing the distance measured between fish, which was used to calculate the overall cohesion value for the whole group. The color-coded lines depict the measurements taken from each focal fish to the other individuals in the screenshot.

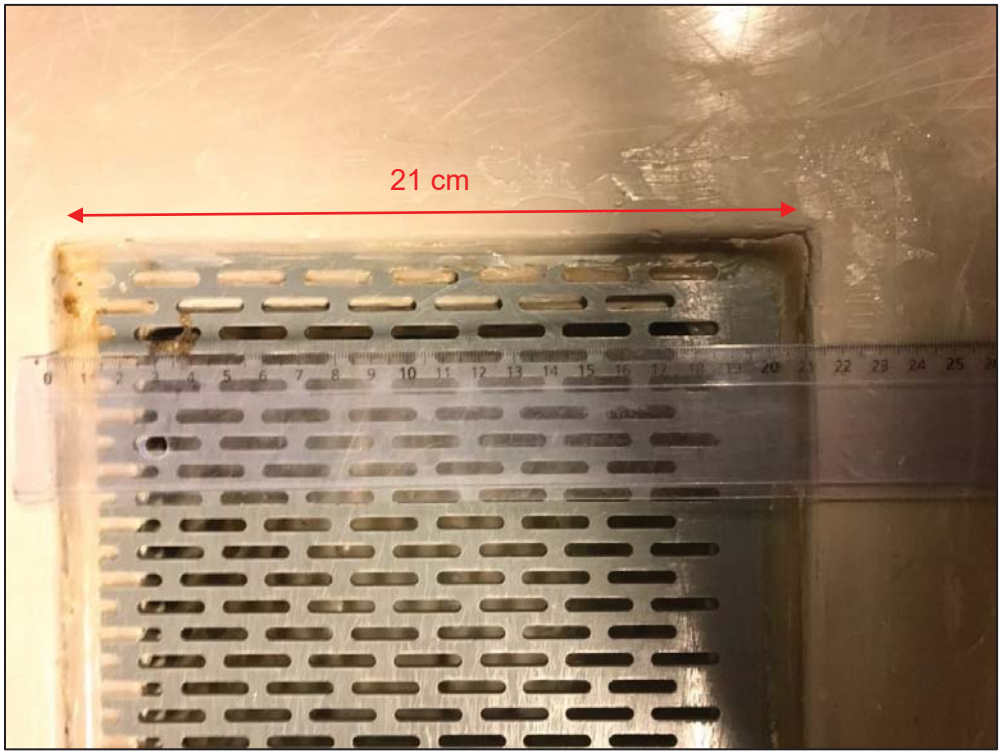

Figure 8: An image of the center grate covering the outflow tube in each tank used to determine the ration of measured pixels (in imageJ) to cm, which was used for converting the cohesion pixel measurements to centimeters.

Individual average measurements from each fish in relation to all others and finally the average for all fish was used as a general proxy for cohesion for each treatment throughout the given timepoints. In order to standardize the visible area between all tanks

and account for slightly different camera angles, all visible area for each tank was measured and the cohesion values were divided by the total area (in cm^2, see table 2).

Table 2: Tank area conversion to square centimeters

Tank Areas	
Tank	**Area (cm2)**
1	5,649.69
2	6,371.46
3	6,299.78
4	5,193.25
5	4,549.40
6	6,799.95

2.4.4 Aggression

To quantify agonistic behavior, we chose to focus on interactions conducted during the early morning hours, shortly after the lights were turned on, since fish were the least disturbed at this timepoint. Within this period, aggressive acts were quantified during one-minute intervals every two min for a total of 10 min. A total of five screenshots were captured for each spanning a period of ten minutes. In total, seven days' worth of early morning footage were analyzed. Territorial and aggressive agonistic behavior was quantified based on parameters for these behaviors described by Keenleyside and Yamamoto (1962). These behaviors were described as charging, chasing, nipping, and fleeing. Charging is a direct attack wherein the aggressor makes a direct line towards another fish. Chasing is the repeated, prolonged pursuit of a retreating fish. Nipping is typically the result of both charging and chasing behaviors and is classified as biting at the body of the target fish, typically on or around the tail fin area. Fleeing is how subordinate fish respond to attacks, retracting their fins and rapidly retreating from an aggressor. Instances of aggression were pooled together and the total number of aggressive acts were used for further analysis.

2.4.5 Locomotion/Activity Levels

Locomotion was measured as a proxy for activity. This was done by selecting a 12-minute period in the early morning, once again chosen since fish were the least disturbed at this timepoint. Within this 12-min period, one min intervals were analyzed starting with the first min and continuing with five min intervals. During these min intervals fish were individually tracked and were considered to be active if they moved more than one body length (which is a common measurement for locomotion *e.g.* Vindas et al. 2019). These times were recorded in seconds. Once these adjustments were made, treatment and control were graphed across all four (nonconsecutive) days.

2.5 Plasma Sample Analysis

2.5.1 Cortisol Analysis

Cortisol in plasma from EDTA-treated blood was analyzed using a commercially available DetectX® cortisol enzyme immunoassay kit (Arbor Assays, Ann Arbor, MI, USA) previously validated for Atlantic salmon (see manufacturer`s website for further details: https://www.arborassays.com/product/k003-h-cortisol-eia-kit/#publications), following the manufacturers protocol. The absorbance of the prepared ELISA plate was read in a plate reader at 450 nm and the concentrations were calculated using the four-parameter logistics curve.

2.5.2 Chloride Analysis

Chloride in plasma from EDTA-treated blood was analyzed using an ABL90 flex Pluss analyzer (Radiometer America, Brea, CA, USA) following the manufacturers protocol.

2.6 Statistical Analysis

Two-way analysis of variance for repeated measures (RMANOVA) was used to compare all behavioral data for the pilot and the DLS reversal by buspirone experiments. For the pilot part 1 and the DLS reversal by buspirone experiment, treatment (buspirone *vs.* control) and time (before, after or end of the bath day for the pilot experiment and selected days for the main buspirone experiment) were used as independent variables. For the pilot experiment part 2, day (*i.e.* bath day 1, 2 or 3) and time (before, after or end of the bath day) were used as independent variables). For the pilot experiment individual fish were included as a random effect, while for the buspirone experiment, tank was included as the random effect variable. A two-way analysis of variance (ANOVA) was used to compare cortisol levels with treatment (buspirone *vs.* control), conditions (basal *vs*. stress) and their interaction as independent variables, with tank as a random effect variable. Similarly, a two-way ANOVA was used to compare growth parameters for the change of environment experiment with treatment (freshwater *vs.* saltwater), time (sampling 1 *vs.* sampling 2) and their interaction as independent variables, with tank as a random effect variable. The chloride plasma data did not achieve normality and was therefore analyzed by a non-parametric Wilcoxon/Kruskal-Wallis test. All models were assessed by their capacity to explain the variability. Differences between all groups were assessed by Tukey–Kramer honestly significant difference post hoc test. Before final acceptance of the model, diagnostic residual plots were examined to ensure that no systematic patterns occurred in the errors (*e.g.* fitted values *vs.* observed values and q–q plots). Data outliers for the cortisol data were determined by the ROUT test. In total 4 values were eliminated based on a 95% confidence interval. All data (except for chloride levels) reached normality and are presented as mean ± SEM.

3. Results

3.1 Pilot Experiment Part 1: Effect of Different Buspirone Dosages on Behavior

3.1.1 Locomotion/Activity Levels

There were significant effects of treatment ($F_{(1,3)}$ = 12.04, p = 0.001), time ($F_{(1,3)}$ = 45.2, p < 0.001) and the interaction between treatment and time ($F_{(1,3)}$ = 11.8, p < 0.001) on the time fish spent moving longer than a single body length. A Tukey post-hoc test revealed that while untreated control fish generally maintained a low level of movement throughout all time points, treatment with buspirone significantly increased locomotion 10 min after the start of the buspirone bath for both doses. While fish treated with the lower dose of buspirone (3mg/L) maintained high locomotion levels also at the end of the bath, the higher dose (5mg/L) fish significantly decreased their activity by the end of the bath hour (Figure 9A). Curiously, we observed (but didn't quantify) that while the fish who received 3mg/L exhibited normal swimming behavior, the 5mg/L group's movements were erratic and spastic in nature starting soon after the buspirone treatment. See Table 1 in Appendix 1 for detailed information on Tukey post-hoc tests.

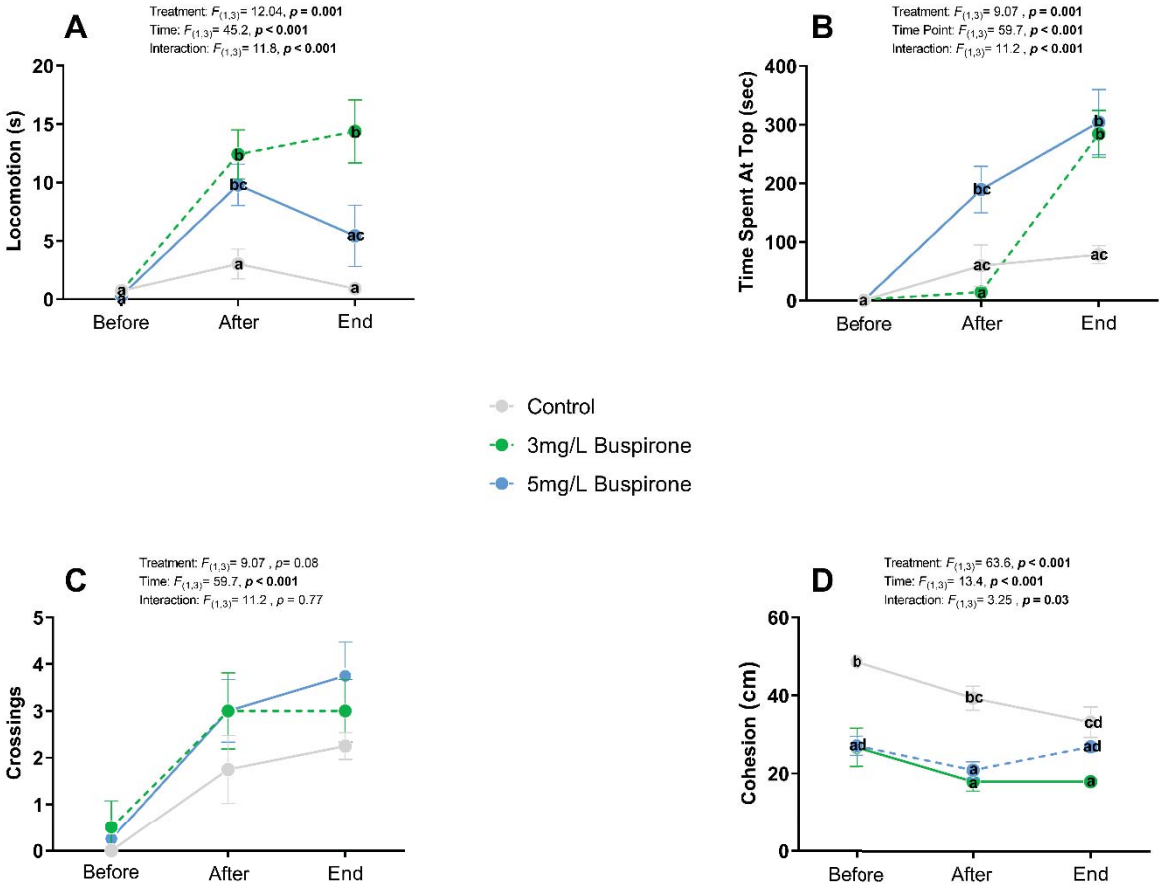

Figure 9: Mean ± SEM of anxiety-like parameters measured for buspirone-treated (3 mg/L and 5 mg/L) and control fish. Measurements were taken 10 min before, 10 min after they were exposed to the buspirone/sham bath and the last 10 min after 1 h in the bath. The measured parameters were: locomotion (i.e. time spent moving more than one body length; A), time spent at the top half of the water column (B), the number of times fish crossed between top and bottom halves (C), and overall group cohesion (measured as average distance between all fish within the group; D). Repeated measures ANOVA statistics are given within each panel and small letters symbolize Tukey post-hoc differences.

3.1.2 Vertical Positioning

There were significant effects of treatment ($F_{(1,3)} = 9.07$, $p = 0.001$), time ($F_{(1,3)} = 59.7$, $p < 0.001$) and the interaction between treatment and time ($F_{(1,3)} = 11.2$, $p < 0.001$.) on vertical positioning of the fish. Tukey post-hoc analyses showed that while all fish increased their time spent in the top half of the water column as the bath hour progressed, the control group did not differ significantly between timepoints, as seen in Figure 9B. The 3mg/L group however, showed no notable increase between the timepoint before the bath and right after it, but spiked up significantly by the end of the bath hour, showing that the fish in this group spent most of the 10-minute timepoint at the top of the tank. Meanwhile, the 5mg/L group gradually increased their time spent at the top of the tank, but in a far more gradual manner in comparison to the rapid spike of the lower dose group. Both treated groups spent significantly more time at the top of the tank by the end of the hour than the control group, showing a general increase in the use of the top half of the tank due to buspirone treatment. See Table 2 in Appendix 1 for detailed information on Tukey post-hoc tests.

3.1.3 Vertical Crossings

There was a significant effect of time ($F_{(1,3)} = 59.7$, $p < 0.001$), in which all groups show a tendency to gradually increase the number of crossings with time. Although treatment was not significant, it showed a tendency for significance in which buspirone groups show an overall higher number of crossings, compared to control fish ($F_{(1,3)} = 9.07$, $p = 0.08$). No significant interaction effect was found for number of crossings ($F_{(1,3)} = 11.2$, $p = 0.77$).

3.1.4 Group Cohesion

There were significant effects of treatment ($F_{(1,3)} = 63.6$, $p < 0.001$), time ($F_{(1,3)} = 13.4$, $p < 0.001$) and the interaction between treatment and time ($F_{(1,3)} = 3.25$, $p = 0.03$). Cohesion between individuals generally decreased over the course of the bath hour. Overall, the control group maintained higher cohesion than the two treated groups and significantly decreased gradually from the before bath period to the end of the hour (Figure 9D). See Table 3 in Appendix 1 for detailed information on Tukey post-hoc tests.

3.2 Pilot Experiment Part 2: Effect of Buspirone Over Time

3.2.1 Locomotion/Activity Levels

There were significant effects of day ($F_{(1,3)} = 60.1$, $p < 0.001$), time ($F_{(1,3)} = 731$, $p < 0.001$) and the interaction between day and time ($F_{(1,3)} = 160$, $p < 0.001$). A Tukey post-hoc test showed that the first bath day of buspirone treatment caused a significant increase in locomotion between the immediate post-bath period and the end of the hour (Figure 10A). Notably, the second bath day showed no change in locomotion throughout the hour, and the final bath showed a significant increase at a steadier rate of increase in comparison to the first day. See Table 4 in Appendix 1 for detailed information on Tukey post-hoc tests.

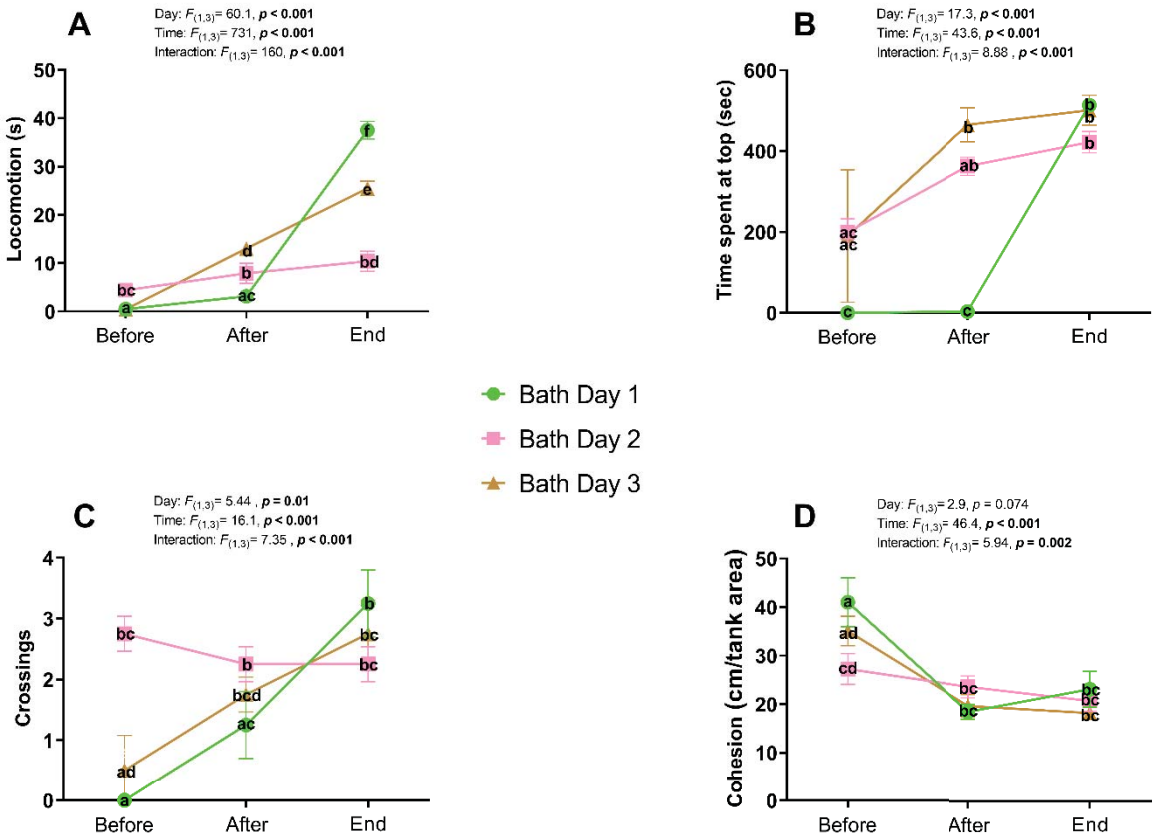

Figure 10: Mean ± SEM of anxiety-like parameters measured for buspirone-treated (3mg/L, chosen for its efficacy in the previous pilot experiment part 1) fish across three treatment days. Measurements were taken 10 min before, 10 min after they were exposed to the buspirone bath and the last 10 min of the 1 h long bath. The measured parameters were: locomotion (i.e. time spent moving more than one body length; A), time spent at the top half of the water column (B), the number of times fish crossed between top and bottom halves (C), and overall group cohesion (measured as average distance between all fish within the group; D). Repeated measures ANOVA statistics are given within each panel and small letters symbolize Tukey post-hoc differences.

3.2.2 Time Spent at Top

There were significant effects of day ($F_{(1,3)}$ = 17.3, p < 0.001), time ($F_{(1,3)}$ = 43.6, p < 0.001) and the interaction between day and time ($F_{(1,3)}$ = 8.88, p < 0.001). Prior to the first buspirone treatment, all individuals spent no time at the top of the tank, a trend that continued immediately after the treatment was administered. However, by the end of the bath hour, time spent at the top had significantly increased, compared to control as shown in Figure 10B. The two subsequent bath days also showed an increase in time spent at the top of the water column by the end of the bath hour, but the magnitude of this increase appears to be less than that of the first day due to the fact that fish were spending more time at the top already, prior to the bath as opposed to the first day where all fish remained at the bottom half of the tank. See Table 5 in Appendix 1 for detailed information on Tukey post-hoc tests.

3.2.3 Vertical Crossings

There were significant effects of day ($F_{(1,3)}$ = 5.44, p = 0.01), time ($F_{(1,3)}$ = 16.1, p < 0.001) and the interaction between day and time ($F_{(1,3)}$ = 7.35, p < 0.001). Tukey post-hoc analyses showed that the number of midline crossings increased sharply on the first and third bath days, as shown in Figure 10C. Meanwhile, the second bath day shows a slight, statistically insignificant decrease in crossings over the three timepoints. See Table 6 in Appendix 1 for detailed information on Tukey post-hoc tests.

3.2.4 Group Cohesion

No significant effect was found for day ($F_{(1,3)}$ = 2.9, p = 0.074), though it showed a tendency for significance. However, time ($F_{(1,3)}$ = 46.4, p < 0.001) and the interaction of time and day ($F_{(1,3)}$ = 5.94, p = 0.002) had a significant effect. Overall cohesion generally decreased over all days, with a significant decrease between before and the end of the bath period in days 1 and 3. There were no significant differences between timepoints for day 2. See Table 7 in Appendix 1 for detailed information on Tukey post-hoc tests.

Notably, all these results show that while the effect of buspirone in the measured parameters showed a similar direction for days 1 and 3, results for day 2 did not show the same pattern. Furthermore, even though days 1 and 3 show the same response direction, the magnitude of the response measured in day 1 is much greater than that of day 3. Altogether, this indicates that repeated treatment with buspirone without sufficient resting times between bath treatment days, leads to a lower efficiency of the buspirone effect.

3.3 DLS Reversal by Buspirone

3.3.1 Diurnal Patterning

There was a significant effect of time of day ($F_{(1,3)}$ = 23.89, p = 0.0001) on group cohesion, while treatment ($F_{(1,2)}$ = 0.09, p = 0.77) and their interaction ($F_{(1,3)}$ = 0.61, p = 0.62) were found to have no significant effects. As shown on Figure 11, cohesion was increased for both treated and control groups during the day and decreased during the night. This trend remained consistent for both groups on all four timepoints, although the magnitude of this trend appears to be higher for buspirone-treated fish.

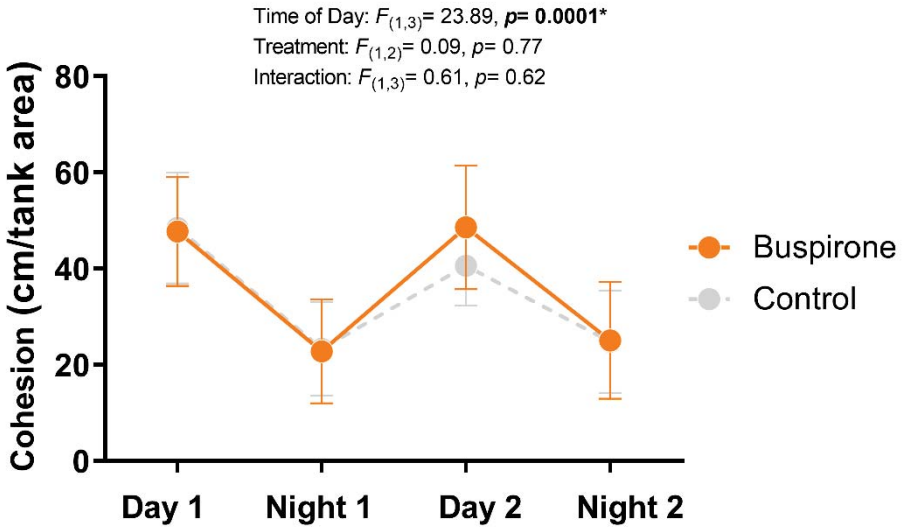

Figure 11: Mean ± SEM of group cohesion of DLS profile Atlantic salmon, during night and day of two buspirone (5 mg/L) and sham treatment days. Cohesion was analyzed immediately following bath treatments (i.e. Day time points) and approximately 7 hours later, before the lights were turned off (i.e. night time points). Repeated measures ANOVA statistics are given at the top of the graph.

3.3.2 Locomotion

There was a significant effect of date ($F_{(1,3)}$ = 28.32, p = 0.0001) found for locomotion, but both treatment ($F_{(1,2)}$ = 0.31, p = 0.60) and their interaction ($F_{(1,3)}$ = 1.97, p = 0.17) had no significant effects. The general trend is that locomotion decreased after bath treatment (Figure 12).

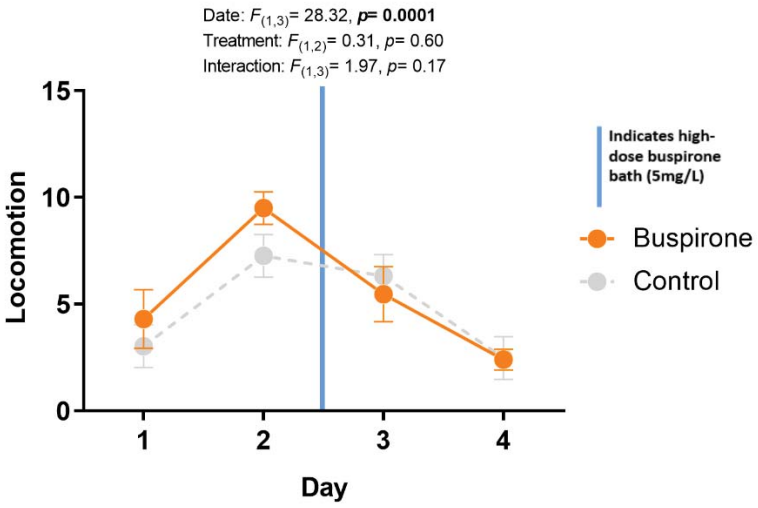

Figure 12: Mean ± SEM of buspirone (5mg/L) or control treatment on locomotion of DLS profile Atlantic salmon over a period of four non-consecutive days. Bath treatment day is indicated with a vertical blue line. Repeated measures ANOVA statistics are given at the top of the graph.

3.3.3 Feeding Cohesion

Date ($F_{(1,3)}$ = 4.88, p = 0.019) was the only significant effect found in the before feeding timepoint, while treatment ($F_{(1,2)}$ = 0.0085, p = 0.93) and their interaction ($F_{(1,3)}$ = 0.85, p = 0.48) did not show any significance (Figure 13A). At the after feeding timepoint, neither date ($F_{(1,3)}$ = 2.9, p = 0.078), treatment ($F_{(1,2)}$ = 0.13, p = 0.73), nor their interaction ($F_{(1,3)}$ = 0.15, p = 0.92) had any significant effect on cohesion (Figure 13B). Similarly, the post-feeding timepoint had no significant effect for date ($F_{(1,3)}$ = 3.12, p = 0.06), though it is trending towards significance, with both treatment groups showing an overall increase of cohesion throughout the analyzed days (Figure 13C). Treatment ($F_{(1,2)}$ = 3.4, p = 0.58) and the interaction effect ($F_{(1,3)}$ = 0.086, p = 0.96) showed no significance.

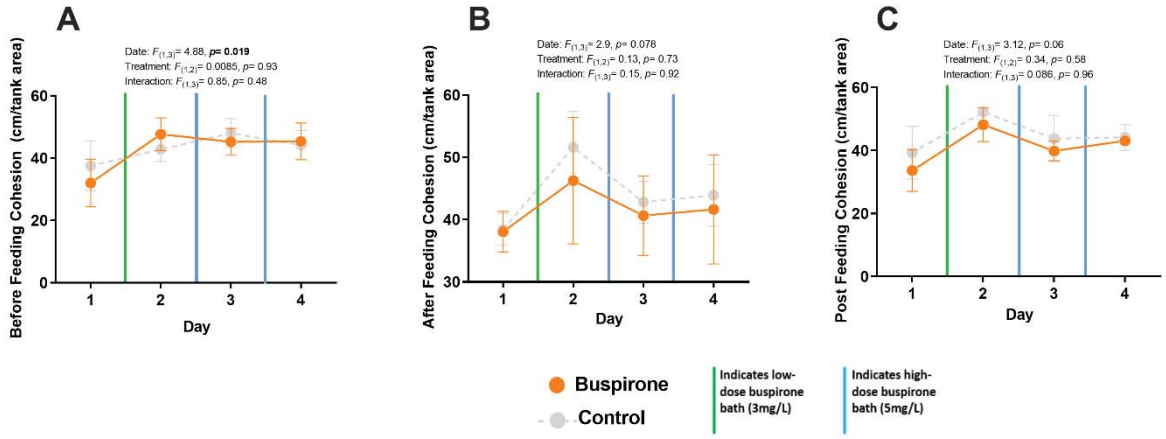

Figure 13: Mean ± SEM of group cohesion (measured as average distance between all fish within the group) at and around feeding time for the control and buspirone-treated DLS profile Atlantic salmon. Bath treatment days are indicated with a vertical green line for the 3mg/L dose and a vertical blue line for the 5mg/L dose. The timepoints analyzed were 10 minutes before feeding (A), 10 minutes after feeding (B), and 10 minutes after the end feeding period, described as post feeding (C). Repeated measures ANOVA statistics are given within each panel.

3.3.4 Undisturbed Cohesion

Neither date ($F_{(1,6)}$ = 1.96, p = 0.11), treatment ($F_{(1,2)}$ = 0.02, p = 0.88), nor their interaction ($F_{(1,6)}$ = 0.33, p = 0.92) show any significance for the early morning footage analyses. Although there were no significant differences between treatments, it appears that buspirone-treated fish show a general trend of decline prior to the first bath, which then reverses into a trend of general increase after the bath. While control mirrors this trend on the first two (non-consecutive) days, cohesion for this group remains generally unchanged throughout subsequent days (Figure 14). Due to disparities in the amount of footage available at different times of day as a result of technical issues, the undisturbed cohesion data was gathered from more timepoints than those which were available for the analysis of cohesion during feeding.

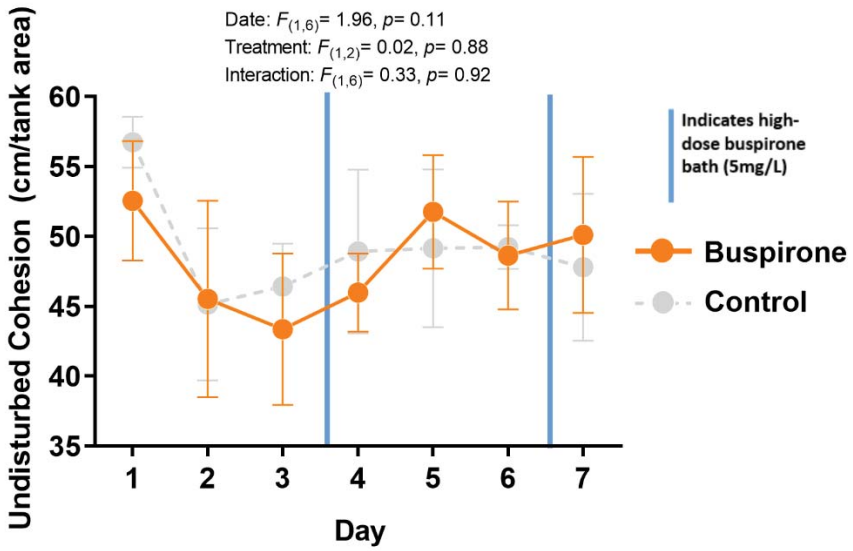

Figure 14: Mean ± SEM of group cohesion of buspirone (5mg/L) or sham treated DLS profile Atlantic salmon (measured as average distance between all fish within the group) during early mornings immediately after all lights turned on to ensure all fish were undisturbed. Seven non-consecutive days were chosen for analysis of footage from the early morning and bath treatment days are indicated with vertical blue lines. Repeated measures ANOVA statistics are given at the top of the graph.

3.3.5 Instances of Aggression

Date ($F_{(1,6)}$ = 4.03, p = 0.0061) was found to have a significant effect on total instances of aggression while treatment ($F_{(1,2)}$ = 0.05, p = 0.83) and their interaction ($F_{(1,3)}$ = 0.28, p = 0.94) had no significant effect. As shown in Figure 15, aggression did not vary significantly between treated and control fish, but generally decreased in a steady trend throughout the progression of the experiment for both groups.

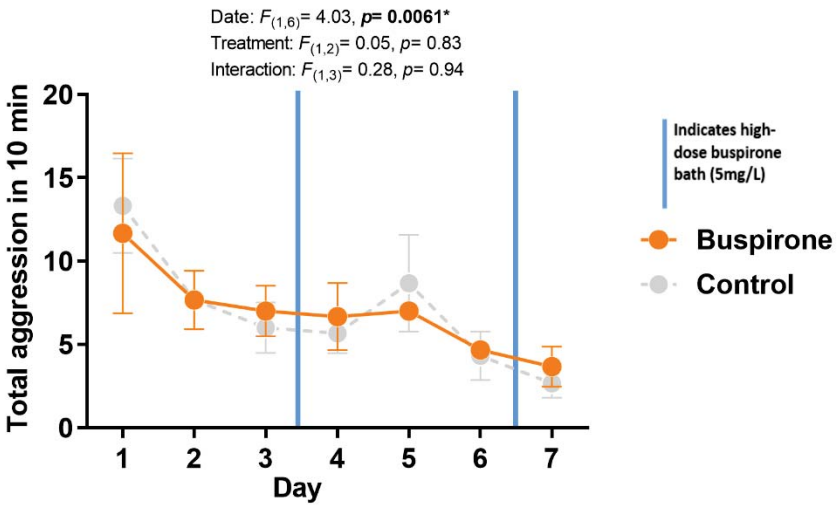

Figure 15: Mean ± SEM of total instances of aggression quantified as total amount of aggressive acts (i.e. nipping, chasing, charging, and/or biting) in a 10-minute section of footage over a non-consecutive period of seven days for buspirone- (5mg/L) or sham-treated DLS profile Atlantic salmon. Bath treatment days are indicated with vertical blue lines. Repeated measures ANOVA statistics are given at the top of the graph.

3.3.6 Cortisol

Stress level ($F_{(1,2)}$ = 210, p < 0.001) was found to have a significant effect, while treatment ($F_{(1,2)}$ = 1.48, p = 0.29) did not. The interaction between the two ($F_{(1,2)}$ = 4.95, p = 0.03) was also found to be statistically significant. Tukey post-hoc analysis shows that while fish analyzed at basal levels had low plasma cortisol regardless of treatment with buspirone, both fish groups increased their cortisol levels post-stress, and there were no significant differences between treatment groups. Interestingly, the buspirone group shows a large variation with some individuals showing very low post-stress cortisol, and others very high. Meanwhile, the control group remains more homogenous in their post-stress results (Figure 16). See Table 8 in Appendix 1 for detailed information on Tukey post-hoc tests.

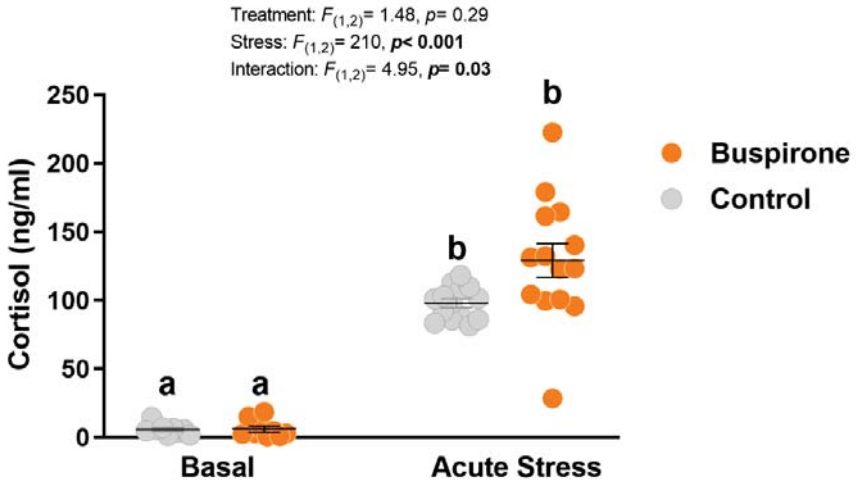

Figure 16: Mean ± SEM of plasma cortisol levels taken at both basal and post-acute stress conditions for buspirone-treated (i.e. fish were treated twice with 3mg/L and twice with 5mg/L throughout the course of the experiment) and control (sham-treated) DLS profile Atlantic salmon. Blood samples were taken either immediately after euthanization for basal results or after a confinement stress test. Repeated measures ANOVA statistics are given within the figure and small letters symbolize Tukey post-hoc differences.

3.4 Change of Environment Experiment

3.4.1 Length, Weight, and Condition Factor

Time ($F_{(1,2)}$ = 575.456, p = 0.0001) was found to have a significant effect on the length, while treatment ($F_{(1,2)}$ = 0.0107, p = 0.9272) and their interaction ($F_{(1,3)}$ = 0.3195, p = 0.5721) had no significant effect (Figure 17A). Similarly, for mass (Figure 17B), time ($F_{(1,2)}$ = 0.1206.54, p = 0.0001) showed a significant effect while treatment ($F_{(1,2)}$ = 0.00, p = 0.9952) and their interaction ($F_{(1,3)}$ = 0.4488, p = 0.5031) had no significant effect. Finally, condition factor (Figure 17C) also reflects this pattern, with time ($F_{(1,2)}$ = 178.95, p = 0.0001) having a statistically significant effect while treatment ($F_{(1,2)}$ = 0.2720, p = 0.6540) and their interaction ($F_{(1,2)}$ = 0.0180, p = 0.8933) show no significant effects. Generally, both groups increased their length, weight, and condition factor from Sampling 1 to Sampling 2 regardless of treatment.

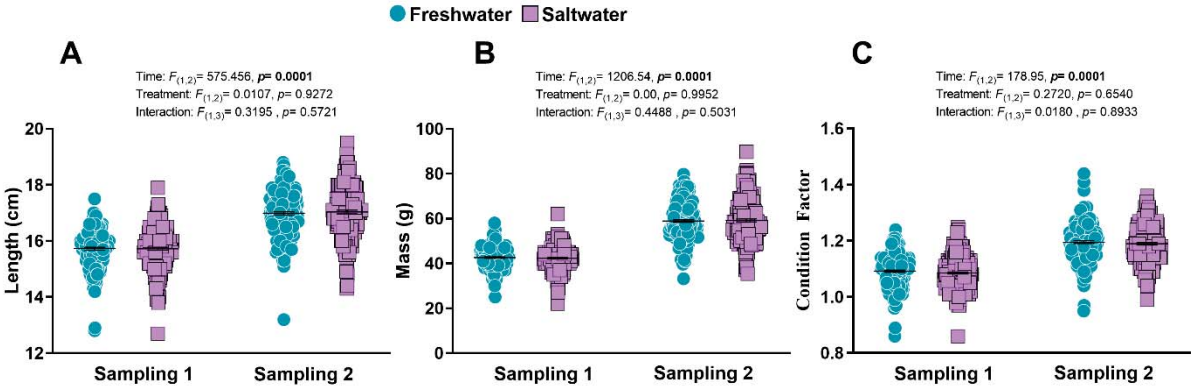

Figure 17: Mean ± SEM length (cm; A), mass (g; B), and condition factor (C, calculated by dividing weight by length) taken at the beginning (Sampling 1) and end (Sampling 2) of the novel environment experiment for fish groups kept in either freshwater or saltwater. Repeated measures ANOVA statistics are given within each panel.

3.4.2 Plasma Chloride Analysis

Time ($\chi 2_{(2)}$= 30.86, p< 0.0001) was found to have a significant effect on changes in plasma chloride. Both freshwater and saltwater groups significantly changed in plasma chloride levels at the end of the experiment in comparison to the beginning. While the group in Sampling 1 appears to show more variation, by Sampling 2 both groups had become more homogenous overall. See Table 9 in Appendix 1 for detailed information on Tukey post-hoc tests.

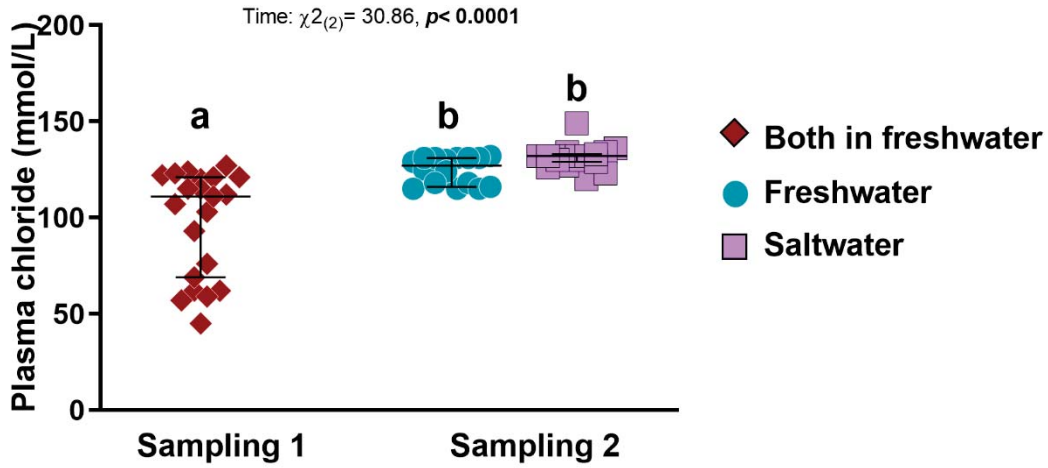

Figure 18: Median values of plasma chloride in fish at the start of novel environment experiment, when all individuals were in freshwater (Sampling 1) and at the end of the experiment, where groups had been separated into freshwater and saltwater for a period of a month (Sampling 2). Non-parametric Wilcoxon/Kruskal-Wallis test statistics given within the figure and small letters symbolize Tukey post-hoc differences.

4. Discussion

4.1 Experimental Design Considerations

4.1.1 Buspirone Treatments and Behavioral Observations

This experiment focused on the anxiolytic effects of buspirone on DLS profile Atlantic salmon as opposed to alternative medications such as SSRIs. This is due in part to present literature showing fish experiencing serious adverse effects as a result of SSRIs, with previous studies on zebrafish detailing the potential risks and side-effects of administering these medications. Exposure to fluoxetine caused a reduction in the amount of eggs spawned and affected ovarian conditions in female zebrafish (Lister et al., 2009) while causing reproductive dysfunction and reduction in sperm count and motility in male zebrafish, and other medications such as citalopram have been shown to cause hypoactivity after treatment (Prasad et al., 2015; Bachour et al., 2020). Buspirone also offers a degree of specificity that some broader drugs lack in terms of control over targeted structures by specifically targeting the 5-HT1A receptor, but it can target both pre-synaptic and post-synaptic 5-HT1A receptors in a way that decreases or increases serotonergic activity (Loane & Politis, 2012). Activating autoreceptors that are returning serotonin back to the pre-synaptic cell decreases serotonergic activity, specifically inhibiting anxiety-related behaviors as seen in zebrafish trials (Gebauer et al., 2011). This ability to specifically target and reverse anxious behaviors associated with the DLS profile while also lacking unwanted side effects associated with benzodiazepines such as lethargy or physical dependence made buspirone the preferred choice in this study (Mohler, 2002).

All experimental fish were treated with buspirone through a bath treatment in both the pilot and main experiments. A bath was chosen in favor of dosing through injection for a number of logistical reasons. Injections are physically taxing for fish, in order to receive an injection a fish must be anesthetized beforehand and allowed to recover from the anesthetic before results of the treatment can be analyzed. The injection site also causes physical trauma, and prolonged handling heightens stress reactions. DLS fish are also particularly vulnerable to further stress, to the point where handling has been known to cause death in severely affected individuals. Therefore, minimizing handling was integral to maintaining survival for fish in this experiment. While an injection can guarantee exact dosage across all individuals, lasts longer in the body and also acts faster upon injection, the immense amount of stress the injection process incurs alongside the impracticality of injecting a large amount of fish meant that dosing via bath was the most humane and practical option for this study.

The pilot experiments consisted of only one tank per treatment. Ideally, this should have been extended to at least three tanks per treatment for a wider breadth of results. However, because this experiment was aimed at simply determining the dosage and method of administering buspirone for the later experiment, we wanted to minimize the amount of experimental fish. In accordance with aiming to reduce the number of experimental animals, refining the experiment as a whole, and replacing the usage of animals where possible, we decided to run this portion of the study with a minimal amount of experimental fish in the interest of ethics and welfare (Combes & Balls, 2014).

For the video footage collected in this experiment, all behavioral observation was performed manually. While utilization of a tracking software minimizes observer error and bias, this software requires very specific conditions, meaning that insufficient light, the presence of bubbles, or obscured tank areas rendered tracking software unusable in this scenario. Instead, we attempted to minimize observer bias by selecting portions of the footage and results to be analyzed by two independent observers to ensure the results were consistent. Additionally, there is another master project currently aiming at creating a custom

automated software to analyze these videos, which could then be used to corroborate current results, this work is still ongoing.

4.1.2 DLS Reversal by Buspirone

An important factor to note in this experimental design is that the experimental fish were not absolutely confirmed to have the DLS profile, as described by Vindas et al. (2016). Previously, the DLS profile has been found in fish in the saltwater stage while all fish in this experiment were in freshwater. However, preliminary data from our group (Vindas et al. unpublished) shows that this profile can already be found in freshwater as well, particularly when individuals are subjected to repeated chronic stress. Based on this information, we chose to analyze fish in the freshwater stage. Details will be discussed later in the section when discussing results.

Due to logistical issues and technical problems with the recording device, most of the footage from early in the experiment was lost. Therefore, we were unable to match days between analyses (*i.e.* undisturbed days to the locomotion days) and had to choose different selections of dates to work around missing footage. Regardless of this lack of standardization with the initial footage, we were still able to obtain data on the general changes in behavior and activity for all fish throughout the course of the experiment.

All footage from this portion of the study was filmed from above as opposed to the pilot, which was filmed horizontally. Due to the larger volume of total fish in the experiment, larger tanks were required. The facility where these fish were kept also did not have any glass aquaria. Hence, large plastic containers were used for this experiment. These containers were all opaque plastic that did not allow for side filming. However, with minor adjustments made to the method of analyzing behavior, different behavioral parameters were still able to be examined. Also, due to the nature of the shared space at this facility, a dedicated experimental room for 6 large tanks was not a feasible or practical idea, and the experiment ran its course in a room with other holding tanks and regular traffic. This did not allow us to minimize the disturbances that fish were subjected to in terms of other activities being conducted in this room. However, we tried to compensate for this by analyzing video footage for timepoints in which there were either no disturbances, or the disturbances were caused by activities related to our own experiment, such as feeding or bath treatment.

4.1.3 Change of Environment Experiment

The purpose of this experiment was mainly to determine how well fish exhibiting a DLS profile grow and survive if one changes their environment (*i.e*. removing bigger conspecifics and decreasing the tank area), meaning that it was not meant to be a behavioral comparison and no video footage was taken. However, in the scope of this study as a whole a behavioral component would have been fitting.

Ultimately, this study is lacking physiological information such as analysis of serotonin, gene expression, and brain physiology that was initially meant to accompany the present analyses of this thesis. Due to the circumstances surrounding COVID-19 and the resultant restrictions put into place, these additional analyses could not be performed for this study and have been taken over by another master's student.

4.2 Discussion of Results

4.2.1 Pilot Experiment

In both segments of the pilot experiment, we found that buspirone treatment generally decreased anxiety symptoms and associated behaviors in salmon. Overall findings show increased movement as a result of treatment in the first part of the experiment, with treated groups significantly increasing their overall locomotion and number of crossings

while decreasing group cohesion over time. Stressed fish, typically described as behaviorally inhibited as a result of a prolonged stress response, stereotypically show low locomotor activity and a tendency to remain either at the surface or remain motionless at the bottom in comparison to normal behaviors described in salmon such as active swimming in search of food (Øverli et al., 1998; Vindas et al., 2019; Orlov et al., 2006).

These results show that as predicted, fish were experiencing lessened behavioral inhibition as a result of buspirone treatment, they began utilizing more of the aquaria space by spending more time in the top half of the water column in comparison to stressed fish. Decreased group cohesion in treated fish demonstrates more activity and willingness to explore novel environments. These results are in accordance with behavior previously described in fish buspirone trials (Escobedo & Gould, 2012).

In general, these results are in accordance with previous studies on buspirone's effect on 5-HT1A receptors and the subsequent reduction in anxiety-associated behaviors in zebrafish and mice (Bencan et al., 2009; Escobedo & Gould, 2012).

The dosing parameters in this segment of the study were also relevant to the behavior displayed by the groups in each part of the pilot. The initial method of dosing was treating the water with either 3mg/L, 5mg/L, or a sham bath and leaving the fish in this treated water overnight. Results were observable nearly immediately after treatment in the hour these fish were filmed, though the difference in doses resulted in markedly different behaviors. While the 3mg/L group showed a rapid reversal of anxious behaviors into increased seemingly normal movement and exploration of the area, the 5mg/L group was displaying clear adverse effects from the increase in dosage. Instead of the smooth active swimming movement observed in the lower dose group, the movement of fish treated with 5mg/L buspirone was erratic and jerky, showing spastic and unpredictable behavior as a result of the increased dose. After a full night in the bath treatment, all treated fish were either found dead or displaying aberrant behavior that led to immediate euthanization, showing that high or prolonged doses of buspirone can quickly have negative physiological effects. Additionally, while the subsequent segment of the pilot experiment was done with the remaining group of fish receiving only hour-long baths in treated water, repeated buspirone treatment after only one-day rest periods between baths may reduce the efficacy of buspirone treatment. Therefore, it would have been ideal, if bath treatments would have been spaced further apart to reduce physiological stress on these fish and it is something that was taken into consideration in the DLS reversal by buspirone experiment.

4.2.2 DLS Reversal by Buspirone

The experimental fish used in this portion of the study could possibly differ from those in the pilot study by having experienced chronic, prolonged stress as opposed to a short-term stress response, a potential reason for the markedly different outcomes between experiments. While the pilot experiment fish were experiencing stress as a response to handling, moving, and a novel environment, the DLS reversal by buspirone fish fit a profile of chronic stress which is similar to the DLS profile. Traits of the DLS profile have been described in previous literature as stunted growth, anorexia from lack of feeding, behavioral inhibition, and remaining listless at the top or sides of a sea cage (Vindas et al., 2016). Fish under continued stress have also been observed to have altered neuroendocrine responses as well as a suppressed immune system (Vindas et al., 2019; Larson et al., 2006; Barton, 2002). These chronically subordinated fish also suffer from impaired cognitive function and an overall decline in physical health that increases their risk of mortality (Barton et al., 1987; Juell, 1995). Fish described as fitting the DLS profile are typically found in saltwater, with most investigations into their physiology done by selecting growth-stunted fish matching this

profile from pens or sea cages in saltwater environments. However, unpublished data from our group shows that DLS profile can occur in the freshwater environments as well. That is, Vindas et al. (unpublished) found that chronically stressed salmonids in freshwater show a chronically elevated 5-HT response at basal levels which is incapable of responding further to a novel acute stressor, which is exactly the same neurochemical profile which has been characterized for DLS fish in saltwater cages (Vindas et al. 2016). A likely scenario for some of these DLS fish is one where the growth-stunted, depression-like profile begins to emerge when the fish is still in freshwater, worsening with time as they transition into seawater and experienced cumulative stress. Alternatively, DLS fish fail to adapt after the transition to saltwater, stop eating and become growth-stunted, an effect that could be purely genetic or due to stressors associated with transfer into saltwater.

Another important background aspect of this behavioral profile is the specific social dynamics of aquaculture paired with the hierarchical relationships of salmon. In the wild, these fish fall into hierarchies of dominants and subordinates, wherein the dominant individuals maintain easier access to food resources and in turn grow larger and more efficiently (Harwood et al., 2003). However, wild environments also allow for socially subordinate individuals to use varied strategies to acquire food resources without having to field conflicts with aggressive, socially dominant individuals (Adams & Huntingford, 2005). In aquaculture, the benefits of different strategies disappear in favor of aggressive and confrontational traits accidentally artificially selected for in conjunction with intentional traits such as rapid growth (Adams & Huntingford, 2005; Cubitt et al., 2008). The resultant situation in aquaculture is one where individuals who do not match the ideal socially dominant and aggressive type selected for in such a setting falls behind on growth due to being unable to access resources and consistently is subordinated in confrontations, progressively falling lower down the social hierarchy (Fernö et al., 2011). The emergent growth differential typically resolves itself in the wild with larger fish leaving to migrate downriver, creating an opportunity for smaller fish to have unfettered access to resources and therefore catch up on growth. However, this opportunity is not present in aquaculture, where fish are transferred through different environments in large groups, and the ramifications for smaller fish here are potentially life-threatening (Harwood et al., 2003). Total instances of aggression gradually decreased throughout the course of this experiment (see Figure 15, section 3.3.5), but both treated and control fish showed essentially the same level of decline, implying that regardless of treatment aggressive acts decreased as a group of unfamiliar fish established a social hierarchy and maintained it. Because all these fish were essentially the same size, instances of aggression between them were not as extreme as confrontations between fish with a large size disparity, where smaller fish can possibly get killed. In this experiment, all bigger fish were removed, so this decrease in aggression shows a more normalized establishment of social rank among equally-matched individuals.

A concurrent issue that may influence social hierarchies in aquaculture and the emergence of a subset of the population that is showing DLS behavior is the presence of different coping styles and the advantages of certain styles over others in a confined environment (Koolhaas, 2008; Øverli et al., 2007; Vindas et al., 2017). Certain behavioral and physiological responses are better suited to the specific nature of aquaculture, and the challenging environment rewards proactive individuals that respond to stressors in a confrontational manner rather than a submissive, reactive response. Reactive individuals prefer to avoid conflict and confrontation altogether if possible, and these typically socially subordinate fish retreat from dominance contests, a response that lowers social status and limits access to food resources (Metcalfe et al., 2003). Proactive individuals also prefer predictable and stable environments, a further benefit in the structured setting of an aquaculture farm that could procure an advantage in comparison to the wild (Ruiz-Gomez et

al., 2011). The continuous lowering of social rank through these failed dominance contests and the inability to successfully avoid conflict situations in a confined space potentially causes these reactive, submissive fish to experience chronic stress which as stated previously, has possible health risks such as decreased immunity and impaired neuroendocrine function (Nettle, 2004; Vindas et al., 2019). Therefore, freshwater fish that were considerably smaller in comparison to similarly aged conspecifics were chosen as potential DLS profile fish for this experiment.

The disparity in responses to buspirone treatment between the pilot experiment fish and the DLS reversal by buspirone fish is substantial. While the pilot experiment fish experienced behavioral changes and cessation of anxiety-like symptoms within a few minutes of treatment even at the lower (3mg/L) buspirone dose, this same dose elicited no change in behavior from the chronically stressed potential DLS fish. Some analyses showed general trends of buspirone-treated fish behaving slightly different, such as differences in cohesion between treatments on specific days in the undisturbed and bath days analyses, but none of these differences were significant. This response is in stark contrast to the near-immediate effect the 3mg/L dose had on the pilot experiment fish, and even more surprising considering that the DLS reversal by buspirone fish were treated with the high 5mg/L buspirone dose during the final two baths of the experiment and still we did not observe any significant behavioral changes. This is very surprising since the pilot experiment fish that received a 5mg/L treatment started showing adverse effects from the high dose within a few minutes of treatment such as erratic swimming and jerky movements. However, when this dosage was administered to the DLS reversal by buspirone fish, no identifiable changes in behavior were observed. The lack of any significant changes in behavior even after multiple buspirone treatments presents the possibility that this profile cannot be reversed in these fish, at least by pharmacological manipulation with buspirone, and that they are effectively beyond the point of returning to a normal behavioral profile.

Even though behavior was not changing in any observable or significant way as a result of buspirone treatment of the potential DLS profile fish, analysis of plasma cortisol levels for both control and treated fish at both basal and post-stress levels showed that the treatment was still having some kind of physiological effect. While the post-stress response of the control group was relatively grouped together and homogenous, the buspirone treated fish showed an inconsistent response to stress with a wider range of values. These unexpected results imply the possibility that there is a physiological component to this treatment even while behavioral results are remaining unchanged, and that this physiological response is varied, possibly due to buspirone acting on different receptors. Because buspirone acts on 5-HT1A receptors both pre- and post-synaptically, the response to drug treatment can vary depending on the location and either decrease or increase serotonergic signaling, which in turn, affects physiology and behavior in opposing manners (Winberg et al., 1997; Loane & Politis, 2012). This difference in response can increase or reduce the efficacy of treatment, and excessive repeated treatment has been shown to induce restless, agitated behavior, meaning that the behavioral outcome of treating with buspirone is potentially unpredictable (Loane & Politis, 2012; Jann, 1988). Given this unexpected result, especially in the context of the pilot experiment resulting in predicted behavioral changes, new questions about the general effectiveness of targeting the serotonergic system to reverse the DLS profile arise. Checking the 5-HT1A receptor levels and 5-HT neurochemistry levels of fish that react with high compared to low cortisol response would help explain the variation in the response to stress seen in this experiment, a project that is planned to be executed by another master's student. Further study on the actual precision of serotonergic system manipulation as well as more investigation on physiological analysis of the main experiment samples would potentially shed light on the source of this disparity

between experimental trials and possibly pinpoint if the DLS profile in this stage can even be reversed at all.

4.2.3 Change of Environment Experiment

This experiment showed that for fish in the 30-50cm range, the removal of bigger competitors in a space that allows for easier access to food and a general change in environment resulted in these fish catching up in growth with their previously age-matched bigger conspecifics, which implies that this environmental change effectively reverses the potential for a DLS profile. As discussed previously in section 1.1 Study Species, a driving factor behind the growth disparity that sometimes emerges in groups of farmed salmon is unequal access to resources. In the wild, growth differentials resolve themselves over time when larger salmon grow enough to begin migration, thus leaving the riverine environment and removing difficult competition for resources (Harwood et al., 2003). This experiment essentially creates this scenario in captivity by removing these potential DLS profile fish into their own separate environment to catch up on growth. After a month in these tanks without the presence of larger competitors, the fish selected for this experiment actually grew better than the ones who had been taken directly taken into the sea cages (Ole Folkedal 2019, personal communication). Interestingly, the fish who were growth-stunted and most at risk for displaying a DLS profile ended up not only growing as well as their conspecifics who were continued under regular husbandry protocol, but surpassed them as well. A change in environment aided these fish immensely in catching up in growth with their conspecifics and not falling further into a behaviorally inhibited state. At the time of vaccination and the start of the experiment, these fish were considered to be potentially exhibiting the DLS profile, but by the conclusion of the experiment this profile was not present at all in terms of growth rate and mortality.

Currently, growth-stunted fish are thought to not tolerate saltwater well, if at all. It was generally accepted that due to their small size, their saltwater tolerance would be lower. However, as shown by our results in the change of environment experiment, fish between 30-50 cm (usually considered to be too small to tolerate slat water) showed normal plasma chloride levels in saltwater, which indicates that they show a normal seawater tolerance and had no issue with transitioning environments. After this experiment concluded, these fish were moved to sea cages and continued growing well, with survival levels matching other fish that were not part of the experiment and had been directly moved to the sea cages (Ole Folkedal 2020, personal communication).

An important element to consider when comparing results between the DLS reversal by buspirone and the change in environment experiments, is that the fish used in the latter experiment were bigger, being in the range between 30 and 50cm while the fish in the former were all ≤ 30cm. A likely scenario to explain the relative success of the change in environment fish is the advantage they had in terms of size. Being further along in growth and development could potentially have allowed these fish to recover and reverse the DLS profile while it was still reversible, whereas smaller fish were past the point of being able to recover at all. Another factor to note in this experiment is that these experimental fish were never confirmed as DLS fish, and this information was extrapolated based on their growth being relatively stunted in comparison to their age-matched conspecifics. Further investigation would be beneficial to supplement this experiment with behavioral and physiological data corroborating the DLS profile prior to the experiment start and its reversal at the end.

4.3 Impact and Future Perspectives

To summarize the outcomes of this study in short: while buspirone had the expected effect on behavior of temporarily stressed individuals, the behavior of DLS-type fish in the main experiment remained largely unaffected. A possible explanation is that these fish had incurred long-lasting damage from stunted growth, and that this type of behavioral inhibition cannot be reversed through drug treatment affecting the 5-HT system. Notably, this fish also experienced a change in environment, since all bigger conspecifics were removed and they were transferred to smaller tanks. However, a reverse of their profile to more active and bigger individuals, similar to that seen in the change of environment medium fish. Another possibility inhibiting the reversal of this profile is the inherent difference in physiology that would affect how an individual copes with stress, whether these differences cause or influence the response to DLS is unknown. A high (5mg/L) dose of buspirone that was large enough to induce erratic behavior in the pilot experiment fish elicited no response or change in behavior from these DLS reversal by buspirone fish assumed to be chronically stressed fish. The comparison between the pilot fish who successfully experienced a reversal of their stress-related behavioral inhibition, and the chronically stressed, growth-stunted individuals in the main buspirone experiment, and the slightly larger behaviorally inhibited individuals in the change of environment experiment, show an interesting range in responses from fish in very different situations. A change in environment allowed for significant growth and improvement of survival for the change of environment fish, but a similar change of environment had no effect on the smaller buspirone-treated fish.

This variation in response raises the question of whether manipulating the serotonergic system is the best approach for attempting to reverse this profile, or even if the chronically stressed fish that were under 30cm were amenable for reversal of their profile and were this irreversibly behaviorally inhibited. Physiologically, there was a varied response from these fish after receiving buspirone treatment, as reflected in the plasma cortisol results. However, this variation was not reflected behaviorally. A possible avenue for further investigation would be looking closer at the different subtypes of serotonin receptors found in the brain and how they are affected by buspirone. The results of the buspirone experiment on potential DLS fish indicates that buspirone is affecting the HPI axis by altering parts of the serotonergic system responsible for this axis, but leaving the parts affecting behavior unaltered (Kaufman et al., 2016). Posing these questions as potential areas of further research would elucidate the behavioral or physiological reasons behind such a diversity of results across this study. Further experimentation on the pharmaceutical component of this study would provide interesting perspectives on whether serotonergic system manipulation is a possible candidate for DLS reversal or not or if alternate neurophysiological targets would have provided a more decisive result. Additionally, comparing the results obtained by administering buspirone through different methods such as comparing injections and treatment baths would offer more insight into the cause of such varied plasma cortisol levels in the buspirone-treated fish and their concurrent lack of any behavioral response. Finally, behavioral analysis of DLS profile fish allowed to grow in saltwater in an environment with reduced competition can further elaborate on how exactly this profile reverses so efficiently in such a situation.

5. Conclusion

Given the varied findings from each portion of this study, I would like to briefly revisit the initial hypothesis and aims stated previously in the introduction of this thesis with the added perspective of the subsequent results and outcomes. We hypothesized that **it is possible to reverse the DLS profile through either a total change in environment or through manipulation of the serotonergic system with buspirone**, but this manipulation of the serotonergic system did not go as planned, chronically stressed fish potentially displaying a DLS profile did not show any real behavioral change after treatment, though physiologically there was a clear difference in the individual post-stress levels, indicating an individual variating physiological response to buspirone, meaning there was clearly some effect internally, as shown in Figure 16, Section 3.3.6. Given the results of the change of environment experiment in comparison to the buspirone experiment, reversibility of the DLS profile seems to only be feasible up to a certain point, beyond which no amount of environmental changes or pharmacological manipulation can reverse it. In order to more thoroughly examine these elements further research and more neurophysiological data is needed, possibly with more experimentation testing different methods of administering buspirone or even medications that have entirely different targets overall.

- Aim 1: Determine how buspirone affects salmon in terms of concentration, dosage, and the method of administration.

We found that the optimal method of administering buspirone to groups of fish was through a bath treatment limited to one hour. While 3mg/L was a very effective dose in terms of reversing stress-induced behaviors, the 5mg/L dose caused erratic behavior and was considered to be too high. These fish that were not at risk of fitting a DLS profile had their potential behavioral inhibition as a result of temporary stress successfully reversed through buspirone treatment.

- Aim 2: Assess if it's possible to reverse a potential DLS profile for fish in freshwater by repeated treatment with buspirone.

These fish that potentially fit the DLS profile did not respond to buspirone treatment in the same way as healthy, temporarily stressed fish did. There was a quantifiable physiological response as seen in the highly varied plasma cortisol results for post-stress buspirone-treated fish, but no behavioral response at all, a result that suggests these fish are seriously affected by their condition. Dosages that were high enough to adversely affect the pilot study fish had no discernible effect on these fish, presenting a possibility that the DLS profile is irreversible on these individuals.

- Aim 3: Test if it is possible to reverse a potential DLS profile by changing social and environment structure during freshwater phase and after saltwater exposure.

These fish experienced a reversal of a potential DLS profile through a change in environment and social dynamics, showing that this profile can easily be reversed by removing bigger conspecifics and reducing the holding area so that food is easily available. While the first sampling (sampling 1) was taken when all fish were being held in a large tank in the presence of larger competitors, the second sampling (sampling 2) was done after the change in environment to smaller tanks and groups, which resulted in better growth, comparable to bigger conspecifics, regardless of water salinity. The main difference between these experimental fish and those in the buspirone experiment is the size advantage the change of environment fish had. Both groups were assumed to fit the description of the DLS profile, but reversing this profile was only possible for the larger fish.

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Appendix

Table 1: The full results of a Tukey post-hoc analysis on the locomotion data from the first part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
3mg/L,End	5mg/L,Before	0.0002*
3mg/L,End	3mg/L,Before	<.0001*
3mg/L,End	Control,Before	0.0004*
3mg/L,End	Control,End	0.0004*
3mg/L,After	5mg/L,Before	0.0015*
3mg/L,After	3mg/L,Before	<.0001*
3mg/L,After	Control,Before	0.0022*
3mg/L,After	Control,End	0.0027*
3mg/L,End	Control,After	0.0031*
5mg/L,After	5mg/L,Before	0.0001*
3mg/L,After	Control,After	0.0188*
5mg/L,After	3mg/L,Before	0.0244*
5mg/L,After	Control,Before	0.0002*
3mg/L,End	5mg/L,End	0.0280*
5mg/L,After	Control,End	0.0002*
3mg/L,After	5mg/L,End	0.1428
5mg/L,After	Control,After	0.0054*
5mg/L,End	5mg/L,Before	0.0587
5mg/L,End	3mg/L,Before	0.5776
5mg/L,End	Control,Before	0.0994
3mg/L,End	5mg/L,After	0.6179
5mg/L,End	Control,End	0.1250
5mg/L,After	5mg/L,End	0.1491
Control,After	5mg/L,Before	0.6920
3mg/L,After	5mg/L,After	0.9690
5mg/L,End	Control,After	0.8016
Control,After	3mg/L,Before	0.9846
Control,After	Control,Before	0.8356
Control,After	Control,End	0.8870
3mg/L,End	3mg/L,After	0.9233
Control,End	5mg/L,Before	1.0000
Control,Before	5mg/L,Before	1.0000
3mg/L,Before	5mg/L,Before	1.0000
Control,End	3mg/L,Before	1.0000
Control,End	Control,Before	1.0000
Control,Before	3mg/L,Before	.

Table 2: The full results of a Tukey post-hoc analysis on the time fish spent at the top of the water column from the first part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
5mg/L,End	5mg/L,Before	<.0001*
5mg/L,End	3mg/L,Before	<.0001*
5mg/L,End	Control,Before	<.0001*
3mg/L,End	5mg/L,Before	<.0001*
5mg/L,End	3mg/L,After	<.0001*
3mg/L,End	3mg/L,Before	<.0001*
3mg/L,End	Control,Before	0.0001*
3mg/L,End	3mg/L,After	<.0001*
5mg/L,End	Control,After	0.0002*
5mg/L,End	Control,End	0.0006*
3mg/L,End	Control,After	0.0006*
3mg/L,End	Control,End	0.0015*
5mg/L,After	5mg/L,Before	0.0020*
5mg/L,After	3mg/L,Before	0.0037*
5mg/L,After	Control,Before	0.0085*
5mg/L,After	3mg/L,After	0.0070*
5mg/L,After	Control,After	0.0638
5mg/L,End	5mg/L,After	0.0688
5mg/L,After	Control,End	0.1524
3mg/L,End	5mg/L,After	0.2996
Control,End	5mg/L,Before	0.4977
Control,End	3mg/L,Before	0.5422
Control,End	Control,Before	0.5600
Control,After	5mg/L,Before	0.7640
Control,End	3mg/L,After	0.7417
Control,After	3mg/L,Before	0.8232
Control,After	Control,Before	0.8401
Control,After	3mg/L,After	0.9467
3mg/L,After	5mg/L,Before	0.9996
5mg/L,End	3mg/L,End	0.9997
Control,End	Control,After	0.9996
3mg/L,After	3mg/L,Before	1.0000
Control,Before	5mg/L,Before	1.0000
3mg/L,After	Control,Before	1.0000
3mg/L,Before	5mg/L,Before	1.0000
Control,Before	3mg/L,Before	.

Table 3: The full results of a Tukey post-hoc analysis on group cohesion from the first part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
Control,Before	5 mg/L,After	<.0001*
Control,Before	5 mg/L,End	<.0001*
Control,Before	3 mg/L,After	<.0001*
Control,Before	5 mg/L,Before	<.0001*
Control,Before	3 mg/L,End	<.0001*
Control,Before	3 mg/L,Before	<.0001*
Control,After	5 mg/L,After	<.0001*
Control,After	5 mg/L,End	<.0001*
Control,After	3 mg/L,After	0.0002*
Control,Before	Control,End	0.0015*
Control,End	5 mg/L,After	0.0018*
Control,End	5 mg/L,End	0.0018*
Control,After	5 mg/L,Before	0.0136*
Control,After	3 mg/L,End	0.0148*
Control,End	3 mg/L,After	0.0166*
Control,After	3 mg/L,Before	0.0185*
Control,Before	Control,After	0.1261
3 mg/L,Before	5 mg/L,After	0.1311
3 mg/L,Before	5 mg/L,End	0.1332
3 mg/L,End	5 mg/L,After	0.1567
3 mg/L,End	5 mg/L,End	0.1591
5 mg/L,Before	5 mg/L,After	0.1677
5 mg/L,Before	5 mg/L,End	0.1703
Control,End	5 mg/L,Before	0.5392
Control,End	3 mg/L,End	0.5621
3 mg/L,Before	3 mg/L,After	0.5631
Control,After	Control,End	0.5914
Control,End	3 mg/L,Before	0.6211
3 mg/L,End	3 mg/L,After	0.6221
5 mg/L,Before	3 mg/L,After	0.6449
3 mg/L,After	5 mg/L,After	0.9881
3 mg/L,After	5 mg/L,End	0.9888
3 mg/L,Before	5 mg/L,Before	1.0000
3 mg/L,Before	3 mg/L,End	1.0000
3 mg/L,End	5 mg/L,Before	.
5 mg/L,End	5 mg/L,After	.

Table 4: The full results of a Tukey post-hoc analysis on locomotion from the second part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
Day 1,End	Day 3,Before	<.0001*
Day 1,End	Day 1,Before	<.0001*
Day 1,End	Day 1,After	<.0001*
Day 1,End	Day 2,Before	<.0001*
Day 1,End	Day 2,After	<.0001*
Day 1,End	Day 2,End	<.0001*
Day 3,End	Day 3,Before	<.0001*
Day 3,End	Day 1,Before	<.0001*
Day 1,End	Day 3,After	<.0001*
Day 3,End	Day 1,After	<.0001*
Day 3,End	Day 2,Before	<.0001*
Day 3,End	Day 2,After	<.0001*
Day 3,End	Day 2,End	<.0001*
Day 3,After	Day 3,Before	<.0001*
Day 3,After	Day 1,Before	<.0001*
Day 3,End	Day 3,After	<.0001*
Day 1,End	Day 3,End	<.0001*
Day 2,End	Day 3,Before	<.0001*
Day 2,End	Day 1,Before	<.0001*
Day 3,After	Day 1,After	<.0001*
Day 3,After	Day 2,Before	<.0001*
Day 2,After	Day 3,Before	<.0001*
Day 2,After	Day 1,Before	<.0001*
Day 2,End	Day 1,After	<.0001*
Day 2,End	Day 2,Before	0.0002*
Day 3,After	Day 2,After	0.0019*
Day 2,After	Day 1,After	0.0041*
Day 2,Before	Day 3,Before	0.0246*
Day 2,Before	Day 1,Before	0.0268*
Day 2,After	Day 2,Before	0.0602
Day 1,After	Day 3,Before	0.2576
Day 1,After	Day 1,Before	0.2740
Day 3,After	Day 2,End	0.2921
Day 2,End	Day 2,After	0.3661
Day 2,Before	Day 1,After	0.9559
Day 1,Before	Day 3,Before	.

Table 5: The full results of a Tukey post-hoc analysis on the time fish spent at the top of the water column from the second part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
Day 1,End	Day 1,Before	<.0001*
Day 1,End	Day 1,After	<.0001*
Day 3,End	Day 1,Before	<.0001*
Day 3,End	Day 1,After	<.0001*
Day 3,After	Day 1,Before	<.0001*
Day 3,After	Day 1,After	<.0001*
Day 2,End	Day 1,Before	<.0001*
Day 2,End	Day 1,After	<.0001*
Day 2,After	Day 1,Before	0.0003*
Day 2,After	Day 1,After	0.0003*
Day 1,End	Day 3,Before	0.0012*
Day 1,End	Day 2,Before	0.0018*
Day 3,End	Day 3,Before	0.0020*
Day 3,End	Day 2,Before	0.0028*
Day 3,After	Day 3,Before	0.0075*
Day 3,After	Day 2,Before	0.0106*
Day 2,End	Day 3,Before	0.0346*
Day 2,End	Day 2,Before	0.0476*
Day 2,Before	Day 1,Before	0.0983
Day 2,Before	Day 1,After	0.1086
Day 3,Before	Day 1,Before	0.1310
Day 3,Before	Day 1,After	0.1441
Day 2,After	Day 3,Before	0.2161
Day 2,After	Day 2,Before	0.2762
Day 1,End	Day 2,After	0.3689
Day 3,End	Day 2,After	0.4788
Day 3,After	Day 2,After	0.8090
Day 1,End	Day 2,End	0.8837
Day 3,End	Day 2,End	0.9449
Day 2,End	Day 2,After	0.9900
Day 1,End	Day 3,After	0.9974
Day 3,After	Day 2,End	0.9989
Day 3,End	Day 3,After	0.9997
Day 1,End	Day 3,End	1.0000
Day 2,Before	Day 3,Before	1.0000
Day 1,After	Day 1,Before	.

Table 6: The full results of a Tukey post-hoc analysis on the number of midline crossings from the second part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
Day 1,End	Day 1,Before	<.0001*
Day 2,Before	Day 1,Before	0.0005*
Day 1,End	Day 3,Before	0.0005*
Day 3,End	Day 1,Before	0.0005*
Day 2,End	Day 1,Before	0.0051*
Day 2,Before	Day 3,Before	0.0051*
Day 2,After	Day 1,Before	0.0051*
Day 3,End	Day 3,Before	0.0051*
Day 1,End	Day 1,After	0.0162*
Day 2,End	Day 3,Before	0.0484*
Day 3,After	Day 1,Before	0.0484*
Day 2,After	Day 3,Before	0.0484*
Day 2,Before	Day 1,After	0.1316
Day 1,End	Day 3,After	0.1316
Day 3,End	Day 1,After	0.1316
Day 1,After	Day 1,Before	0.3089
Day 3,After	Day 3,Before	0.3089
Day 2,End	Day 1,After	0.5883
Day 2,Before	Day 3,After	0.5883
Day 2,After	Day 1,After	0.5883
Day 1,End	Day 2,After	0.5883
Day 3,End	Day 3,After	0.5883
Day 1,End	Day 2,End	0.5883
Day 1,After	Day 3,Before	0.8612
Day 2,Before	Day 2,After	0.9848
Day 2,End	Day 3,After	0.9848
Day 2,Before	Day 2,End	0.9848
Day 3,After	Day 1,After	0.9848
Day 2,After	Day 3,After	0.9848
Day 1,End	Day 3,End	0.9848
Day 3,End	Day 2,After	0.9848
Day 3,Before	Day 1,Before	0.9848
Day 1,End	Day 2,Before	0.9848
Day 3,End	Day 2,End	0.9848
Day 2,End	Day 2,After	1.0000
Day 2,Before	Day 3,End	1.0000

Table 7: The full results of a Tukey post-hoc analysis on group cohesion from the second part of the pilot experiment. Significant differences are shown in red/orange with a star.

Level	- Level	p-Value
Day 1,Before	Day 3,End	<.0001*
Day 1,Before	Day 1,After	<.0001*
Day 1,Before	Day 3,After	<.0001*
Day 1,Before	Day 2,End	<.0001*
Day 1,Before	Day 1,End	<.0001*
Day 1,Before	Day 2,After	<.0001*
Day 3,Before	Day 3,End	0.0001*
Day 3,Before	Day 1,After	0.0002*
Day 3,Before	Day 3,After	0.0005*
Day 3,Before	Day 2,End	0.0011*
Day 1,Before	Day 2,Before	0.0020*
Day 3,Before	Day 1,End	0.0087*
Day 3,Before	Day 2,After	0.0131*
Day 2,Before	Day 3,End	0.0828
Day 2,Before	Day 1,After	0.0995
Day 3,Before	Day 2,Before	0.1901
Day 2,Before	Day 3,After	0.2209
Day 2,Before	Day 2,End	0.3891
Day 1,Before	Day 3,Before	0.5204
Day 2,After	Day 3,End	0.6234
Day 2,After	Day 1,After	0.6792
Day 1,End	Day 3,End	0.7294
Day 1,End	Day 1,After	0.7801
Day 2,Before	Day 1,End	0.8712
Day 2,After	Day 3,After	0.8954
Day 2,Before	Day 2,After	0.9321
Day 1,End	Day 3,After	0.9480
Day 2,After	Day 2,End	0.9801
Day 2,End	Day 3,End	0.9923
Day 1,End	Day 2,End	0.9940
Day 2,End	Day 1,After	0.9963
Day 3,After	Day 3,End	0.9998
Day 3,After	Day 1,After	1.0000
Day 2,End	Day 3,After	1.0000
Day 2,After	Day 1,End	1.0000
Day 1,After	Day 3,End	1.0000

Table 8: The full results of a Tukey post-hoc analysis on plasma cortisol levels at both basal and post-stress states from the DLS reversal by buspirone experiment. Significant differences are shown in orange with a star.

Level	- Level	p-Value
Buspirone,Stress	Buspirone,Basal	<.0001*
Buspirone,Stress	Control,Basal	<.0001*
Control,Stress	Buspirone,Basal	<.0001*
Control,Stress	Control,Basal	<.0001*
Buspirone,Stress	Control,Stress	0.1214
Control,Basal	Buspirone,Basal	1.0000

Table 9: The full results of a Tukey post-hoc analysis on plasma chloride levels for the change in environment experiment fish. Significant differences are shown in orange with a star.

Level	- Level	p-Value
Saltwater	Start	<.0001*
Freshwater	Start	0.0001*
Saltwater	Freshwater	0.5359

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