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SELECTIVE SEROTONIN REUPTAKE INHIBITOR EFFECTS ON HYBRID STRIPED BASS PREDATORY BEHAVIOR AND INTERNAL CHEMISTRY

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Environmental Toxicology

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by
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August 2017

Accepted by:
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Dr. Thomas Schwedler

ABSTRACT

Prescription drug use continues to increase across the United States. An important part of these medications are selective serotonin reuptake inhibitors (SSRIs) that function as anti-depressants, and include drugs as citalopram (Celexa) and sertraline (Zoloft). SSRI's main mode of action is the inhibition of the serotonin reuptake transporter, causing a buildup of extracellular serotonin, one of the neurotransmitters in the central and peripheral nervous system. SSRIs can be considered persistent pollutants due to their continuous release from wastewater treatment effluent, drug manufacturing effluent, and agricultural runoff. Aquatic organisms can become non-target organisms when subjected to sub-lethal concentrations (low ppb to high ppm) of antidepressants. Behavioral tests provide sensitive endpoints for determining whether aquatic organisms have been subjected to antidepressants, causing changes in their ecological fitness. The goal of this research was to determine whether SSRIs cause sublethal effects in fish populations through a change in feeding behavior, supported by brain and plasma chemistry and changes in serotonin-related gene expression in the intestine. We hypothesized a decrease in feeding behavior, a decrease in serotonin levels, and a change in gene expression after exposure to sertraline and citalopram. Hybrid striped bass (HSB) exposed to citalopram (6 day exposure at 50-150 µg/l) and sertraline (4-100 µg/l, 6 days exposure, 6 days recovery) were fed every three days to determine effects on behavior. Blood, brain, and intestine samples collected from euthanized fish every three days were analyzed for concentrations of citalopram, sertraline and serotonin. Both sertraline and citalopram

caused a change in predatory behavior during exposure, with sertraline having a more dramatic effect than citalopram. The sertraline recovery period showed that the bass were able to rapidly return to normal feeding behavior, even while the antidepressant was still located in the brain and plasma. Citalopram and sertraline were both detected in brain and plasma samples, but in different levels during the exposure and recovery period. Serotonin levels also differed between each SSRI treatment. Our results showed that SSRIs may cause an upregulation of both the serotonin reuptake transporter and cholecystokinin, a satiation signaling protein. From an ecological standpoint an increased feeding time could make exposed bass populations less ecologically fit compared to other populations that are not as affected by antidepressants.

DEDICATION

I dedicate this thesis to my parents:

David and Donna Stoczynski

I also dedicate this thesis to Romeo M. Schwartz

You three pushed me to achieve success through thick and thin.

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I would first like to thank my committee, Dr. Peter van den Hurk, Dr. Joseph Bisesi, and Dr. Thomas Schwedler. You have all helped me through many trials and tribulations in order to get where I am today. My success at Clemson would not have been possible without your direction and unending patience to work with me during the difficulties that not only research brought but life as well. I would like to thank my late advisor Dr. Stephen Klaine. Without you I never would have made it to Clemson, and would have never been given this challenging project.

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Finally thank you to my parents and my loving boyfriend for helping me through the trials that have been graduate school so far. You have seen me cry during my failures and smile during my successes. To all my friends, thank you for your support. The journey is not over yet.

TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

Page

CHAPTER ONE

LITERATURE REVIEW

Pharmaceutical usage

As the benefits of old and new pharmaceuticals becomes realized, the number of drug prescriptions written continues to grow at an increasing rate. From 1999-2000, 44% of the population took one prescription drug. By 2008, this proportion increased to 48% [1]. In 2006, six of the most dispensed pharmaceuticals included the antidepressants: citalopram, sertraline, duloxetine, venlafaxine, paroxetine, and bupropion [2]. Spending on prescription drugs increased to 250 billion dollars in 2009 and accounted for roughly 12% of total personal health care expenses [1]. The number of prescriptions increased from 254 million in 2010 to 314 million in 2015 [3].

Antidepressants include several classes including selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), norepinephrine and dopamine reuptake inhibitors (NDRIs), monoamine oxidase inhibitors (MAOIs), and tricyclic. SSRI sales started in the mid-1980s. SSRIs were developed to increase extracellular serotonin, which would beneficially help patient's mood and behavior. SSRI are prescribed to treat clinical depression, attention deficit disorder, panic disorder, and obsessive-compulsive disorder [2]. In 2005, a Denmark study reported 5% of adults were prescribed an antidepressant with 57% taking citalopram and 14% taking sertraline [4]. The world economic co-operation and development department reported a 60% increase in antidepressant use from 2001-2011 [5]. Sertraline has seen a substantial increase in consumption from 2007-2013 becoming

the 60th most widely prescribed pharmaceutical overall [6]. In 2013, sertraline became the second most dispensed psychiatric drug [7].

Pharmaceuticals entering the environment

Pharmaceuticals enter the environment from wastewater treatment effluent containing drugs from human excrement, unmetabolized drugs, or disposal of unused or expired drugs in toilets [2, 8, 9]. Livestock can be administered pharmaceuticals which end up in solid or liquid wastes that run off agricultural fields [10]. Finally, pharmaceutical manufacturing plants could release compounds into the environment through their contribution of contaminated waters entering sewage treatment plants [10, 11]. Concentrations of pharmaceuticals in surface waters can range from ng/L to µg/L levels.

Wastewater treatment facilities have limited ability to remove pharmaceuticals from sewage [12]. Different wastewater treatment processes end in varying removal efficiencies resulting in a discharge into surrounding waters [13]. Citalopram has the highest removal efficiently of the SSRIs at 98%, while sertraline has the lowest at 60% [4]. Studies in several countries reported antidepressants in wastewaters (0.15-84,000 ng/L), surface/ground waters (0.5-8,000 ng/L), and drinking waters (0.5-1,400 ng/L) [5, 14]. Three different sewage treatment plants found citalopram in a range of concentrations from 382 ng/L to 612 ng/L [15]. Many treatment plants use primary and secondary removal systems such as activated sludge and biological degradation to clean wastewaters [16]. Installation of advanced tertiary removal systems (nanofiltration, reverse osmosis, UVC photolysis, ozonation) may result in increased removal efficiencies

[13, 16, 17]. Doctors chronically administer drugs for the upkeep of therapeutic effect for patients, resulting in frequent enough discharges to make pharmaceuticals persistent pollutants leading to continuous exposure over an aquatic organism's life cycle [15, 18, 19].

SSRIs occur in a variety of different environmental matrices making them the most common detected human pharmaceuticals in aquatic habitats [5]. Sertraline has been analyzed around the United States and showed an average wastewater concentration range of 78-120 ng/L [20]. A small creek in Texas registered sertraline levels averaging 4.27 ng/L [21]. Downstream from a wastewater treatment plant in Iowa, researchers detected ten different antidepressants in two streams including sertraline (0.7-37.5 ng/L) and citalopram (4.58-205 ng/L) [9]. Antidepressants had high sorption coefficients (reported as K_{oc} values which are organic carbon normalized sorption coefficients) in soils and sediments. Research reported the antidepressant fluoxetine has the lowest sorption rate while citalopram had the highest [5]. Scientists observed high brain antidepressant concentrations at wastewater treatment outflows, while concentrations remained elevated in the brain further downstream from the effluent pipe [9]. SSRI mixtures in natural environments showed combined concentrations up to 3,000 ng/L near treatment plant outflows [3].

Most pharmaceuticals that enter the environment could potentially be persistent in aquatic ecosystems. Persistence of such pollutants could pose life-cycle problems to the aquatic biota [22]. Research shows lethal effects of antidepressant pharmaceuticals on aquatic life occur at concentrations over 1 mg/L, far from environmental relevance [23].

These compounds appear at low levels, so observation of chronic sublethal effects occur more than acute effects [12, 15]. Sublethal effects from pharmaceutical exposure could result in long-term changes from the individual to population level after several generations of exposure [11, 15].

Pharmaceutical partitioning

When looking at several pharmaceutical compounds, log K_{ow} accounted for less than 50% of the variation suggesting more than the K_{ow} is needed to understand environments favorable for some compounds [13]. Different properties of a drug dictated partitioning behavior such as dissociation constant, molecular weight, lipophilicity, and pH of adsorption site [24]. K_{ow} cannot be solely relied on for understanding partitioning of antidepressants due to the ionizable drug characteristics. Compounds that showed ionizable functional groups tended to move away from lipid environments whereas non-ionizable compounds tended to move towards lipid environments. Relying on just K_{ow} values could lead to inaccurate fish tissue partitioning estimates for antidepressants [13].

Researchers investigated toxicity and bioaccumulation of ionizable drugs took into account pH to increase the success of ecological risk assessments [24]. The bioavailability of ionizable xenobiotics', including pharmaceuticals, changes depending on the compound's ionization state. Neutral compounds can cross cellular membranes with ease compared to their ionized counterparts, making the neutral compounds more bioavailable [25]. Pharmaceuticals distributed throughout the body depend on the ionization state created by the pH ranges found in the body [24]. Ionization state alone cannot explain pharmaceutical toxicity. Fish models investigated the problem by

measuring how excretory products changed pH in the fish's immediate environment. The pH change could ionize compounds making them more likely to be absorbed or prevent compounds from crossing cellular membranes [25]. SSRIs are weak base pharmaceuticals with pKa values ranging from 9.05-10.5 [5]. SSRIs in neutral pH aqueous solutions will be found in their ionic form more than their un-ionic form. Sertraline (a SSRI) showed increased toxicity to fathead minnows as the percentage of un-ionized drug increased [25].

Antidepressants concentrate in different tissues within fish. Lipophilic xenobiotics including SSRIs primarily enter fish through the gills while exposure through feeding and water intake is less significant [15]. Low water temperatures decreased ventilation rate and metabolism causing decreased water and pharmaceutical intake [26]. Pharmaceuticals that entered the fish via the gills bypassed first pass metabolism in the liver before systemic distribution. Antidepressants and their metabolites may distribute to the liver, brain, plasma, and muscle of fish, but more research is needed to determine the relative levels getting into these target tissues [27].

European agencies outlined a process to determine the environmental risk of human pharmaceuticals. The first phase predicted environmental concentrations using the market percentage, max daily dose, wastewater per inhabitant per day, and dilution factors [28]. Some examples of human daily doses for SSRIs are 0.01, 0.01, 0.02 and 0.02g for escitalopram, citalopram, fluoxetine, and paroxetine respectively. Sertraline and fluvoxamine have higher daily doses of 0.05 and 0.1g, respectively [5]. A predicted environmental concentrations below 0.01 µg/L resulted in no further tests due to the low

environmental threat of that compound. A predicted environmental concentration higher than 0.01 µg/L led to more tests (second phase) due to a potential environmental threat. The second phase used biodegradability tests to investigate wastewater treatment facilities and the environment. These tests investigated sorption behavior of the drug to sewage sludge, soil, water, and octanol [28].

Microcosms provided an excellent tool for analyzing the environmental risk of pharmaceuticals over several trophic levels that could not be completed with single-species tests [29]. Results from microcosm experiments suggested antidepressants could potentially bioaccumulate in aquatic species at distances up to 1 km from effluent discharge in the St. Lawrence River; showing antidepressants can be transported great distances in surface waters [9, 12]. Difficulties can arise when trying to make comparisons between multiple studies since the medium of exposure (microcosms, lakes, and rivers), ambient concentrations of antidepressants, exposure time, and fish species were different [12].

Serotonin and the serotonin reuptake transporter

Serotonin, a common neurotransmitter, was found in several species of fish, and serotonin synthesis and function shows high conservation between invertebrates and vertebrates [5, 8, 15]. Tryptophan hydroxylase synthesizes serotonin from tryptophan in the central nervous system and gastrointestinal tract [4, 15]. Tryptophan concentration limits the rate of serotonin synthesis in the fish brain [30]. Invertebrate research hypothesizes serotonin controls gonadal maturation and induction of spawning and metamorphosis along with metabolism and behavior [5, 29]. Developmental biology

confirmed serotonin appeared first during brain development and helped in the regulation of cell proliferation, differentiation, and apoptosis [31].

Both mammals and teleost fish exhibit homologies in the hypothalamic-pituitary-adrenal axis, resulting in the use of neurotransmitters to maintain autonomic, behavioral, and stress responses [32]. Serotonin can both, directly and indirectly, stimulate the release of gonadotropin releasing hormone and gonadotropin luteinizing hormone from the hypothalamus and pituitary gland [19]. Research hypothesizes stimulation of the hypothalamus-pituitary axis by serotonin controls the release of corticotrophin releasing hormone during non-stressful conditions and adrenocortical releasing hormone during stressful conditions [30]. Delayed elevation of serotonin in some brain areas could cause changes in behavior, endocrine, and reproductive systems [8, 30]. Research suggests serotonin influences behavioral changes in feeding, appetite, and locomotion [11, 32]. Compounds in addition to serotonin might explain behavioral changes seen in the literature. A study by Conners et al. (2009) demonstrated decreased foraging behavior in tadpoles after serotonin controlled brain activity which regulated corticotrophin-releasing factor (CRF) changed behaviors in the hypothalamus [33].

The serotonin reuptake transporter (SERT) is an enzyme responsible for controlling intra and extracellular serotonin concentrations. SERT is the main target for selective serotonin reuptake inhibitors (SSRIs) which have structural differences but work by the same mode of action [18]. SSRIs therapeutic effect increases serotonin in the synapse by blocking SERT ATPase activity [12, 34].

Available information on mammalian pharmacology may be helpful for predicting fish responses if the species have similar target receptors to previously studied pharmaceuticals [21]. Research has shown similarities exist between enzymes and receptors in aquatic species and humans, suggesting pharmaceuticals have the ability to affect aquatic non-target organisms [35, 36]. *Danio rerio*, *Daphnia pulex*, and *Chamydomonas reinhardtii* (green algae) showed 86%, 61%, and 35% similarities, respectively when compared to 1,318 human drug targets. The study showed lower invertebrates and plant species have the potential to respond to pharmaceuticals in the environment due to conserved drug target receptors [37]. Serotonin receptors have been detected in several fish species including Japanese medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), goldfish (*Carassius auratus*), hybrid striped bass (*Morone chrysops x Morone saxatilis*), and zebrafish (*Danio rerio*) [8, 21, 38]. Studies that compared SSRI receptors in different species, at the amino acid level, determined fish, on average, have more highly conserved receptors for SSRIs than other species more closely related to humans [38]. Zebrafish (*Danio rerio*) have two genes which code for SERT with 66-69% and 75% conserved compared to human SERT [34]. SERT sequence conservation jumped to 93% when amino acid residues were compared [3]. SERT binding affinity showed similarities between model organisms such as fathead minnows, zebrafish, and laboratory rats [34]. The hybrid striped bass SERT showed 72% homology with human SERT [38]. Sweet (2015) determined the conservation of the functional domain for SERT was similar between the hybrid striped bass and mammals. Different SSRIs were tested to determine which had the highest binding affinity for the hybrid

striped bass SERT. The antidepressants rank similarly from highest to lowest binding affinity in fish and humans: sertraline > fluoxetine > citalopram > venlafaxine [38, 39].

Ecotoxicology of pharmaceuticals

Non-target organisms with similar drug receptors may be at heightened risk for exposure to pharmaceuticals even at low concentrations [25]. Pharmaceutical companies performed numerous tests to develop nonclinical safety profiles for their drugs. The amount of mammalian data is extensive but only a few proposed models investigated ecotoxicological effects using mammalian data to prioritize pharmaceuticals [38]. Researching different behavioral endpoints for toxicity could improve our understanding of pharmaceuticals on fish populations [40]. Behavior correlates with ecological fitness at individual and population levels. A change in behavior can result in tradeoffs that cause changes in individual fitness or population increase/decrease/local extinctions [8, 11, 41]. Population-level changes depend on the trophic level first affected leading to potential negative consequences in other trophic levels [11].

When a drug's chemical half-life exceeds effluent release rate, characterization of chronic and sublethal effects on aquatic organisms should be examined [23]. Sublethal effects include changes in behavior. A behavior describes a sublethal response of an organism to biotic and abiotic stimuli [34]. The responses can vary in type, intensity, and time of occurrence depending on physiological signals and environmental or social tolerance ranges [22, 34]. Behaviors describe necessary mechanisms organisms use to react and adapt to changes in the environment, such as exposure to pharmaceuticals [22].

Behavioral endpoints provide the most sensitive ecotoxicological approach to studying pharmaceuticals in the environment [22, 25]. Past research reported behavioral responses could be 10 to 100 times more sensitive than standard endpoints such as survivorship [22, 25]. Out of several different endpoints used in determining ecological risk assessment, feeding rate showed more sensitivity as a toxicological response than standardized endpoints such as growth or survivorship [25, 34]. When exposed to a toxicant organisms try and reverse their effects, and changes in behavior can be the first to show [22]. Studies have linked other biomarkers, including neurotransmitters, plasma enzymes, oxidase activity, hormones, and energy metabolism, to behavior [5, 22].

Many antidepressants work by effecting neurotransmitters in the brain to cause their therapeutic effects. The dose of antidepressants and the time exposed can have profound effects on physiological endpoints [11]. Research showed neurotransmitters such as serotonin, dopamine, and norepinephrine influenced locomotion, aggression, and feeding behaviors in fish [22]. Effects included delays in reproductive and physiological development, decreased aggressiveness, and inhibition of feeding responses [5]. Sensitivity in behavioral endpoints could be ranked with locomotor behavior and early life stage behavior as most sensitive followed by reproductive behavioral changes [11]. Feeding changes may not be apparent from growth data depending on food availability [25]. Fluoxetine and venlafaxine research demonstrated changed water column positioning, decreased the ability to capture prey, and delayed escape response at concentrations of 23.2-100.9 µg/L [23, 38]. Another study discovered reduced territorial aggression in coral reef fish along with decreased locomotion and aggression in Siamese

fighting fish [11]. Drummond and Russom (1990) exposed fathead minnows to 300 organic chemicals to determine how to categorize changes in behavior [42]. They determined three acute behavioral toxicity syndromes occured: hypoactivity, hyperactivity, and physical abnormality. Hypoactivity resulted in decreased motion linked to narcotic activity [22]. Hyperactivity described accelerated motion, increased ventilation and changed metabolism. Physical abnormality categorized convulsions and bone deformations happening from damage to the nervous system [22].

Behavioral changes could cause ecological consequences including deviations in predator-prey interactions [22]. Evidence suggests long-term exposures to SSRIs changed hierarchies in fish populations during sensitive times in the reproductive cycle. The ecological health of a population could shift to fish who were originally subordinate compared to the previously dominate fish [15]. The first indication of behavioral changes become apparent between 50-70 minutes to a few days after the start of exposure to antidepressants or tryptophan [43]. Variations in feeding behavior could decrease the nutritional status of the organism affecting their ecological fitness [3]. Deviations in neuromuscular behavior may impair food search and reproductive behavior which increases the risk of predation [44]. Larval fathead minnows showed reduced predator avoidance when in an environment with fluoxetine and venlafaxine at concentrations of 25-250 and 500-5000 ng/L, respectively [9]. Pharmaceuticals may, therefore, cause behavioral changes in the biochemical, individual, and population levels of aquatic ecosystems [34].

Experiments completed with a complex mixture of SSRIs resulted in whole brain serotonin levels decreased by 44% ± 11% by the third day of exposure. By the 9th day of exposure whole brain serotonin levels decreased by 83% ± 7% compared to controls. At day 12, all treatments showed a decrease in whole brain serotonin between 40% - 80% ± 4%. Days 9-12 were a recovery period, but whole brain serotonin levels remained significantly lower than controls [38]. Knowing the plasma concentrations of SSRIs in fish could help predict toxic effects [27]. SSRI levels in fish plasma did not change between 48 and 72 hours after exposure, suggesting concentrations reach their maximum in fish at 48 hours [38]. One study reported pharmaceutical uptake, including antidepressants, in bull sharks (*Carcharhinus leucas*) in river systems. Citalopram was one antidepressant found in the plasma at concentrations of 0.4 ng/mL [45].

The brain is the first organ of interest many times in antidepressant research. Other organs have the potential to be mechanistic sites for SSRIs such as the intestine [46]. Serotonin stimulates and inhibits gut motility depending on the section of the gut [47]. Researched showed higher serotonin concentrations in the whole anterior intestine with lower concentrations in the entire posterior intestine of rainbow trout [46]. Serotonergic neurons in teleost fish can develop into different morphologies and densities with the two most abundant forms being enterochromaffin (EC) cells or enteric neurons [47]. Enteric neurons make up a chain of serotonergic neurons that go up and down the intestine walls [48]. EC cells dominate production of serotonin in the gut. The release of serotonin from EC cells regulates peristalsis, secretion, vasodilation, and perception of pain/nausea [48]. While mammalian intestines contain a large amount of EC cells, some

teleost species have a low number of EC cells or none at all, meaning enteric neurons could be larger producers of serotonin in the fish gut [46]. The serotonin-selective reuptake transporter (SERT) is expressed in the intestinal mucosa serving as a critical molecule for removing serotonin from the intestine space after release from EC cells or enteric neurons [48].

Changes in behavior linked to SSRI exposure may be affecting gene expression within the intestine walls. One hypothesis states changes in feeding behaviors could be a result of up or down regulation of hunger signals from within the gut [49]. Production of cholecystokinin (CCK) takes place in both the brain (CCK-8) and the gut (CCK-A) [50]. CCK has been isolated from the gut of several fish species including trout and cod [49]. CCK appetite control was demonstrated in studies with goldfish and trout [49]. CCK will only be released in the gut in the presence of food and may mediate another protein (leptin) effect on food intake [50]. SSRI exposure could cause a down-regulation in SERT [25]. A pharmacological blockage of SERT could lead to an increase in serotonin in the intestine, where serotonin would move to the blood stream and be removed by platelets expressing SERT [48]. The gene for intestine SERT is expressed downstream from SERT found in brain neurons, making the two serotonin transporters similar [48].

Current citalopram research

Citalopram has two forms; the S (+) enantiomer (escitalopram) represents the more active form [5, 14]. Research showed the S (-) enantiomer (citalopram) could disrupt the effects of escitalopram interacting with the SERT receptor [31]. Citalopram half-life is roughly 36 hours [4]. Excretion of the active drug and its main metabolite

(desmethyl citalopram) contribute to 26% and 19% of the human daily dose (40 mg/day), respectively [15, 27]. The ecotoxicological risk of citalopram to aquatic organisms with nervous systems containing serotonin, such as fish, has not been well studied [4]. Previous research hypothesized citalopram has the lowest toxicity of the SSRI compounds with LC50 of 3.9 mg/L and NOEC of 0.8 mg/L [4, 5].

Previous research on citalopram investigated the effect of exposure through dietary routes. Holmberg et al. (2011) studied citalopram exposure through feeding juvenile rainbow trout citalopram soaked food at environmentally relevant concentrations. The researchers determined whether citalopram caused behavioral changes in aggression or reproduction. No significant changes were found in aggressive behavior or swimming activity between exposure groups [43]. A similar experiment exposed trout to 100 µg/kg of citalopram in pellet food for one week [30]. After seven days of exposure, previously dominant fish became less aggressive and showed decreased cortisol levels compared to controls, but no difference was seen when compared to a tryptophan-supplemented diet [30].

Behavioral experiments exposed aquatic organisms to citalopram via a solely aqueous exposure route. Endler guppy behaviors measured after a chronic exposure for 21 days to 2.3 µg/L or 15 µg/L citalopram saw decreased feeding time and decreased the freezing frequency at 15 µg/L. All the behaviors studied directly correlated with anxiety [19]. A decrease in anxiety can have detrimental effects on the survival of organisms, leaving them more vulnerable to predation and resulting in decreased feeding success [5]. Olsen et al. (2013) hypothesized that SSRI exposure disrupts the hypothalamic-pituitary-

interrenal axis (HPI-axis) causing changes in freezing, exploratory, and feeding behaviors [19]. Feeding behavior of three-spined stickleback exposed to 0.15 or 1.5 µg/L citalopram was investigated for 21 days [14]. The number of attacks on prey (bloodworms) was observed during a 10-minute span, weekly. Fish decreased their number of attacks on prey (by 37.5%) after the first week. This behavior persisted throughout the experiment, and both exposure concentrations showed significant differences from controls, but no differences from each other. The researcher used three fish per tank providing a more realistic experiment since three-spine stickleback are not solitary fish [14].

Current sertraline research

The chemical properties confirmed for sertraline include a pKa of 9.47, a half-life of 37 hours, photodegradation period of 4-11 days, and average therapeutic dose of 50 mg/day with an internal human therapeutic value of 0.19 µg/ml [4, 15, 25, 53]. Sertraline breaks down in the body to desmethylsertraline [21]. In Canada, sertraline detected in incoming wastewaters reported values as high as 47 kg total, annually [27]. Researchers hypothesize sertraline inhibits Na/K ATPase activity in the brain [12]. Research hypothesized sertraline may affect oxidative stress and acetylcholinesterase activity [25]. A decrease in acetylcholinesterase activity suggests dysfunction of ventilator and locomotion as well as inhibition of anti-oxidant enzymes [5]. Some of the antioxidant enzymes (which protect organisms from reactive oxygen species) affected by sertraline include superoxide dismutase, catalase, glutathione peroxidase, and glutathione S-transferase [25].

Sertraline has been documented as the most toxic of the SSRI class. One hypothesis examined the higher log D (used in pharmacology research and quantifies the distribution of a drug at different pH values) value compared to the other SSRIs, resulting in higher bioaccumulation numbers [6, 38]. Sertraline exposure resulted in higher brain concentrations than would be suggested by surrounding water concentrations [23]. Several studies reported sertraline in fish fillet and liver tissues at up to 19 and 545 ng/g, respectively [13]. Subtle changes in the chemical structure of SERT receptors could result in the slight differences in serotonin concentrations seen in the literature [23]. The pH should also be considered with sertraline because aqueous exposure can result in fish plasma levels much higher than human therapeutic doses [51]. Ionization of sertraline relied on the pH of the external and internal environment. Research hypothesizes the un-ionized form of sertraline moves to target sites at a more rapid pace than the ionized form [25].

Studies completed with sertraline documented changes in behavior. Fathead minnows exposed to sertraline showed an anxiolytic effect with fish spending 18-42% less time under shelter compared to controls, leaving fish more vulnerable to predation [34]. Crucian carp exposed to 80 and 300 ng/L sertraline reported increased excessive swimming, decreased feeding rate, decreased food consumption, and decreased shelter seeking. Sertraline was detected in the highest concentrations in the carp liver and brain [40]. Perch feeding was reduced by as much as 60% when exposed to 89 and 300 µg/L sertraline over 7 days [54]. A decrease in feeding could change the balance of predator/prey relations and community structure [5]. Tadpoles exposed to

environmentally relevant concentrations of sertraline and fluoxetine (0.1, 1, and 10 µg/L) showed developmental effects over a 70-day exposure. Sertraline concentrations in tadpoles at day 44 were 6x higher compared to fluoxetine. Growth decreased by 35%, and tadpoles completed tail resorption 10 days earlier than control tadpoles at the 0.1 and 1 µg/L concentrations [33]. Crab populations off Portugal exposed to sertraline reported significant decreases in AChE activity, increased GST biotransformation, and increased oxidative damage [44]. Sertraline exposure to zebrafish embryos resulted in increased abnormalities for tail, yolk-sac, and head at 100 µg/L at 32 hours post fertilization [7]. Sertraline (424 ng/L) exposed crayfish were more aggressive than control crayfish [53].

Few studies involving behavioral endpoints correlate their research to biochemical endpoints, the brain and plasma have been the focus of biochemical research involving sertraline. Sweet (2015) documented a decrease in whole brain serotonin levels for a SSRI mixture involving sertraline. This result is hypothesized to be a result of the SERT receptors not becoming desensitized within the short exposure time. Sertraline exposure may have caused a decrease in the total density of SERT receptors in the tissue suggesting a down-regulation of SERT with long-term exposure [38]. The greatest sertraline concentrations were observed in bull shark livers, mainly thought to be a result of the liver being the place for xenobiotic metabolism [5].

Thesis Goals

Our lab has worked on antidepressant exposures to hybrid striped bass in the past. Sweet (2015) exposed hybrid striped bass to a SSRI mixture including fluoxetine, sertraline, and citalopram, and showed an increase in time to capture prey, with assumed

additivity between compounds [38]. A fluoxetine only exposure was already completed before, leaving a gap with no data on citalopram or sertraline only exposures. All studies used a similar experimental setup consisting of six days of exposure followed by six days of recovery. Bass were fed every three days to gather predatory behavior data. Analytical data collected varied between studies, with whole brain serotonin being the common parameter of interest. Completely understanding mixture data is difficult without having information on the effects of the individual compounds. The main goal of my thesis was to gather data on how sertraline and citalopram, individually, affected feeding behavior and brain chemistry in hybrid striped bass. In addition to brain chemistry analysis, we also investigated gut tissue to see if changes in serotonin transporters and CCK levels in this part of the body can help further explain the changes in predatory behavior. The following objectives helped us meet this goal.

1. Determine if exposure of hybrid striped bass to sertraline causes changes in predatory behavior, brain chemistry, and intestinal gene expression.
2. Determine if exposure of hybrid striped bass to citalopram causes changes in predatory behavior and brain chemistry.
3. Determine how patterns from the previous SSRI mixture exposures compare to the individual SSRI exposures.

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CHAPTER TWO

EFFECTS OF SERTRALINE ON HYBRID STRIPED BASS PREDATORY BEHAVIOR, BRAIN CHEMISTRY, AND GUT GENE EXPRESSION

Introduction

Due to the growing knowledge on the benefits of pharmaceuticals, their use increased in the United States to 314 million prescriptions in 2015 [1]. As a result of this increased use, more pharmaceuticals are finding their way into our wastewater. Unfortunately, wastewater treatment plants do not completely remove pharmaceuticals during treatment and as a result these chemicals can enter the environment [2-5]. Selective serotonin reuptake inhibitor (SSRI) antidepressants are among the most commonly detected compounds and have been detected in the environment at concentrations ranging from low ng/L to µg/L concentrations [4, 6-10]. One of the most commonly prescribed antidepressants, sertraline has a removal efficiency of 60% by wastewater treatment plants [11]. The release of SSRIs in wastewater effluent is frequent enough to consider these drugs pseudo-persistent pollutants [12-14]. Non-target organisms with conserved or similar drug receptors could be affected by exposure to SSRIs into the environment [15, 16].

Sertraline became the second most dispensed psychiatric drug in 2013, and is used to treat many depressive disorders [5, 17]. The mechanism of sertraline, as with all SSRI class drugs, involves blocking the serotonin reuptake transporter (SERT). SSRIs block the reuptake of serotonin back into the pre-synaptic neuron causing a buildup of extracellular serotonin [5, 18]. Research has demonstrated low serotonin causes

behavioral changes in feeding, appetite, and locomotion [19, 20]. Serotonin and SERT are highly conserved between species [3, 21, 22]. SERT has been positively identified in fish species including Japanese medaka (*Oryzias latipes*), three-spined stickleback (*Gasterosteus aculeatus*), goldfish (*Carassius auratus*), hybrid striped bass (*Morone chrysops x Morone saxatilis*), and zebrafish (*Danio rerio*) [18, 21]. In hybrid striped bass, the SERT receptor shows 72% homology with human SERT [21].

Research suggests sertraline as the most toxic of the SSRIs due to its high log D (used in pharmacology research and quantifies the distribution of a drug at different pH values) value resulting in higher bioaccumulation [23]. Sertraline has been reported to have tissue levels as high as 545 ng/g tissue after fish were sampled from five effluent dominate rivers across the United States [10]. Fathead minnows exposed to sertraline demonstrated 18-42% less time seeking shelter compared to controls [8]. Perch exposed to 89, and 300 µg/l sertraline saw feeding decrease by up to 60% after a 7-day exposure [24]. Crayfish showed more aggression towards intruders than controls when exposed to an environmentally relevant concentration of 424 ng/L sertraline [25].

Little research has been done looking at other modes of action for sertraline outside of the brain. Serotonergic neurons are found in high levels in fish intestines [26]. The release of serotonin in the gut may be responsible for regulating peristalsis, secretion, vasodilation, and perception of pain/nausea [27]. Research has hypothesized that SSRIs may be able to regulate the gene expression of SERT [21, 28]. Cholecystokinin (CCK) is a peptide responsible for the hunger signals within the gut and has demonstrated the ability to control appetite in gold fish and trout [29].

Previous research by Sweet (2015) showed decreased serotonin levels in the brain of hybrid striped bass correlated with an increase in time to capture prey using an environmentally relevant SSRI mixture that included sertraline [21]. The goals and hypotheses of this study were to (1) determine the effects of only sertraline on the ability of hybrid striped bass to capture pre; where sertraline was expected to increase bass time to capture prey, (2) quantify the amount of antidepressant reaching the brain and how it affects serotonin levels; where sertraline would be detected in the brain and cause a decrease in serotonin, (3) determine fish plasma antidepressant levels; where sertraline would be detected in the plasma, and (4) investigate whether sertraline causes and up or down regulation of CCK or SERT in the hybrid striped bass intestines; where exposure to sertraline would cause and up regulation in the genes. Few studies using sertraline as a toxicant correlated serotonin levels in the brain with behavioral endpoints. From our knowledge, this study is one of the first to investigate the effects that SSRIs could play in the fish intestine.

Material and Methods

Test chemicals

Sertraline hydrochloride was purchased from TCI chemicals. Acetone, HPLC grade methanol, HPLC grade acetonitrile, glacial acetic acid, trace metals grade HCl, triethylamine, Optima® LC-MS methanol, Optima® LC-MS formic acid, molecular grade ethanol, molecular grade chloroform, molecular grade isopropanol, glycoblue, DEPC water, RNase away, ultrapure water, and RNAsecure (Ambion) were purchased from Fisher Scientific. STAT-60 was purchased from Tel-Test Inc. (Friendswood, TX, USA).

Internal Standard fluoxetine-D5 hydrochloride was purchased from CDN Isotopes (Quebec, Canada). MS-222 was purchased from Pentair aquatic habitats (Apopka, FL, USA). Serotonin creatine sulfate complex and Fluka LC-MS Chromasolv® water were purchased from Sigma Aldrich (St. Louis, USA). Water used for analytical procedures, excluding LC-MS/MS, was ultra-purified using a Milli-Q Super-Q filtration system (Millipore) with a measured resistivity of 18 MΩ x cm.

Fish

All experiments were conducted under the supervision of Clemson University Animal Care and Use Committee using approved animal use protocols (AUP 2015-077, AUP 2014-015). Hybrid striped bass (*Morone saxatilis x Morone chrysops*) were purchased from Keo Fish Farms (Keo, AR, USA) as fingerlings. Bass were kept in 300-450L holding troughs at the Cherry Farm aquatic research lab maintained by Clemson University (Clemson, SC, USA). Troughs were constantly supplied with fresh water (pH 6.45 ± 0.17, hardness 24 mg/L as $CaCO_3$, alkalinity 10 mg/L as $CaCO_3$) as flow through from Lake Hartwell (Clemson, SC, USA). Water coming into Cherry Farm was first filtered through a gravel bed and sterilized with UV radiation. Water temp was maintained between 19 and 24 °C using heated or chilled water depending on incoming water temperature. Air stones and agitators (Boatcycle Inc., Henderson TX, USA) were used to maintain oxygen levels in tanks and troughs. Zeigler Bros, Inc. (Gardners, PA, USA) commercial diet (Finfish Gold crumble, 1.0 mm, 2.0 mm, and 5.0 mm slow sink) was used to feed bass until they reached appropriate size (23 ± 1.81 cm; 131.6 ± 24.8 g).

Fathead minnows (3-4 cm) were purchased from Anderson Minnow Farm (Lonoke, AR, USA). Minnows were kept in 100L troughs with flow through system bringing in the same water as above. Until use, minnows were fed a commercial diet (Tetramin® Tropical Flakes) purchased from Dr's Foster and Smith Inc. (Rhinelander WI, USA).

Bass training

Once bass were of the appropriate size (approximately 6 months to 10 months old), they were trained to capture live prey. Bass were randomly chosen (30-35 bass) and moved to a separate 300L trough for group training. During group training, 5 minnows per bass were dropped into the trough every 3 days over a 6-day period. On the 6th day of group training, bass were moved to individual experimental tanks (1 bass per tank). Bubble aerators were placed in each tank and two grates on top to prevent the bass from jumping out. During individual training, the aerator was removed from the tank and bass were given a few minutes to acclimate. Each bass was fed 4 fat-head minnows, dropped into the tank at the same time, every 3 days over a 6 day span. The time to capture each prey was recorded and used to determine which fish were appropriate to use in the exposures. Bass who ate at least 3 minnows (with comparable feedings on the previous training days) were used in the exposure.

Experimental design

Hybrid striped bass were exposed to sertraline in a static system for 6 days followed by 6 days of recovery time. Exposure tanks purchased from Deep Sea Aquatics were 119L and measured 92.1 cm x 32.4 cm x 40 cm. Each tank had a 1.9 cm PVC

vertical standpipe drilled into the front glass for maintaining water volume when used as a flow through system. Water for the experiments was also taken from Lake Hartwell similar to the holding troughs. Additional filtration through a multi-resin filtration system (Water and Power Technologies, Columbia, SC, USA) was used for additional cleanup before sending water to the tanks. Water quality parameters (pH, DO, and temperature) were measured during the feeding events using a YSI 556 multi-parameter instrument (Yellow Springs Instruments). Work previously completed in this lab demonstrated that a 6 day static exposure did not result in levels of total ammonia nitrogen or free ammonia that was acutely toxic to the fish, thus affecting the behavioral endpoints.

Behavioral tests followed procedures previously done in our laboratory [21, 30, 31]. On the last individual training day (exposure day 0), 4 minnows were dropped into the tank and bass were given 25 minutes to consume all prey. The time each bass took to consume each prey was recorded and then 1500 seconds (25 minutes) was recorded if any minnows were failed to be consumed. The tanks were filled to 80L and marked before turning the water off and spiking the tanks. Each bass was randomly assigned to a treatment (5 bass/treatment/time point) and spiked with the appropriate volume of sertraline to reach nominal concentrations. Feeding days took place on days 3, 6, 9, and 12. After feeding on day 6, the water flow was returned to each tank to start the recovery period of the experiment. After each feeding, 5 bass per treatment were euthanized for brain, plasma, and intestine analysis.

Sertraline exposure

Stock solutions (prepared fresh daily) of sertraline were prepared by dissolving sertraline HCl in methanol and then added to tanks to reach their nominal concentrations (4, 40, and 100 µg/l). Low, medium, and high exposures were performed in addition to a control. The highest concentration equivalent of methanol was added to each control tank to ensure no toxicity form the carrier solvent. Methanol was at a concentration less than 0.1 mg/L; compliant with ASTM international recommendation for experiments involving fish (ASTM1241-05). Two hours after tanks were spiked, appropriate aliquots from each tank were taken to measure sertraline concentrations.

Sertraline analysis

Sertraline concentrations were analyzed on day 0, 2 hours after spiking the tanks. Water samples were acidified with 2-3 drops 2N HCl (pH of 3.0) and extracted using C-18 solid phase extraction cartridges. Cartridges were conditioned with 1 volume methanol, 1 volume acetone, and 2 volumes milli-Q water before running samples. Samples were stored at -20 °C until samples were eluted. Sertraline standards (2-8 mg/l) were made in methanol/1%. Cartridges were eluted with methanol/1% acetic acid and stored in sample vials for HPLC analysis. Samples were run on a Waters HPLC with 1525 Breeze HPLC pump, Waters 717 Plus auto sampler, and Waters 2487 absorbance detector set at 270 nm (Waters, Milford, MA, USA). The mobile phase consisted of 50:40:10:0.3:0.15 water: acetonitrile: methanol: triethylamine: acetic acid set at a flow rate of 1 ml/min and an injection volume of 40 µl. An Alltech Prevail C_{18} column

(150mm, 4.66 I.D.) was used for separation. Approximate run time was 12 minutes with sertraline eluting off the column at 8 minutes.

Brain preparation

Bass were euthanized in MS-222 (1.5 g/L MS222 buffered with $CaCO_3$ (pH 7.0-7.5)). Fish were pithed, brains removed and flash frozen in liquid nitrogen before being transferred to -80°C freezer until processed. Brains were thawed, weighted and transferred to a 1.5 ml micro centrifuge tube. Before sonication, 50 µl of 2.5 mg/L fluoxetine D5 internal standard was added with 50 µl of Milli Q water. Brains were sonicated for 10 seconds at 10% amplitude. Immediately following sonication, 200 µl of freezing acetonitrile was added to each brain, vortexed and placed back in the -80°C to allow proteins to precipitate. Brains were then centrifuged at 17,000 G for 5 minutes at 4°C. Supernatant was removed and placed in a new micro centrifuge tube and placed back in the -80°C again to allow proteins to precipitate. Samples were centrifuged 4 times to ensure all debris was removed. Samples were then placed in LC-MS/MS tubes for analysis. An aliquot of each brain sample was diluted 1:10 in Milli Q water and run using the manufacturer's instructions. Brain antidepressant and serotonin concentrations were normalized to brain tissue weight (g).

Plasma preparation

Bass were euthanized in buffered MS-222. BD Vacutainer tubes (Vitality Medical, Salt Lake City, UT, USA) were used to draw blood from fish caudal artery. Blood was immediately placed on ice, then centrifuged at 3,000 G for 10 minutes at 4°C. Plasma was transferred to a 1.5 ml micro centrifuge tube then placed in -20°C freezer

until processed. Plasma samples were thawed and vortexed. A 20 µl aliquot of plasma was added to a newly labeled micro centrifuge tube with 50 µl of internal standard used above and 150 µl of acetonitrile. Samples were vortexed before being placed back in the -20°C to allow proteins to precipitate. Samples were centrifuged twice at 17,000 G for 5 minutes at 4°C to remove all debris, re-freezing in-between centrifuges. Clear homogenate is placed in an LC-MS/MS tube for analysis.

LC-MS/MS analysis

Table 2.1 shows the parameters used for detecting sertraline and serotonin in the brain and plasma samples. Samples were analyzed on a Shimadzu LC-MS/MS 8030 using a kinetix column (2.6u, C18, 100 x 3.0mm). The mobile phase consisted of 0.01% formic acid in 40% water and 60% methanol. A gradient method ran with 5% methanol for 2 minutes, increasing from 10% methanol for 2 minutes to 95% methanol for 2 minutes, and remaining at 95% methanol for 5 minutes. The total run time was 11 minutes. At the end of the run, the mobile phase reduced back down to 5% methanol over 6 minutes to re-equilibrate the column before the next sample injection. The sample injection volume was 5 µL and the compound retention times were as follows: Serotonin: 2.9 min, fluoxetine-d5: 9.0 min, and sertraline: 9.2 min.

PCR analysis

Bass were euthanized in buffered MS-222. Intestines were removed and divided into proximal, medial, and distal sections. The sections were flash frozen in liquid nitrogen before being placed in the -80°C freezer until processed.

Homogenization

Intestine samples were homogenized with an IKA T10 basic hand homogenizer. Labeled 2 ml centrifuge tubes were filled with 750 µl STAT-60. A small portion of intestine (<75 mg) was placed into the centrifuge tube and immediately homogenized on ice in 30-second segments until all tissue was broken up. Samples then sat for 5 minutes at room temperature to allow for complete dissociation of nucleoprotein complexes before being put back on ice. The homogenizer was cleaned in-between samples using four steps (70% ethanol, RNase away, Diethyl pyrocarbonate (DEPC) water, DEPC water).

RNA extraction

One hundred fifty microliters of molecular grade chloroform were added to each sample after the 5 minutes at room temperature and vortexed for 15 seconds, and was incubated at room temperature for 3 minutes. Samples were centrifuged at 14,000 rpm for 15 minutes at 4°C. Three phases appeared, and the upper aqueous layer was collected making sure not to puncture the middle lipid layer. The upper layer was placed in a new 2 ml tube with another aliquot of 750 µl STAT-60. A second RNA extraction was repeated, and aqueous phase was transferred to a 1.5 ml tube.

RNA precipitation and reconstitution

Glycol blue was added to each sample to co-precipitate with the RNA pellet. Seven hundred microliters of molecular grade isopropanol were added to each sample and placed on ice for 10 minutes. Samples were centrifuged at 14,000 rpm for 45 minutes at 4°C. Supernatant was poured off, and pellet washed with 750 µl of 75% molecular

grade ethanol. Samples were vortexed and centrifuged at 7,500g for 5 minutes at 4°C. Ethanol wash was repeated. After centrifugation, pellets were air dried until clear. RNAsecure (Ambion) was heated to 60°C with a heating block. Thirty microliters of RNAsecure were added to each pellet, vortexed, and on the heat block for 2-3 minutes. Samples were vortexed and placed back on the heat block for an additional 10 minutes before cooled to room temperature.

RNA quantification

A NanoDrop Lite microliter spectrometer (Thermo Scientific) was used to determine the concentration of RNA in each sample. The machine was initialized and blanked with 2 µl of ultrapure water. Each sample was run on the NanoDrop (the upper limit of this machine was 1,200 ng/µl), additional RNAsecure (Ambion) was added to samples appropriately to make sure they were within the machine limits.

DNAse treatment

One microliter of 10X reaction buffer and 1 µl Perfecta DNase I (2U/µl) (Quanta) was added to 0.2/0.5 ml micro-tubes on ice. The amount of RNA and ultrapure water added were variable depending on the concentration of RNA determined from the NanoDrop. Total volume in the micro-tubes was 11 µl (1 µl 10x stop buffer was added after incubation), and target RNA concentration per tube was 400 ng/µl. Micro-tubes were vortexed and incubated for 30 minutes at 37°C. Stop buffer was added, and tubes were vortexed before incubating for 10 minutes at 65°C.

cDNA synthesis

Four microliters of qScript reaction mix (5x) and 1 µl qScript RT (Quanta) was added to 0.2 ml thin-walled PCR tubes sitting on ice. The amount of RNA and ultrapure water depended on the concentration of stock RNA and the concentration used. The total volume in each tube was 20 µl. The micro tubes were vortexed and placed in a Veriti thermal cycler (Thermos Fisher Scientific) programed for: 1 cycle at 22°C for 5 minutes, 1 cycle 42°C for 30 minutes, 1 cycle at 85°C for 5 minutes, and then held at 4°C.

Quantitative PCR

Table 2.2 gives a detailed description of the primers used. A dilution of 1:20 was completed when preparing the PCR plate for the SERT gene analysis. Undiluted cDNA was used for preparing the CCK PCR plate. A dilution of 1:1000 was completed for the 18S housekeeping gene. Gene primers were made up to an 18 µm concentration. SERT and 18S PCR plates were prepared at a ratio of 10:1:1:8 master mix cyber green (2x), for primer, rev primer, and diluted cDNA. The CCK plate was prepared at ratio of 10:1:1:2:6 master mix cyber green (2x), for primer, rev primer, undiluted cDNA, and ultrapure water. Bio-rad IQ5 software coupled with a Bio-rad IQ5 real-time PCR detection system was used following the method: 1 cycle at 95°C for 3 minutes, 40 cycles of 95°C for 10 seconds and 58°C for 1 minute, 1 cycle at 95°C for 1 minute, and 1 cycle at 55°C for 1 minute.

Statistics

All statistical analysis was performed using JMP Pro 12.0. The data were transformed to log (data) + 1 to reduce data variability for analysis. A model was run

with day, treatment, day*treatment, and tank nested within treatment as a random variable to analyzed all time to capture prey data. Since one HSB was placed in each tank, nesting tank within treatment and treating it as a random variable corrected for the repeated measures of time to capture prey. A least squares mean model run with day, treatment and day*treatment was run to make multiple comparisons for plasma and brain data. PCR data were normalized by transforming fold change to log (data) + 1 before using Student t to determine differences between control and treatment groups in each intestine section.

Results

Sertraline aqueous exposure

Sertraline exposure concentrations were measured (mean ± SE) as 4.5 ± 0.84 µg/L, 35.4 ± 2.18 µg/L, and 96.8 µg/L for low, medium, and high treatment groups, respectively. All exposure groups were within 89-97% of the nominal concentrations. Water quality parameters were measured (mean ± SD) as 6.96 ± 0.22, 21.2 ±1.27, and 8.8 ± 0.2 for pH, water temperature (C°), and dissolved oxygen (mg/L), respectively.

Behavioral assay

Exposure of hybrid striped bass to sertraline caused an increase in time to capture prey at some feeding points over the 12-day experiment. Each prey was analyzed separately, so prey 1 refers to time to capture the first fathead minnow, prey 2 represents time to capture the second fathead minnow, prey 3 refers to time to capture the third fathead minnow, and prey 4 refers to time to capture the fourth fathead minnow.

For the time to capture prey 1 (figure 2.1), bass took significantly longer to capture prey at the highest concentration on day 3 compared to control fish ($p= 0.0255$). The low ($p= 0.0671$) and medium ($p= 0.051$) treatment groups were trending towards an increased time to capture prey, but the statistics were not significant. Day 6 feedings show the medium ($p= 0.0004$) and high ($p= <0.0001$) treatment groups took significantly longer to capture prey compared to control bass. On day 6, high treatment fish time to capture prey was significantly different than low treatment fish ($p= 0.0063$). The medium and low treatment groups ($p= 0.052$) also were trending towards a significant difference in time to capture prey. During the recovery period, day 9 showed no significant difference between treatment groups. On day 12, the medium treatment group took significantly longer to capture prey compared to the control ($p= 0.0086$) and low ($p= 0.0366$) treatment groups and trended toward significance with the high treatment group ($p= 0.0682$).

For time to capture prey 2 (figure 2.2), hybrid striped bass in all sertraline exposed treatment groups took significantly longer to capture prey compared to controls on day 3 ($p= 0.0307, 0.034, 0.0058$). All sertraline treatment groups showed significant increase in time to capture prey compared to controls on day 6 ($p= <0.0001$). No statistically significant time points were observed during the recovery period (days 7-12).

For prey 3 (figure 2.3), hybrid striped bass in all sertraline exposure groups showed significantly longer time to capture prey compared to controls on day 3 ($p= 0.0299, 0.0011, 0.0016$) and day 6 ($p= 0.0004, 0.001, <0.0001$). No statistically significant time points were observed during the recovery period (days 7-12).

A steep decrease in time to capture prey was observed between the last day of sertraline exposure (day 6) and the first feeding day of the recovery period (day 9). This pattern was observed for each prey consumed. No significant differences were seen for time to consume the fourth prey (figure 2.4).

Brain chemistry

Whole brain serotonin levels were analyzed on each feeding day of the experiment and normalized to brain protein concentrations in each sample. A decrease in serotonin was detected in the medium and high treatment groups throughout the experiment (figure 2.5). The medium treatment group showed a significant decrease in serotonin compared to the control on day 6 ($p= 0.0003$). The medium treatment group on days 3, 9, and 12 decreased but p values were just above significance levels ($p= 0.0648$, 0.057, and 0.058, respectively). The high treatment showed significant decrease in serotonin levels compared to the control on days 3-9 ($p= < 0.0001$, 0.0474, and 0.0195, respectively). The high treatment on day 12 decreased but was just non-significant ($p= 0.0627$). Serotonin levels also showed trends with time to capture prey during days 3 and 6 (figure 2.10). Time to capture prey 2 and 3 show a moderate negative relationship with whole brain serotonin levels.

Brain antidepressant concentrations

Sertraline concentrations in the hybrid striped bass whole brain were significantly higher in the medium and high treatments compared to control and low treatments during the exposure period on days 3 and 6 ($p = <0.0001$) (figure 2.6). High and medium treatment groups were significantly different from each other on day 3 ($p = <0.0001$).

During the recovery period, the high treatment group was significantly greater than all other treatment groups on day 9 and 12 ($p = <0.0001, <0.0007$). The medium treatment group was significantly greater than the control and low on day 9 ($p = 0.0188, 0.0375$). The medium treatment group saw a large decrease in brain sertraline levels between days 3 and 6. Sertraline levels in the high treatment increased during days 3-9 before decreasing by day 12, but still significantly increased.

Plasma antidepressant concentrations

Sertraline was detected in plasma samples from each treatment group (figure 2.7). Some fish from the low treatment did not report sertraline detection on days 6 and 9. No dose dependent response was seen between the treatment groups over the duration of the experiment. Each treatment showed decreasing sertraline concentrations in the plasma between days 3 and 9. On day 12, sertraline concentrations increased again for each treatment group. High and low treatment groups were significantly higher than control on days 3 ($p = 0.0071, <0.0001$). High and medium treatments were significantly different on day 3 ($p = 0.0062$). On day 6, the high treatment was significantly higher than control (0.0088). On day 9, the high treatment was significantly higher than all treatment groups ($p = <0.0004$). High and medium treatment groups were significantly different from control plasma ($p = 0.0045, 0.0212$).

Quantitative PCR data

Intestines separated into proximal, medial, and distal from high treatment fish dissected on day 6 were analyzed for two genes (SERT and CCK). Quantitative PCR data

showed a significant upregulation of CCK ($p = 0.0397$) and SERT ($p = 0.0103$) in the medial section of the intestine compared to the control group (figures 2.8 and 2.9).

Discussion

Individual compound exposure can lead to different conclusions when compared to the same compound in a mixture experiment. This study demonstrated that sertraline hindered hybrid striped bass ability to capture prey, caused changes in serotonin levels in the brain, and increased SERT and CCK mRNA expression in the gut. Past studies investigating SSRI effects on hybrid striped bass predatory behavior, when exposed to SSRIs individually or in a mixture, caused an increase in time to capture prey [21, 30-32]. One past study exposed hybrid striped bass to a mixture of SSRIs including: citalopram, fluoxetine, and sertraline. Sweet (2015) saw a significant increase in time to capture prey during the recovery period on days 9 and 12 [21]. In this exposure, the significant increases in time to capture prey occurred during the exposure period on days 3 and 6. Time to capture prey for hybrid striped bass exposed to sertraline did not demonstrate a dose dependent response. When using higher doses of SSRIs in treatment, many different serotonin receptors may be impacted throughout the body helping to explain atypical dose responses in fish [1].

During recovery, all treatment groups showed similar time to capture prey on day 9 and 12 as the control after sertraline was removed on day 6. Sertraline levels in the brain indicated the low and medium treatment group's concentrations decreased as the experiment progressed; while the high treatment concentrations remained elevated even into the recovery period. One hypothesis for the low and medium treatment groups could

be antidepressant metabolism rate in the fish body. Research showed fish metabolize SSRIs with a member of the CYP family [33]. SSRIs can inhibit CYP enzymes, some of which are responsible for their breakdown [34]. While all SSRIs have the same mode of action, not all inhibit the same CYP enzymes with the same strength [34]. CYP2D6 is one of the largest contributors in the metabolism of SSRIs, but CYP3A enzymes metabolize sertraline [34]. Sertraline shows no evidence of inhibiting CYP3A enzymes. We hypothesize sertraline may then be metabolized at a faster rate than other SSRIs because it doesn't inhibit the enzyme that metabolizes it at low enough doses. The high treatment may have been a high enough dose to oversaturate the enzymes preventing quick breakdown of sertraline as seen in the low and medium treatment groups.

Sertraline plasma concentrations showed an interesting trend by decreasing between days 3 and 9 but increasing between days 9 and 12. Fish plasma pH levels range from neutral to basic, leaving the majority of sertraline in its ionized form. Ionized sertraline does not reach the target receptor as quickly as un-ionized sertraline, and un-ionized sertraline has been showed to be more toxic to fathead minnows compared to the ionized form [28]. Ionizable compounds also tend to move away from lipid environments [10]. The initial decrease in sertraline plasma levels from days 3-9 are hypothesized to be the result of the drug moving to target molecules or accumulating in lipids in the fish body, which can be supported in the high treatment with increasing concentrations of sertraline in the brain over days 3-9. When concentration gets low enough, the compound will go from the lipids back into the plasma causing an increase in concentrations in the plasma seen on day 12. Brain sertraline concentrations support this hypothesis with a

decrease between days 9 and 12 which may be due to sertraline remobilizing into the plasma or being metabolized.

Whole brain serotonin levels decreased during the medium and high treatment groups which is the opposite of the expected therapeutic effect to increase extracellular serotonin. Previous research showed short term exposure to fluoxetine caused a decrease in brain serotonin levels in rats and *Betta splendens* injected with fluoxetine showed decreased serotonin in the forebrain [35, 36]. Sweet (2015) exposed hybrid striped bass to a mixture of SSRIs (citalopram, fluoxetine, and sertraline). Serotonin changes were predicted based on previous work with fluoxetine, creating a relationship between serotonin and brain fluoxetine concentrations. Sertraline affinity for SERT is 4x more sensitive than fluoxetine, so fluoxetine predictions were multiplied by 4 [21]. The predictions suggested the highest decrease in percent serotonin would occur on day 6 in the highest treatment. Our exposure saw the largest decrease in the high treatment on day 3 instead of day 6. Serotonin decreases observed during the sertraline treatment loosely followed with the predictions. Decreases continued to be observed in during the recovery period even when time to capture prey had returned to levels similar to the control. Research showed SSRI treatment caused an increase in SERT and serotonin receptor expression which may help compensate for the initial antidepressant exposure [37, 38]. Seven receptor families and 14 receptor subfamilies for serotonin receptors have been identified in mammals. All but one of those receptors has also been identified at the molecular level in fish, though the functions of all have yet to be determined [1]. The diversity of the serotonin receptors gives fish flexibility when responding to

physiological and environmental challenges [39]. We hypothesize that the high treatment may have caused an up regulation of the SERT receptor resulting in some reuptake transporters being unaffected by sertraline which caused a return to normal physiological serotonin levels and feeding behavior seen during the recovery period.

This research has shown that sertraline can cause an upregulation of SERT in the bass gut, helping to support our claim that peripheral serotonergic systems are just as important as the central nervous system when it comes to potential satiety effects [40]. CCK was upregulated during sertraline exposure in the high treatment group. CCK release occurs when food enters the gut [41]. Bass were dissected directly after behavioral assay was completed. Food was not seen past the medial portion of the intestine suggesting we may have observed a change in CCK in the distal intestine if food had been allowed to travel through the entire gut. Past research determined CCK could alter feeding minutes after treatment with corticotrophin releasing factor (CRF) [40]. Changes in serotonin can cause changes in CRF and subsequent changes in feeding behavior [42]. Future research may find trends between CRF and feeding behavior in hybrid striped bass, which may help explain the feeding behavior trends better than just brain serotonin levels. Future research would benefit from differentiating between intra and extra cellular serotonin to prevent masking of effects when whole brain serotonin is analyzed.

Behavioral tests have come under greater scrutiny recently due to lack of consistency and comparability between data. This study follows two important factors which help to make the behavioral endpoints robust. The first factor describes the amount

of time organisms are allowed to acclimate to their experimental environment [43]. Bass are given 9 days to acclimate to exposure tanks during individual training. The second factor involves the time given for observing behavioral endpoints [43]. The 25 minutes given for watching bass consume minnows allows plenty of time to for the bass to chase down prey if it wants to do so. The robustness of our behavioral endpoints can also be seen in the similar trends seen from exposing SSRIs to hybrid striped bass from four different graduate students completing similar tests with different pharmaceuticals.

Conclusions

Exposing aquatic organisms to individual compounds may produce similar or completely different results than what was shown when the individual compound is used in a mixture. This experiment demonstrated sertraline could hinder hybrid striped bass from quickly capturing prey. Serotonin levels in the brain fluctuated during the exposure and recovery period, suggesting more than just serotonin may be responsible for the predatory behavior seen. The evidence up upregulation of two key genes in the gut suggests the peripheral nervous system carries just as much importance in mode of action of SSRIs as the central nervous system does. Serotonin alone may not be the best endpoint to explain the changes in behavior seen after sertraline exposure.

Tables and Figures

Compound	Precursor Ion(s)	Product Ion(s)	Dwell Time (mSec)	Q1 (V)	CE (V)	Q3(V)	Retention Time (minutes)
Serotonin	177.2	160.1	300	-11	-13	-18	2.3
Fluoxetine-d5	315	153.1	25	-14	-10	-17	9.22
Sertraline	307.1	276.05	3	-13	-12	-21	9.27
	307.1	158.95	3	-13	-25	-17	
	307.1	159.95	3	-13	-30	-17	

Table 2.1: LC-MS/MS optimization parameters for detecting serotonin, sertraline, and internal standard in plasma and brain samples.

Gene	Forward Primer	Reverse Primer
18S	5’-TGAAAACATTCTTGGCAAATGC-3’	5’-GCCGCTAGAGGTGAAATTCTTG-3’
SERT	5’-ACTGCTACCTTTCCCTACCT-3’	5’-CTGCCAATCAGGTTTGAGATAGA-3’
CCK	5’-TCTCCTCCAGGAAAGGTTCT-3’	5’-CATGTAGTCCCTGTCTGCTATC-3’

Table 2.2: Primers designed for q-PCR analysis of hybrid striped bass intestine.

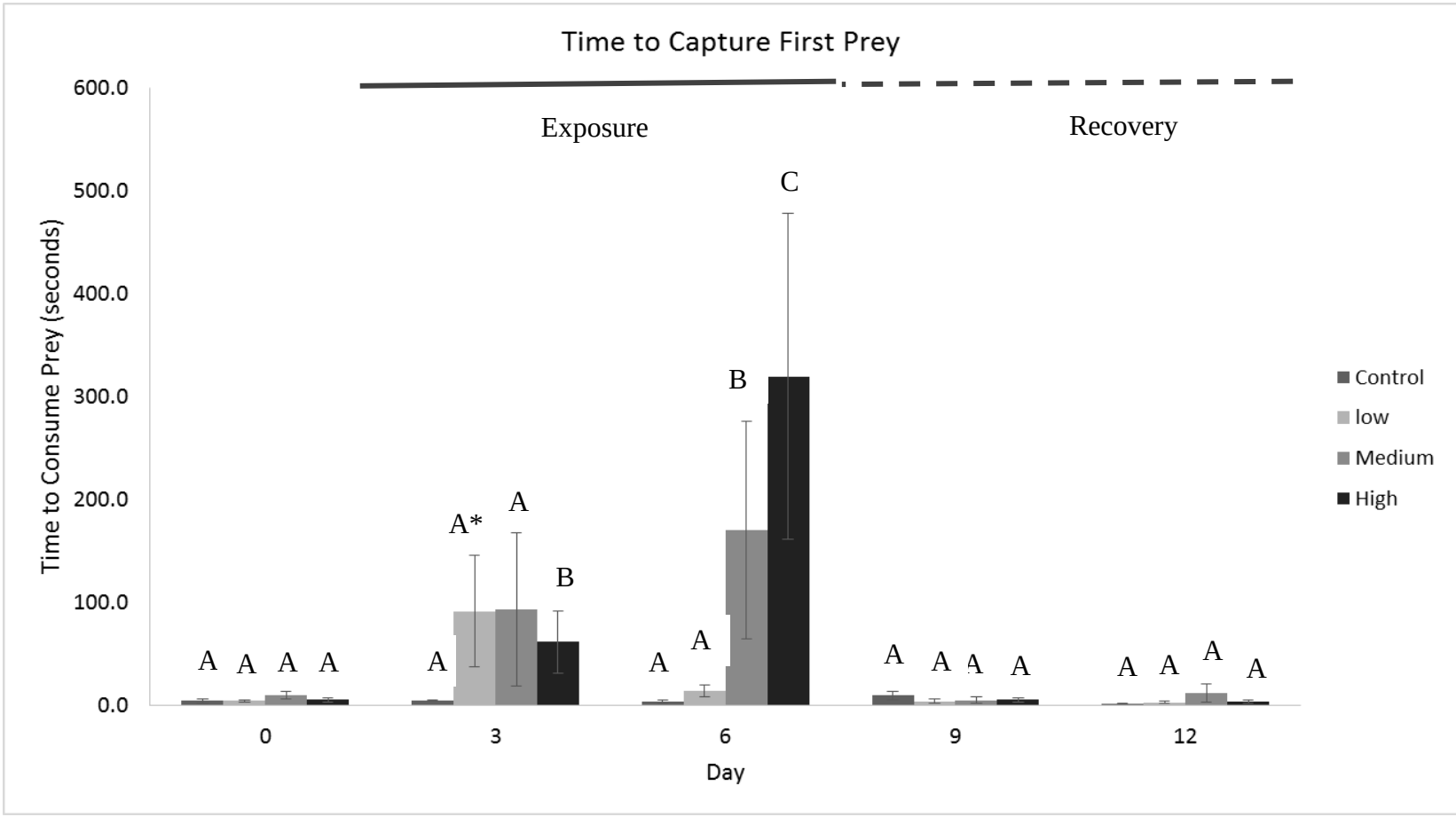

Figure 2.1: Time to capture first fathead minnow (seconds) by hybrid striped bass exposed to sertraline. High treatment was significantly different from control (day 3). * represents low and medium treatment groups p values which suggested significance from control fish (p = 0.051-0.067). High treatment was different from low and control treatments (Day 6). Medium treatment was different from control (Day 6). Bars represent standard error.

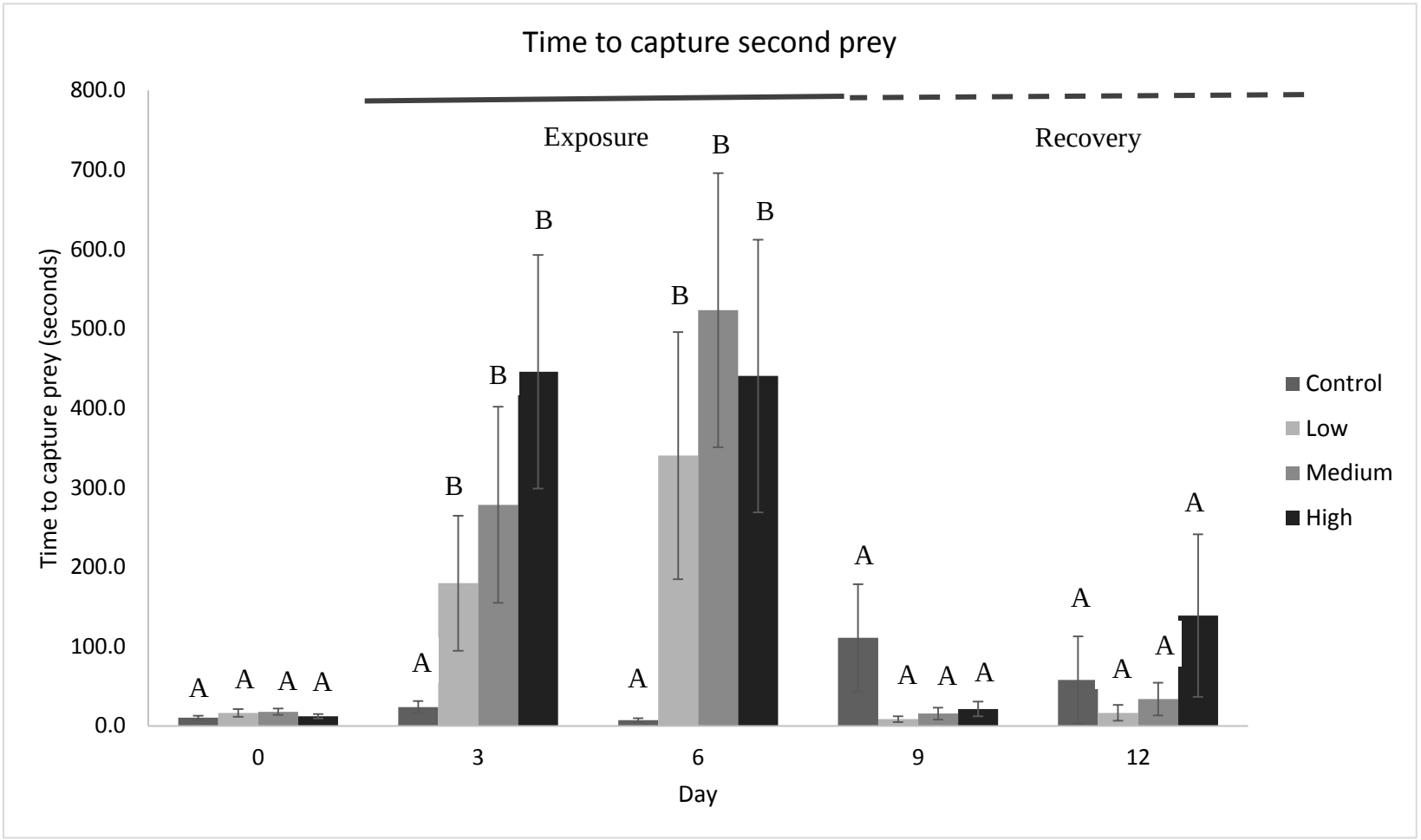

Figure 2.2: Time to capture second fathead minnow (seconds) by hybrid striped bass exposed to sertraline. All treatment groups on days 3 and 6 were significantly different than the control group. Bars represent standard error.

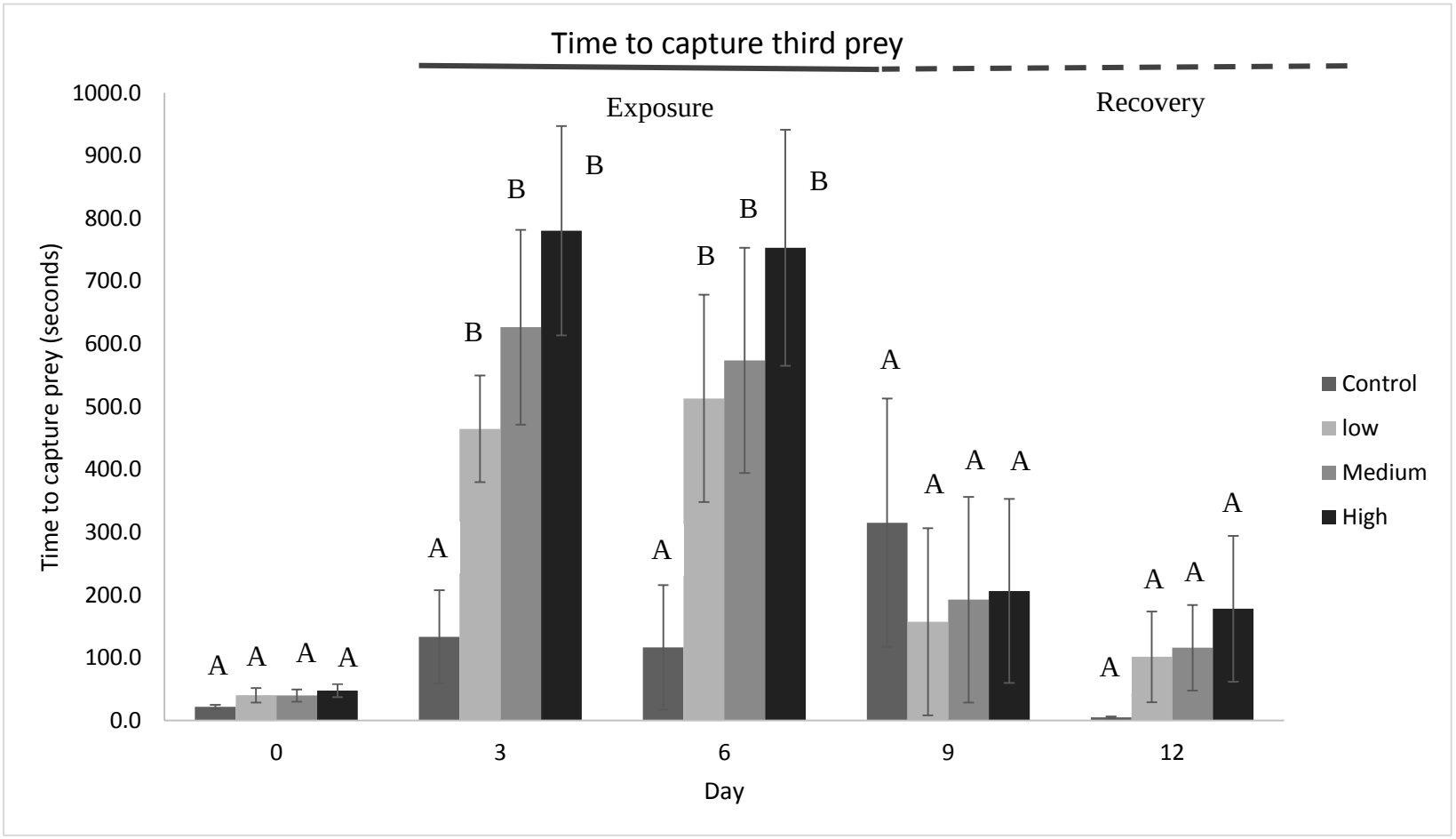

Figure 2.3: Time to capture third fathead minnow (seconds) by hybrid striped bass exposed to sertraline. All treatment groups on days 3 and 6 were significantly different than the control group. Bars represent standard error.

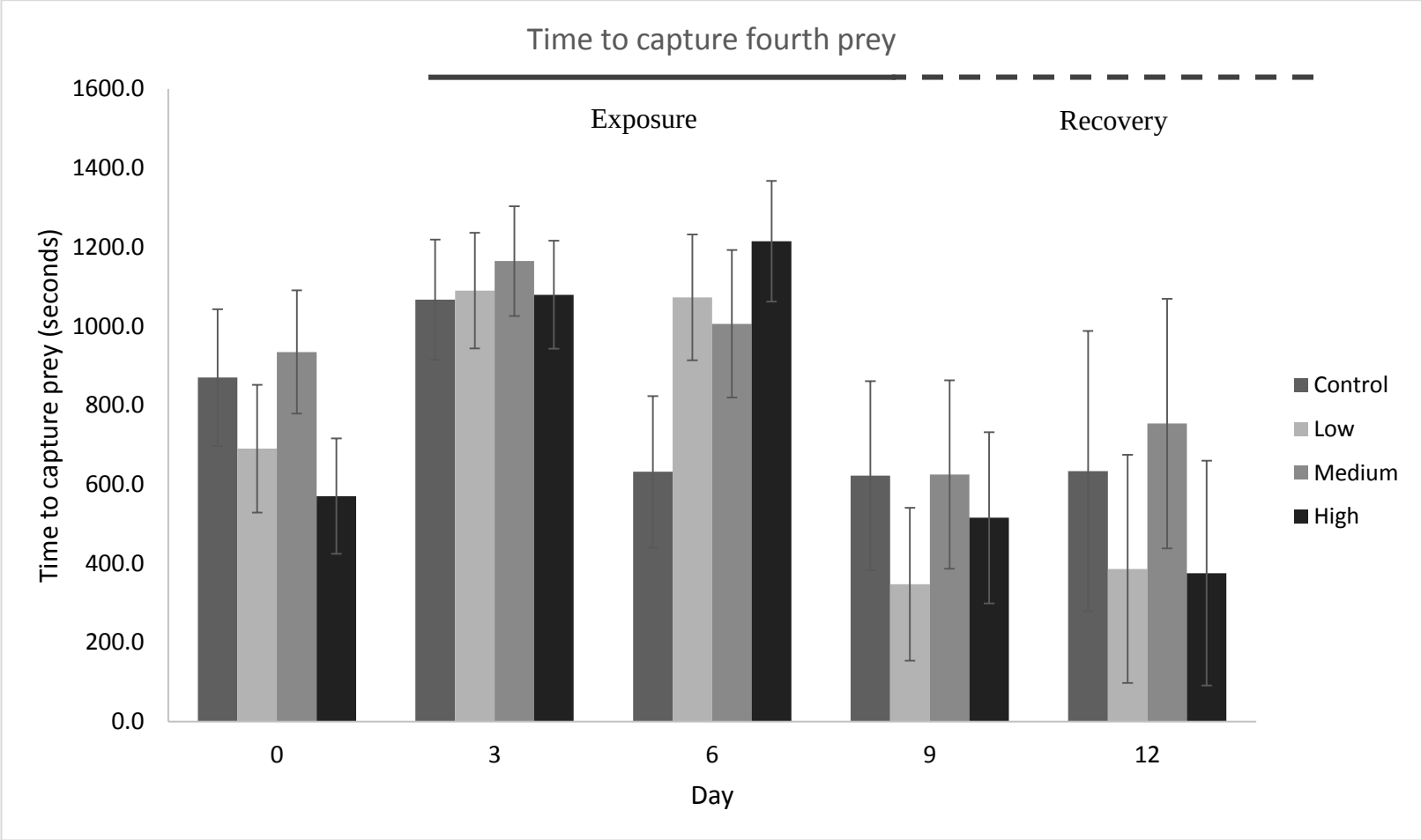

Figure 2.4: Time to capture fourth fathead minnow (seconds) by hybrid striped bass exposed to sertraline. No significant differences were seen through the duration of the experiment.

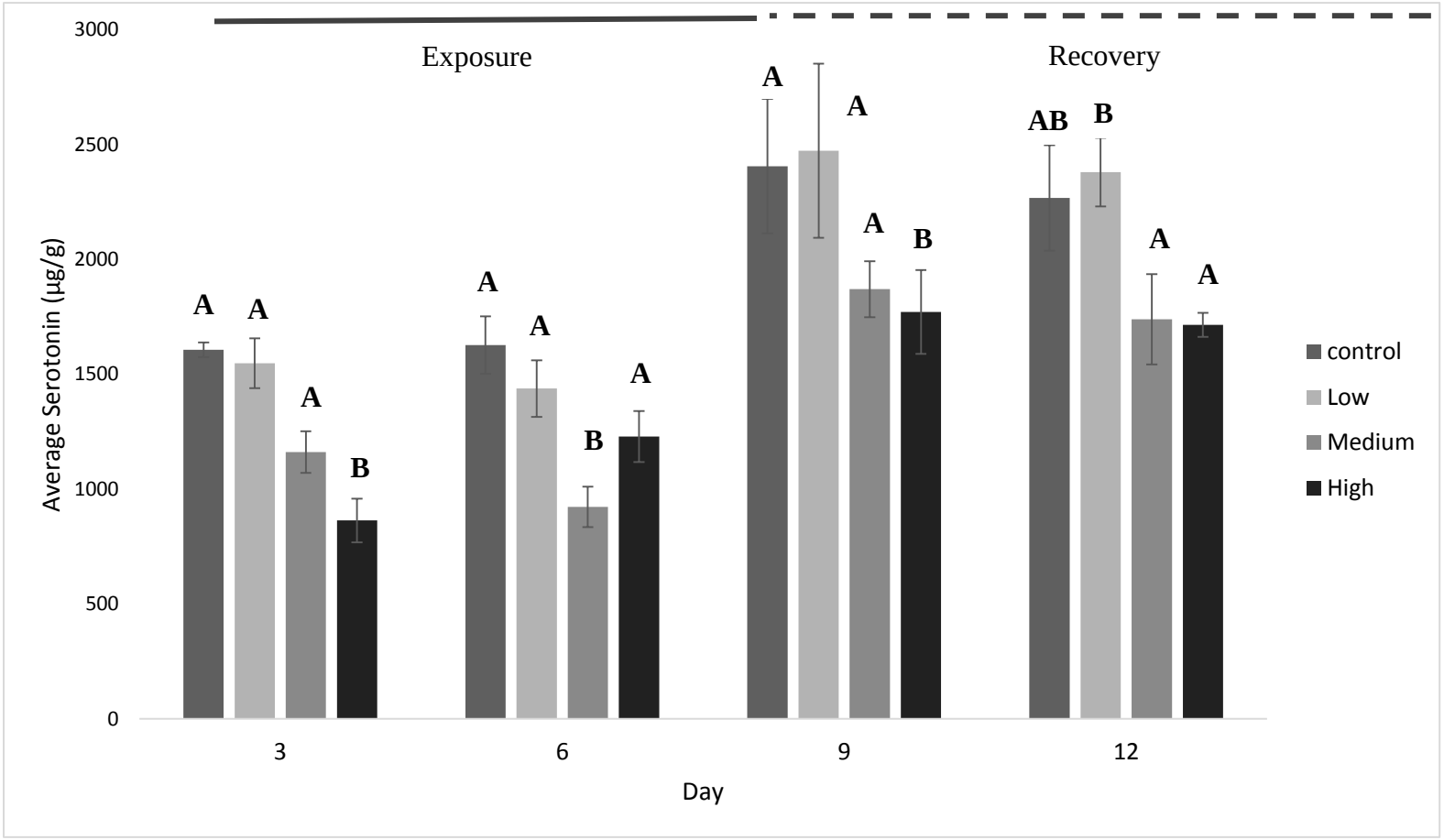

Figure 2.5 Average serotonin concentrations normalized to weight of bass brain. Letters represent significant differences during each day. Bars represent standard error. The medium treatment group was significantly different than the control on day 6. The high treatment group was significantly different than the control on days 3-9. There were several pairing that suggests significance including the medium group from control on day 3, 9, and 12 (p= 0.0648, 0.0662, 0.0580, and between control and high treatment on day 12 (p= 0.0627).

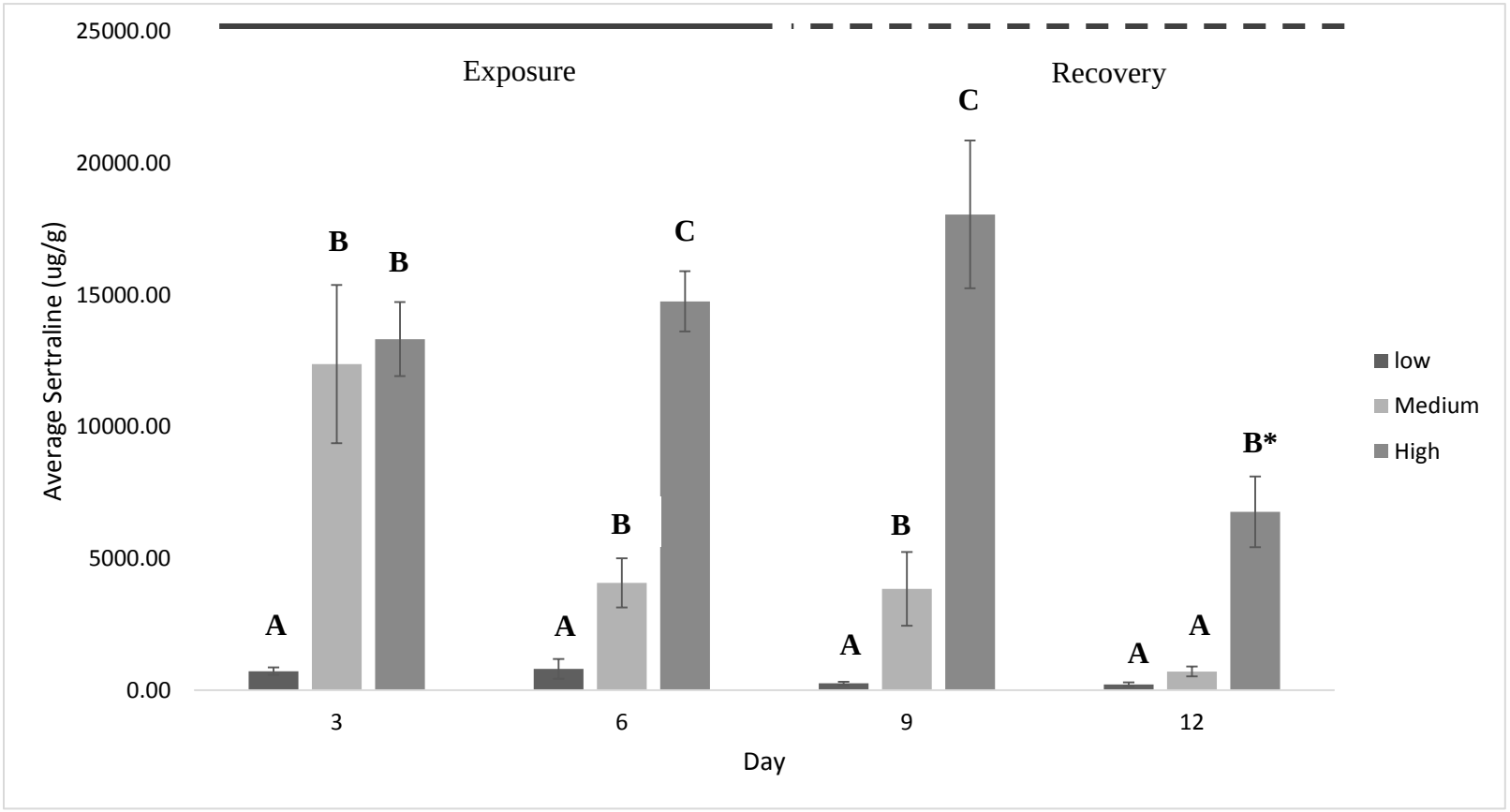

Figure 2.6: Average antidepressant concentration in hybrid striped whole brain during sertraline exposure. Bars represent standard error. * represents treatment groups significantly different from the control bass. Letters represent the statistical differences between treatment groups on any given day. Bars represent standard error.

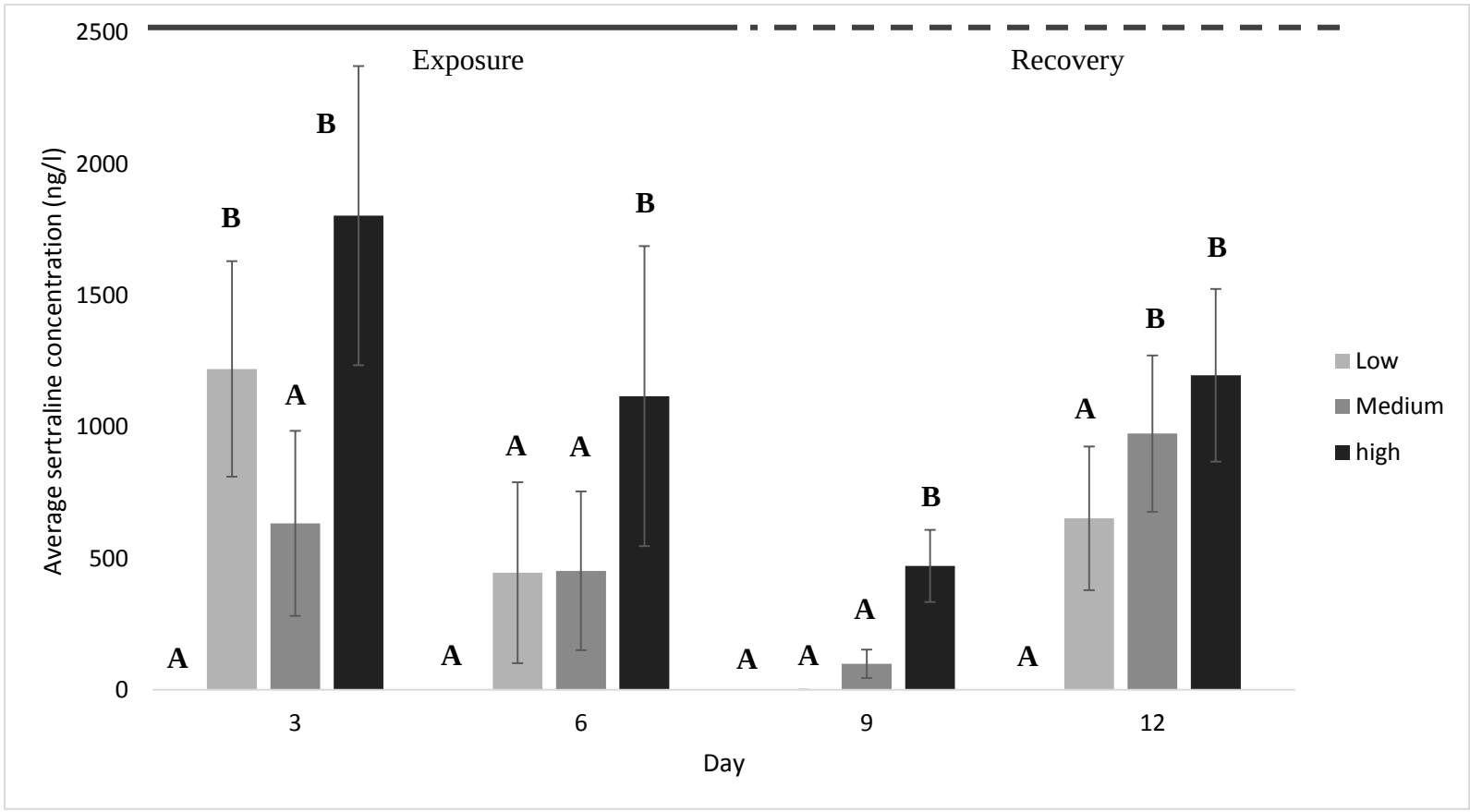

Figure 2.7: Sertraline plasma concentrations from hybrid striped bass exposed to sertraline over 6 days with 6 days of recovery. Plasma concentrations in each treatment group decrease between days 3 and 9. On day 12 sertraline concentrations increase again. The high treatment group was significantly higher than the control group on all days. The medium treatment group was significantly higher than the control on day 12. On day 9, the high treatment group was significantly higher than all other treatment groups. Bars represent standard error.

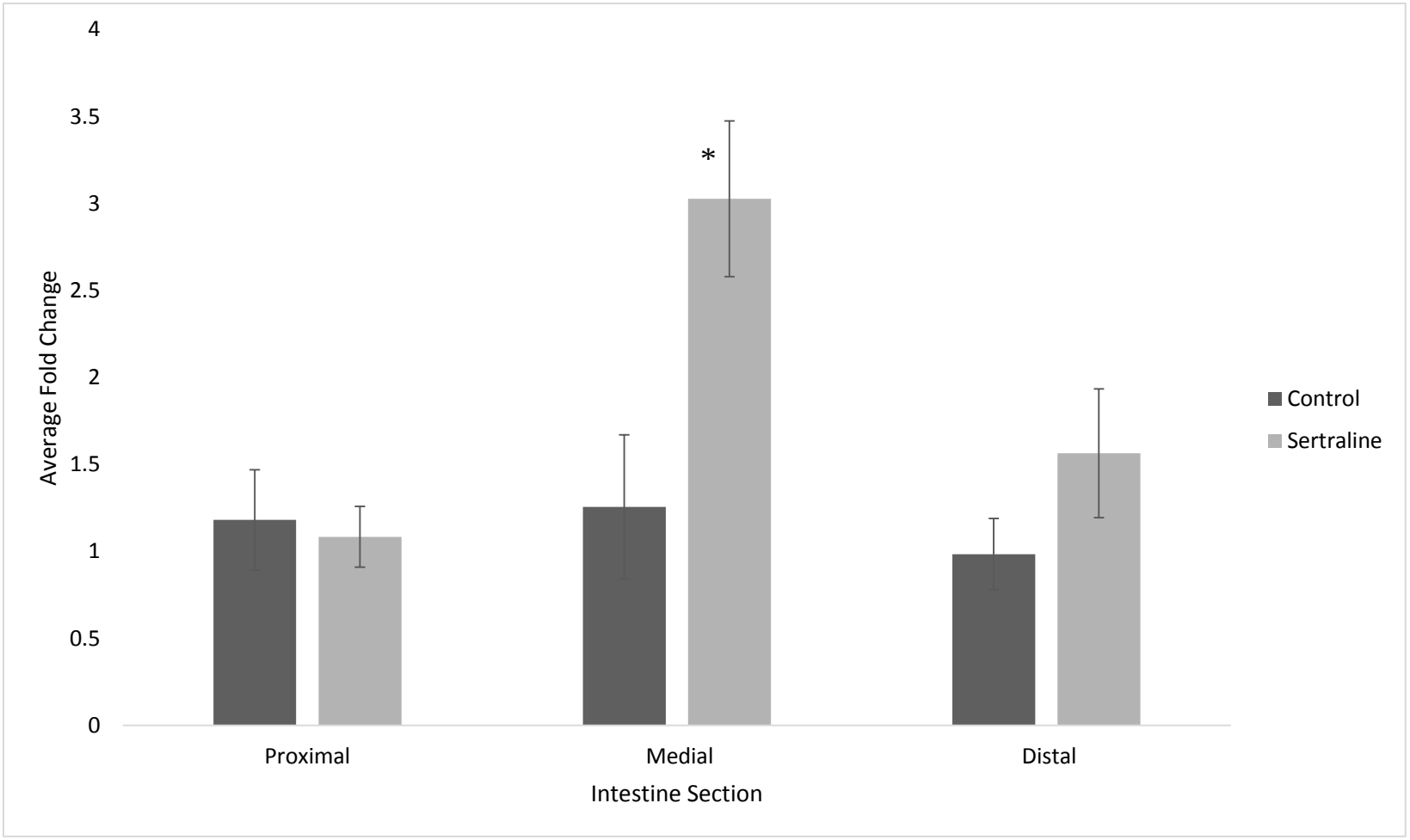

Figure 2.8: Fold change for serotonin reuptake transporter (SERT) in sectioned intestine parts of hybrid striped bass of the high treatment on day 6 of sertraline exposure. In the medial intestine, SERT was significantly upregulated on day 6 of exposure. Bars represent standard error.

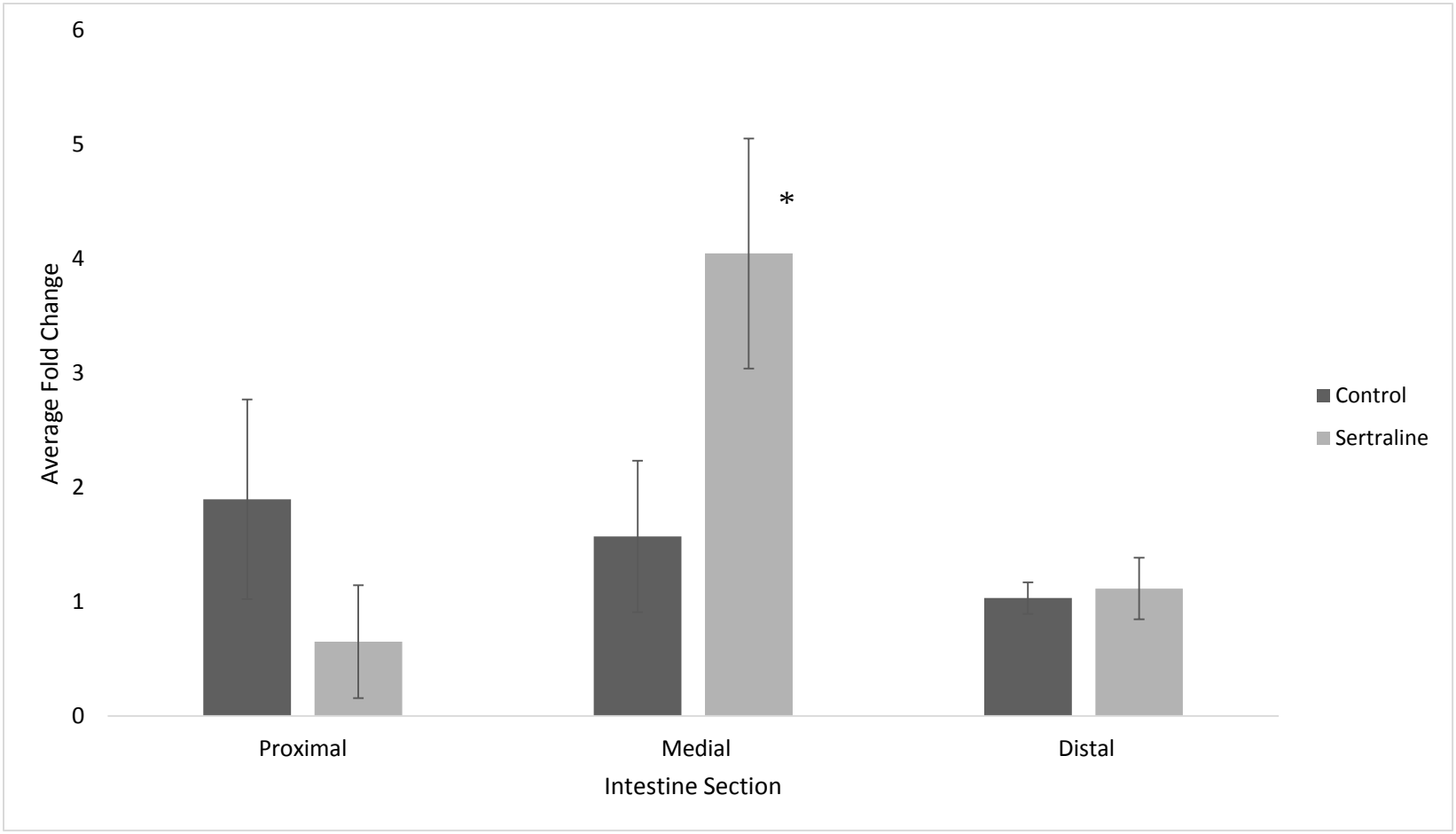

Figure 2.9: Average fold change for cholecystokinin (CCK) in sectioned intestine from hybrid striped bass of high treatment on day 6 of a sertraline exposure. In the medial section of the intestine, CCK was significantly upregulated compared to control fish. Bars represent standard error.

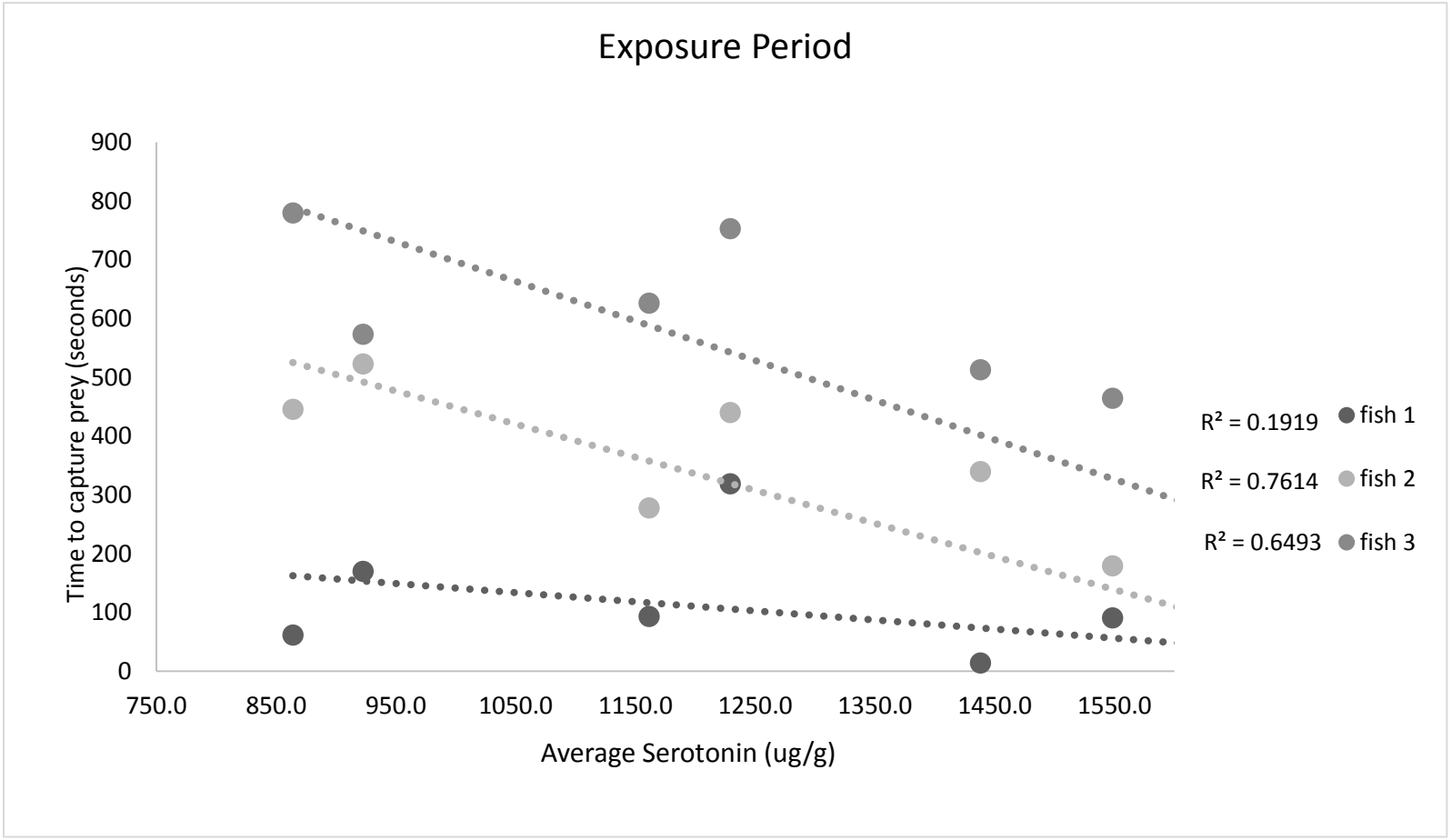

Figure 2.10: Relationships between time to capture prey on days 3 and 6 with whole brain serotonin normalized to brain wet weight. Fish 2 and 3 showed moderate strength negative relationships supporting when serotonin decreases, an increase in time to capture prey is observed.

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CHAPTER THREE

EFFECTS OF CITALOPRAM ON HYBRID STRIPED BASS PEDATORY BEHAVIOR AND BRAIN CHEMISTRY

Introduction

Antidepressants are pharmaceuticals responsible for treating many forms of psychiatric disorders [1]. The number of antidepressant prescriptions in the United States jumped from 254 million in 2010 to 314 million in 2015 [2]. These drugs are often chronically administered to ensure their therapeutic effect. Antidepressants can enter the sewage systems through unused prescription drugs being disposed of improperly and through excretion after human usage [1, 3, 4]. Wastewater treatment plants are not well equipped to totally remove antidepressants from incoming wastewaters [5]. Selective serotonin reuptake inhibitors (SSRIs), a class of antidepressants, removal from wastewaters occurs with different efficiencies, and citalopram has the highest removal efficiency at 98% [6]. This results in consistent discharges into the environment from wastewater treatment plants, making pharmaceuticals pseudo-persistent pollutants [7-9]. The persistence of antidepressants could create life-cycle problems for aquatic organisms [10]. SSRIs have been detected in aquatic ecosystems at concentrations ranging from low ng/l to µg/l [4, 7, 11-14]. Antidepressants have been shown to be transported great distances from point source sites [4, 5].

While SSRIs are not present at overly toxic levels in aquatic environments, it's possible that these compounds may cause sublethal effects [15]. For example, behavioral

endpoints could provide a sensitive ecotoxicological approach to studying SSRIs in the environment, since behavioral mechanisms allow scientists to connect what occurs at the biochemical level to the individual and population level [10, 15, 16]. SSRI's primary mode of action is to increase extracellular serotonin in the brain, and research supports serotonin regulates changes in feeding, appetite, and locomotion [7, 8, 17, 18]. The primary target of SSRIs, the serotonin reuptake transporter (SERT) has high conservation between aquatic species and humans [2, 15, 19]. Hybrid striped bass (*Morone chrysops x Morone saxatilis*) SERT showed 72% homology with human SERT suggesting SSRIs cause similar effects in non-target aquatic organisms [19-21]. Therefore, it's possible that SSRIs present in aquatic environments may interact with SERT in fish, causing similar biochemical and behavioral changes to humans.

Citalopram is an antidepressant in the SSRI class. Evidence suggests citalopram is the least toxic of the SSRI drugs [6, 11]. The risk of citalopram to aquatic organisms such as fish has not been well studied [6]. A dietary exposure study resulted in juvenile rainbow trout showing no changes in aggression or swimming activity, while another similar experiment saw a decrease in aggression and cortisol levels after seven days citalopram exposure [22, 23]. Endler guppies showed a decrease in feeding at an aqueous exposure of 15 µg/l [9]. Three-spined stickleback exposed to 0.15 or 1.5 µg/l citalopram saw a decrease in number of attacks on prey by 37.5% after the first week of a 21 day exposure [12].

Previous research in our lab investigated an environmentally relevant SSRI mixture involving citalopram and observed a decrease in time to capture prey and a

decrease in whole serotonin levels in the brain [19]. However, it is difficult to understand the contribution of citalopram to the behavioral toxicity of this mixture without studying citalopram in isolation. We are collecting data on how citalopram effects hybrid striped bass. The goals of this study were to (1) determine if citalopram exposure causes a change in the time to capture prey for hybrid striped bass, (2) determine citalopram concentration in the brain and plasma, and (3) examine if serotonin concentrations in the brain can help explain the behavioral changes. We hypothesized that citalopram will cause a delay in time to capture prey with a decrease in brain serotonin levels, consistent with previous research.

Materials and Methods

Test Chemicals

Citalopram hydrobromide was purchased from TCI chemicals. Acetone, HPLC gde methanol, HPLC grade acetonitrile, glacial acetic acid, trace metals grade HCl, triethylamine, Optima® LC-MS methanol, and Optima® LC-MS formic acid were purchased from Fisher Scientific. Internal Standard fluoxetine-D5 hydrochloride was purchased from CDN Isotopes (Quebec, Canada). MS-222 was purchased from Pentair aquatic habitats (Apopka, FL, USA). Serotonin creatine sulfate complex and Fluka LC-MS Chromasolv® water were purchased from Sigma Aldrich (St. Louis, USA). Water used for analytical procedures, excluding LC-MS/MS, was ultra-purified using a Milli-Q Super-Q filtration system (Millipore) with a measured resistivity of 18 MΩ x cm.

Fish

All experiments were conducted under the supervision of Clemson University Animal Care and Use Committee using the approved animal use protocols (AUP 2015-077, AUP 2014-015). Hybrid striped bass (*Morone saxatilis x Morone chrysops*) were purchased from Keo Fish Farms (Keo, AR, USA) as fingerlings. Detailed methods for fish holding can be found in the previous chapter. Briefly, bass were kept in holding troughs at Cherry farm aquatic research lab at Clemson University. Troughs were constantly supplied with water (19-24°C) in a flow through system from Lake Hartwell (Clemson, SC, USA). Bass were fed a commercialized slow sink diet during holding from Zeigler Bros, Inc. (Gardners, PA, USA).

Fathead minnows were purchased from Anderson Minnow Farm (Lonoke, AR, USA). Minnows were kept in 100L troughs with flow through system bringing in the same water as above. Until use, minnows were fed a commercial diet (Tetramin® Tropical Flakes) purchased from Dr's Foster and Smith Inc. (Rhinelander WI, USA).

Bass Training

Once bass were of the appropriate size (23.4 ± 1.7 cm, 131 ± 28.8g), they needed to be trained to capture live prey. Detailed methods for training can be found in previous chapter. Briefly, bass were randomly chosen and moved to a trough for group training. During group training, 5 minnows per bass were dropped into the trough every 3 days over a 6 day period. On the 6th day of group training, bass were moved to individual experimental tanks (1 bass per tank). Each bass was fed 4 fat-head minnows every 3 days over a 6 day span. The time to capture each prey was recorded and used to determine which fish were appropriate to use in the exposures.

Experimental design

Hybrid striped bass were exposed to citalopram in a static system for 6 days. Detailed experimental design can be found in the earlier chapter. Briefly, water quality parameters (pH, DO, and temperature) were measured during the feeding events using an YSI 556 multi-parameter instrument (Yellow Springs Instruments). On the last individual training day (exposure day 0), 4 fathead minnows were dropped into the tank. Bass who ate at least 3 fat-head minnows (with comparable feedings on the previous training days) were used in the exposure. Water in the tanks was filled to 80L and marked before turning the water off and spiking the tanks. Each bass was randomly assigned to a treatment (5 bass/treatment/time point), and spiked with the appropriate volume of sertraline to reach nominal concentrations. Feeding days took place on days 3 and 6. After each feeding, 5 bass per treatment were euthanized for brain, plasma, and intestine analysis.

Citalopram exposure

Stock solutions (prepared fresh daily) of citalopram were prepared by dissolving citalopram HBr in methanol and the added to tanks to reach their nominal concentrations. Low, medium, and high exposures were performed in addition to a control. The highest concentration equivalent of methanol was added to each control tank to ensure no toxicity from the carrier solvent. Methanol was at a concentration less than 0.1 mg/L; compliant with ASTM international recommendation for experiments involving fish (ASTM1241-

05) [24]. Two hours after tanks were spiked, appropriate aliquots from each tank were taken to measure citalopram concentrations.

Citalopram analysis

Citalopram concentrations were analyzed on day 0, 2 hours after the spiking of tanks. Water samples were acidified with 2-3 drops 2N HCl (pH of 3.0) and extracted using C-18 solid phase extraction cartridges. Cartridges were conditioned with 1 volume methanol, 1 volume acetone, and 2 volumes milli-Q water before running samples. Samples were stored at -20 °C until analysis preparation. Cartridges were eluted with methanol/1% acetic acid and stored in sample vials for HPLC analysis. Samples were run on a Waters HPLC with 1525 Breeze HPLC pump, Waters 717 Plus auto sampler, and Waters 2475 multi wavelength fluorescence detector set to excitation of 250 nm and emission of 325 nm (Waters, Milford, MA, USA). The mobile phase consisted of 50:40:10:0.3:0.15 water: acetonitrile: methanol: triethylamine: acetic acid set at a flow rate of 1 ml/min and an injection volume of 40 µl. An Alltech Prevail C_{18} column (150mm, 4.66 I.D.) was used for separation. Approximate run time was 12 minutes with citalopram eluting off the column at 7.2 minutes.

Brain and plasma preparation

Detailed methods for tissue preparation can be found in the earlier chapter. Briefly, bass were euthanized in buffered MS-222. Brains were removed and kept at -80°C until processed. Brains were thawed, weighed, and sonicated with the addition of an internal standard, Milli Q water, and acetonitrile. Brains were refrozen to allow proteins to precipitate. Samples were centrifuged up to 4 times at 17,000 G for 5 minutes at 4°C

before being placed in LC-MS/MS tubes for analysis. Brain antidepressant and serotonin concentrations were normalized to brain tissue weight (g).

Blood was removed from fish caudal artery and placed on ice until centrifugation to collect plasma. Samples were kept at -20°C until processed. A 20 µl aliquot of plasma was added to a centrifuge tube with internal standard and acetonitrile. Samples were vortexed before being placed back in the -20°C to allow proteins to precipitate. Samples were centrifuged twice at 17,000 G to remove all debris. Clear homogenate is placed in a LC-MS/MS tube for analysis.

LC-MS/MS analysis

Table 3.1 shows the parameters used for detecting citalopram and serotonin in the brain and plasma samples. Samples were analyzed on a Shimadzu LC-MS/MS 8030 using a kinetix column (2.6u, C18, 100 x 3.0mm). The mobile phase consisted of 0.01% formic acid in 40% water and 60% methanol. An isocratic method ran with 5% methanol for 2 minutes, increasing from 10% methanol for 2 minutes to 95% methanol for 2 minutes, and remaining at 95% methanol for 5 minutes. The total run time was 11 minutes. At the end of the run, the mobile phase reduced back down to 5% methanol over 6 minutes to re-equilibrate the column prior to the next sample injection. The sample injection volume was 2 µL and the compound retention times were as follows: Serotonin: 2.9 min, citalopram: 8.8 min, fluoxetine-d5: 9.0 min.

Statistics

All statistics were performed using JMP Pro 12.0. The data was transformed to log (data) + 1 to reduce data variability for analysis. A model was run with day,

treatment, day*treatment, and tank nested within treatment as a random variable to analyzed all time to capture prey data. Since one HSB was placed in each tank, nesting tank within treatment and treating it as a random variable corrected for the repeated measures of time to capture prey. A least squares mean model run with day, treatment and day*treatment was run to make multiple comparisons for plasma and brain data.

Results

Aqueous citalopram concentrations

Water concentrations (mean ± SE) for citalopram were measured as 70.8 ± 5.19 µg/L, 126.3 ± 3.84 µg/L, and 190.0 ± 9.81 µg/L for low, medium, and high treatments, respectively. All aqueous water concentrations were within 80% of the nominal concentrations. Water quality parameters were measured (mean ± SD) as 6.98 ± 0.25, 22.5 ± 1.43, and 8.81 ± 0.21 for pH, water temperature (C°), and dissolved oxygen (mg/L), respectively.

Behavioral assay

Exposure of hybrid striped bass to citalopram caused an increase in time to capture prey at some feeding points over the 6 day experiment. Each prey was analyzed separately, so prey 1 refers to time to capture the first fathead minnow, prey 2 represents time to capture the second fathead minnow, prey 3 refers to time to capture the third fathead minnow, and prey 4 time to capture the fourth fathead minnow.

The medium treatment group showed a significant increase in time to consume prey 1 compared to the low ($p= 0.0292$) and high ($p= 0.0167$) treatment only on day 6 (figure 3.1). The high treatment took significantly longer to eat prey 1 compared to the

low treatment on day 3 (p= 0.0128). The medium treatment took significantly longer to capture the second prey from all the other treatment groups on day 3 (p= 0.0491, 0.0062, 00048) and day 6 (p= 0.0019, 0.0022, 0.0023) (figure 3.2). The medium treatment bass took significantly longer to eat the third prey compared to all other treatment groups on day 3 (p= 0.0034, 0.0041, 0.0009) and day 6 (p= 0.0431, 0.0117, 0.0012) (figure 3.3). The medium and high treatments took significantly longer to eat the fourth prey compared to the low treatment on day 3 (p = 0.0028 and 0.0452) (figure 3.4). The medium treatment took significantly longer to capture the fourth prey compared to the low treatment on day 6 (p = 0.0249).

Brain chemistry

No significant decreases in serotonin levels were seen during the 6 day citalopram exposure (figure 3.5).

Brain antidepressant concentrations

Citalopram concentrations in the brain increased with treatment group in a dose dependent fashion (figure 3.6). Medium and high treatments had significantly higher values than the control treatment on days 3 (p= 0.0361, 0.0048) and 6 (p= 0.007, < 0.0001). Medium and high treatments had significantly higher values than the low treatment on days 3 (p= 0.0227, 0.002) and 6 (p= 0.0194, < 0.0001). Medium and high treatment groups differed on day 6 (p= 0.0083). There was an increase in brain citalopram concentrations between days 3 and 6. Some low treatment bass had no citalopram detected in their brain.

Plasma antidepressant concentrations

Citalopram detected in the plasma on day 3 of the exposure showed increasing antidepressant concentration in the plasma with increasing aqueous antidepressant concentrations (figure 3.7). Plasma concentrations at the low treatment were significantly different from those at the high treatment ($p= 0.04$). Day 6 plasma concentrations decreased from those on Day 3 for the low and high treatments. The medium treatment group plasma levels increased from day 3 to 6. The medium and high treatments were significantly higher than the low treatment ($p= 0.001$ and 0.002, respectively).

Discussion

Previous research suggested citalopram caused changes in behavior but failed to show a dose dependent response typical of some toxicants [6, 11]. The medium treatment was the only group to show an increase in time to capture prey during the six day exposure (figures 3.1-3.3). Kellner et al. (2015) saw citalopram effect the three-spined stickleback in a non-dose dependent fashion. The three spined stickleback was exposed for 21 days to citalopram where a decrease in attacks on prey occurred in the 0.15 and 1.5 µg/l concentrations [12]. The Endler guppy was chronically administered citalopram for 21 days during which time the 15 µg/l group showed decreased feeding behavior [9]. Two different experiments were completed with trout, where one (using fry) showed no changes in behavior while the other (using two year old fish) demonstrated a decrease in aggression [22, 23]. Differences in citalopram exposure may be a result of age, species, and food web status. Fish intermediate in the food web (guppies and stickleback) have different behaviors compared to larger predatory fish (trout and bass). Previous research

does show citalopram can have an effect on fish behavior, but few studies have been completed with larger predatory fish which would be a more beneficial comparison.

Citalopram plasma concentrations leveled off over the 6-day exposure. Previous research with a SSRI mixture did not see plasma levels change between 48 and 72 hours [19]. A maximum concentration in the plasma may have been reached at 72 hours during our citalopram exposure, with any additional citalopram moving to lipid tissues or being metabolized.

The brain showed a dose dependent increase in citalopram on both days 3 and 6 of the exposure. Changes in behavior were only seen in the medium treatment when larger concentrations of citalopram were detected in the brain during the high treatment. One hypothesis could be an unfavorable ratio of citalopram enantiomers in the high treatment. Citalopram is a racemic mixture where the S- (citalopram) form can interact with the S+ (escitalopram) form, blocking interaction with SERT [25]. Even though a higher concentration of citalopram was detected in the high treatment brains compared to the medium, the medium may have had a more favorable ratio for causing an effect on feeding behavior. A second hypothesis to explain high citalopram concentrations in the brain is the drug has poor blood brain penetration [26]. Serotonergic neurons in the brain are located in large concentrations in the telencephalon and brainstem raphe nuclei. The serotonergic neurons branch off into the hippocampus, hypothalamus, and isthmus [2, 7]. Citalopram may not be reaching the interior portions in the brain at high enough concentrations to cause behavioral changes. When brains were dissected blood was also collected with the brains, and high citalopram concentrations may have resulted from the

drug in the blood surrounding the brain or just past the blood brain barrier. A third hypothesis to explain citalopram in the brain but no change in behavior is binding affinity. Citalopram was ranked third behind fluoxetine and sertraline in binding affinity to hybrid striped bass SERT [19]. At the low treatment, citalopram may not have been prevalent enough to bind tightly to SERT causing a downscale change in feeding behavior.

No significant changes in brain serotonin were seen over the 6 day citalopram exposure. Sweet (2015) predicted no large percent change in citalopram only exposure to hybrid striped bass based on the binding affinity of the compound [19]. Rainbow trout exposed to citalopram for seven days did not show any changes in aggression compared to controls. Researchers suggested that the seven day exposure may not have been enough time for an effect to be observed [23]. Due to the low toxic nature of citalopram and low binding affinity to the hybrid striped bass SERT, a longer exposure period may have been needed to see an effect on serotonin levels [19]. One review suggested that SSRI exposure should take place for 21 days or longer because the therapeutic effects occur weeks after administration of the drug has begun [2]. Serotonin effects many hormones within the brain, including corticotrophin releasing factor, which are suggested to cause a decrease in feeding behavior [18, 27]. Measuring additional endpoints may have led to a better explanation of why the medium treatment group showed a decrease in time to capture prey without a change in serotonin.

Our whole brain analysis of serotonin may have masked any serotonin changes since we did not differentiate between different parts of the brain or intra and extra

serotonin levels [2]. Evidence has shown that serotonin levels changed in different parts of the brain when exposed to fluoxetine [28, 29]. Fluoxetine in one experiment showed no differences in biochemical activity in the telencephalon and hypothalamus [29]. Differences in serotonin were detected after chronic fluoxetine exposure to Siamese fighting fish in the forebrain and hindbrain [28]. Fluoxetine levels in these studies were measured in whole brain vs separate sections. Because we did whole brain analysis of serotonin and citalopram, we could be overlooking evidence where citalopram is not traveling to the right parts of the brain to cause a behavioral effect in the low and high treatments.

Sublethal changes in behavior such as feeding could alter the ecological fitness of an individual or population if exposure continues for several generations which could happen with continued release of SSRIs into the environment [7-9, 17]. Citalopram research on aquatic organisms has not been sufficiently completed to the same extent as fluoxetine [6]. Behavioral tests are useful for seeing sublethal effects because the most sensitive endpoints include locomotion behaviors [17]. Our study met two standards for having robust behavioral endpoints. Our fish were allowed to acclimate for six days prior to exposure and behavioral tests were completed for 25 minutes per feeding time [30]. Industrial contaminants are used for making ecological regulations, but antidepressants work differently than industrial compounds and are not good substitutes [31]. Research needs to continue looking into less studied compounds such as citalopram. Many organisms are exposed to multiple compounds in the environment, understanding

individual compounds could give insight as to what combined effect (antagonistic, additive, and synergistic) occurs within the mixtures.

Figures and Tables

Compound	Precursor Ion(s)	Product Ion(s)	Dwell Time (mSec)	Q1 (V)	CE (V)	Q3(V)	Retention Time (minutes)
Serotonin	177.2	160.1	300	-11	-13	-18	2.3
Citalopram	325.2	109.05	3	-14	-26	-23	8.94
	325.2	262.05	3	-14	-21	-19	
	325.2	116.05	3	-14	-28	-25	
Fluoxetine-d5	315	153.1	25	-14	-10	-17	9.22

Table 3.1: LC-MS/MS optimization parameters for detecting serotonin, citalopram, and internal standard in plasma and brain samples.

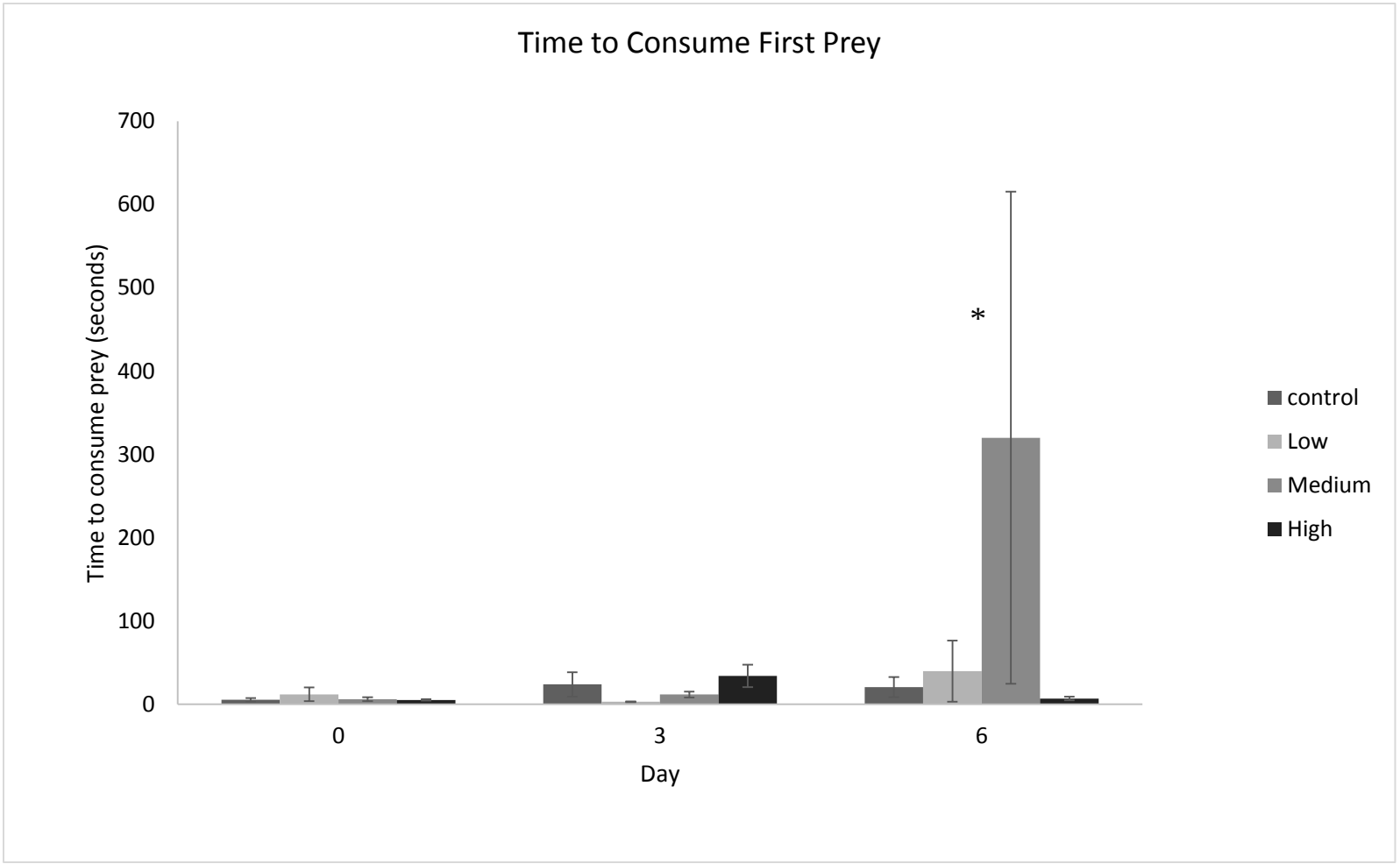

Figure 3.1: Time to capture the first fathead minnow (seconds) for hybrid striped bass when exposed to citalopram. The medium treatment group took significantly longer to capture prey compared to the medium and high treatment groups on day 6.

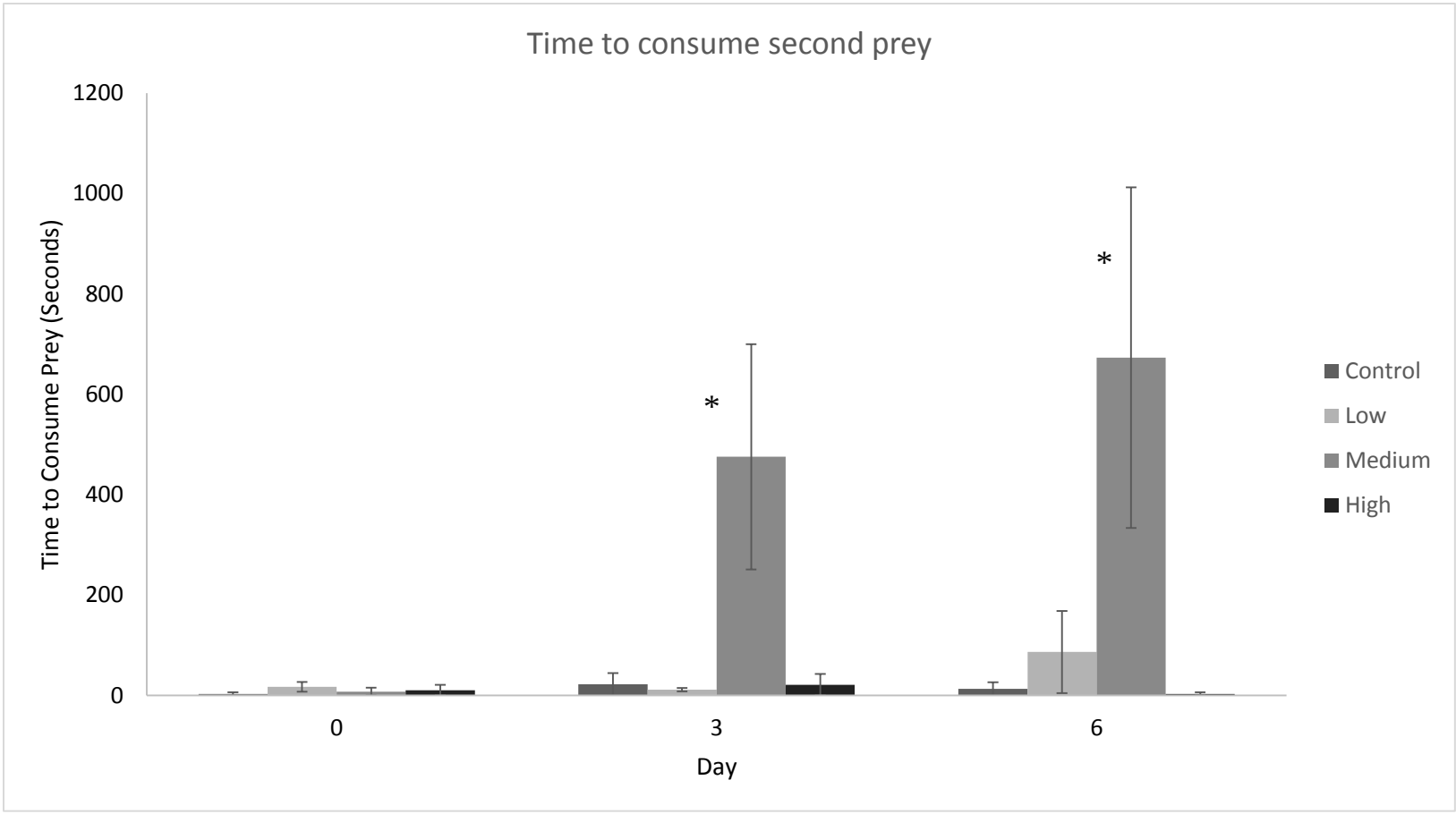

Figure 3.2: Time for hybrid striped bass to consume the second prey (seconds) when exposed to citalopram. The medium treatment group took significantly longer to capture prey compared to all other treatment groups on days 3 and 6.

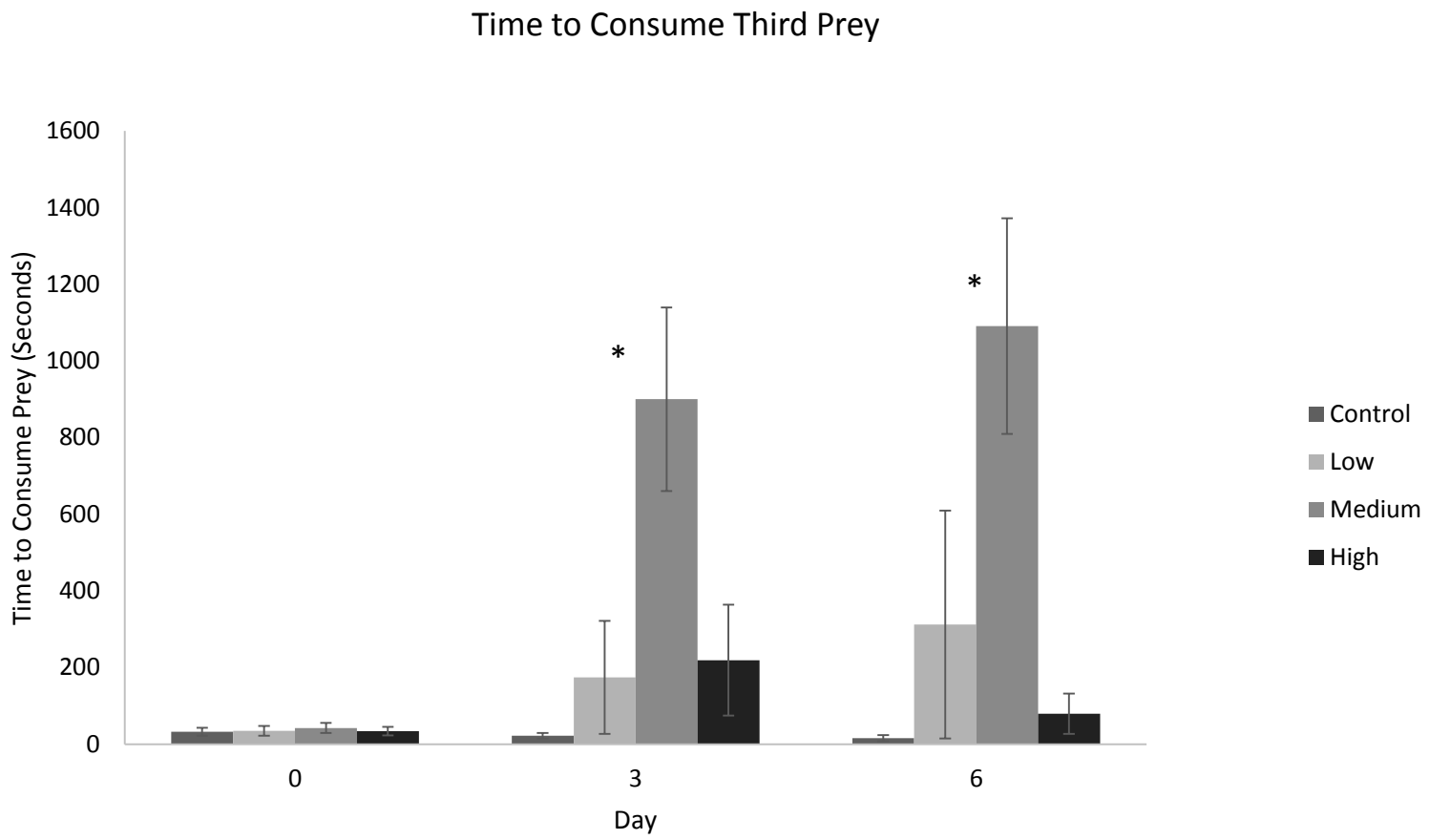

Figure 3.3: Time for hybrid striped bass to consume the third prey (seconds) after exposure to citalopram. The medium treatment group took significantly longer to capture prey compared to all other treatment groups.

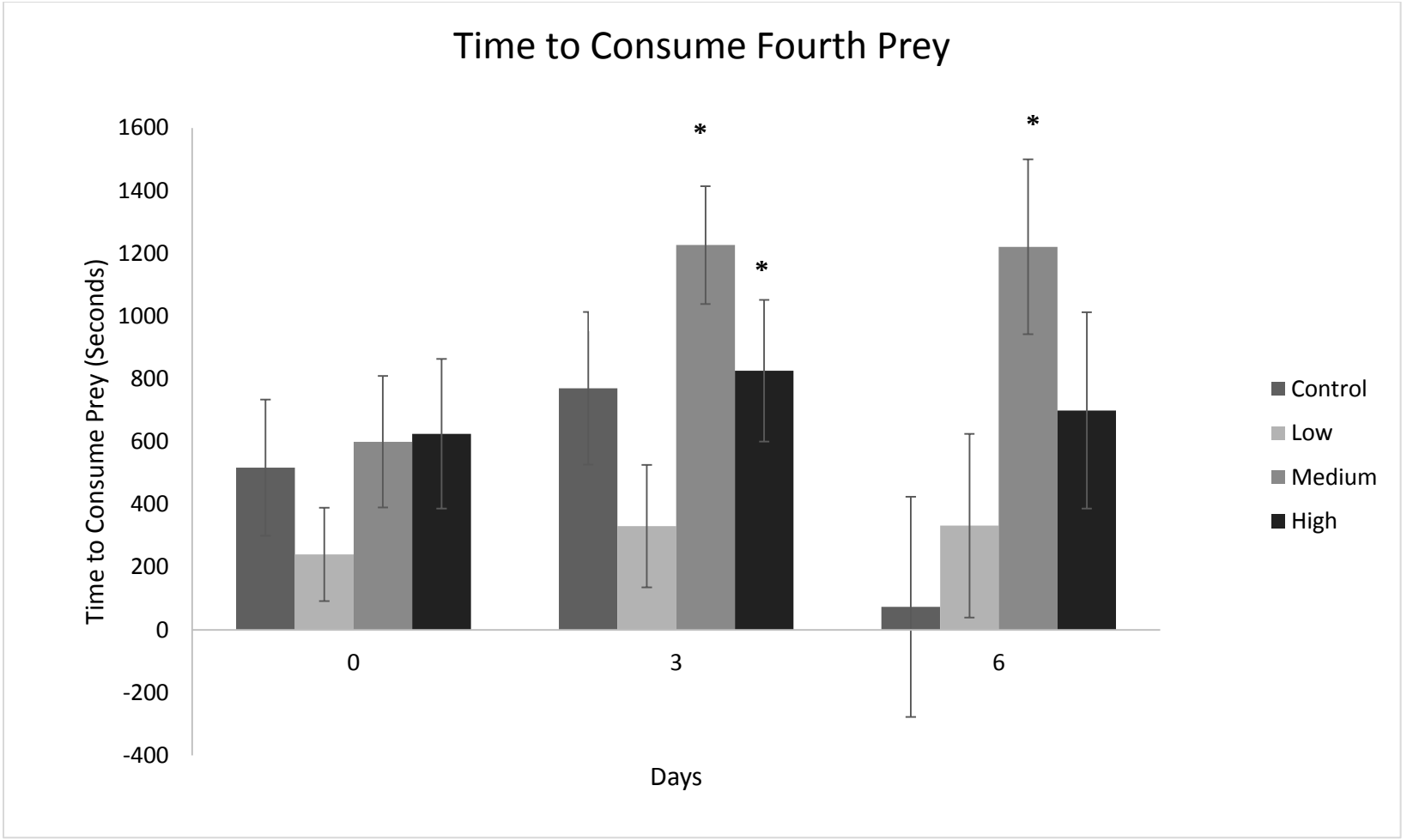

Figure 3.4: Time for hybrid striped bass to consume the fourth prey (seconds) after exposure to citalopram. The medium and high treatment took significantly longer to capture prey compared to the low treatment on the 3rd day. The low and medium treatments were significantly different on the 6th day.

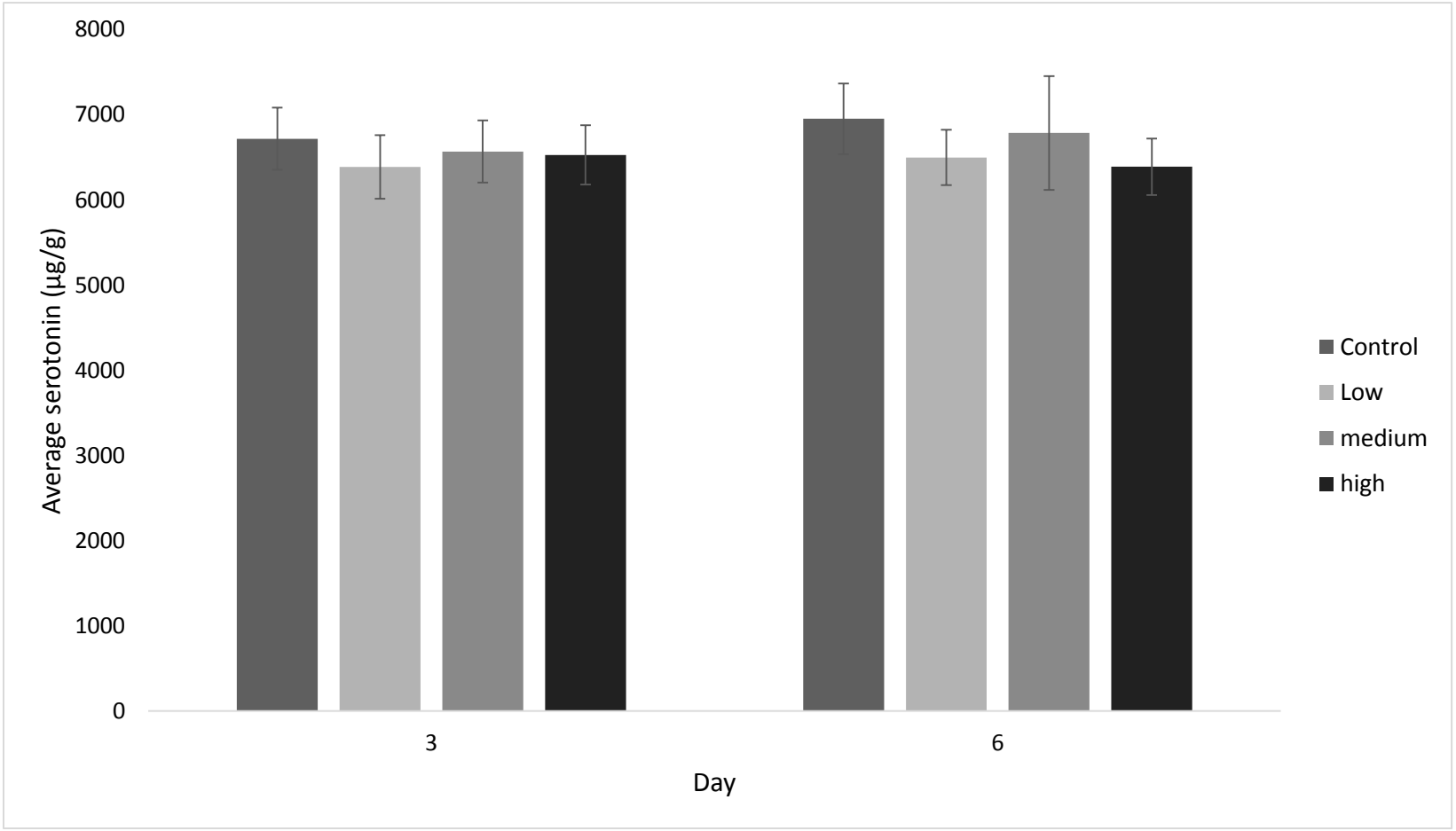

Figure 3.5: Whole brain serotonin levels in hybrid striped bass after sertraline exposure normalized to wet weight of brains (g). No significant differences were seen in serotonin levels.

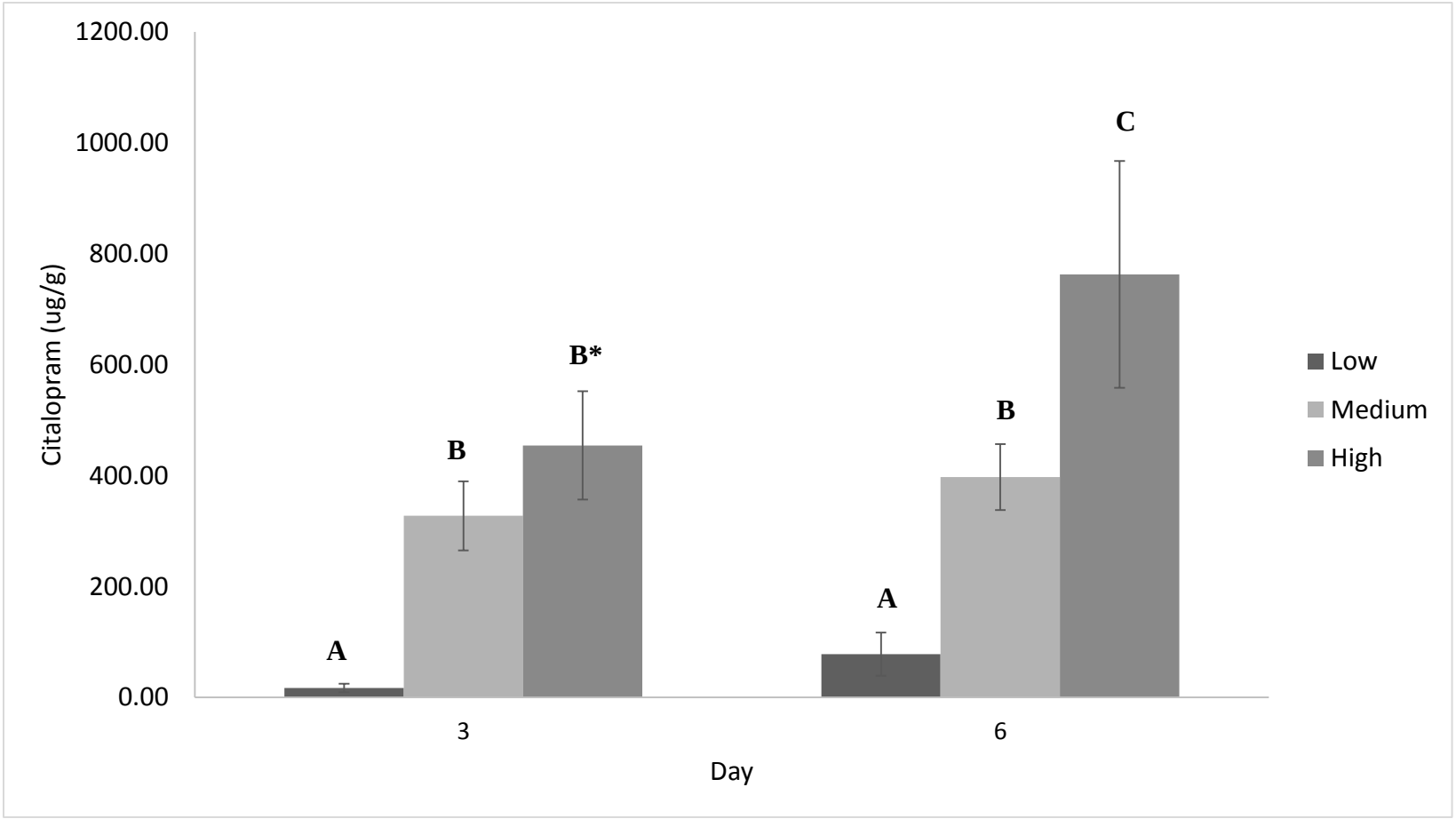

Figure 3.6: Citalopram levels in the whole brain of hybrid striped bass exposed to citalopram. On day 3, medium and high treatments were significantly higher than the low treatment. On day 6, each treatment group was significantly different from each other. The medium and high treatments were also significantly higher than the control groups on both days 3 and 6.

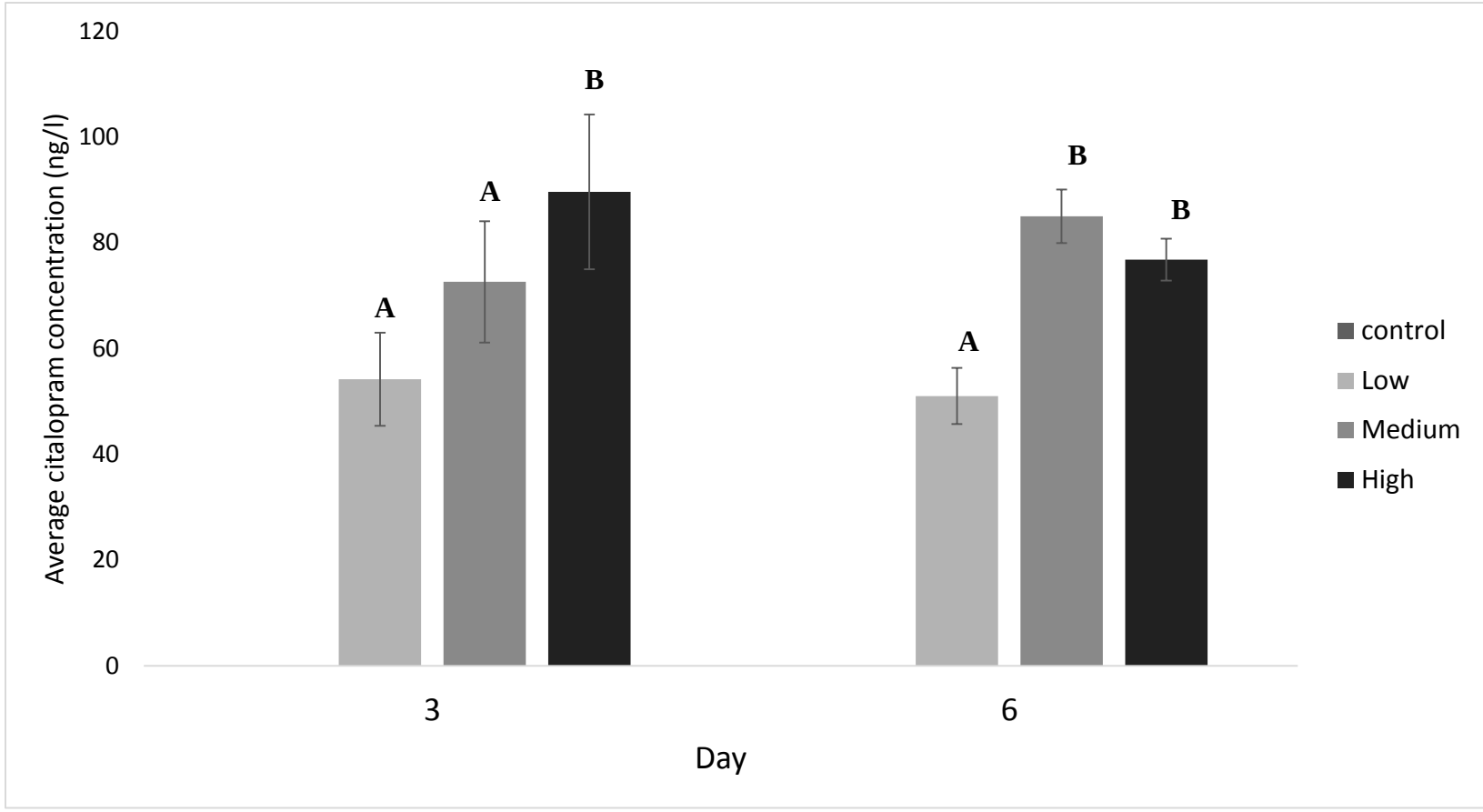

Figure 3.7: Citalopram concentrations found in hybrid striped bass plasma samples during citalopram exposure. On day 3, the low and high treatment groups showed significantly different plasma citalopram concentrations. On day 6, medium and high treatment groups were significantly higher than the low treatment group.

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CHAPTER FOUR

FINAL DISCUSSION AND CONCLUSIONS

Comparison of SSRI mixtures and individual SSRI exposures

This thesis is an extension of previous work in which Sweet (2015) investigated changes in hybrid striped bass predatory behavior after exposure to a mixture of selective serotonin reuptake inhibitors (SSRIs) including citalopram, fluoxetine, and sertraline [1]. Gaworecki et al. (2008) completed a study that exposed hybrid striped bass to fluoxetine only [2]. This thesis studied citalopram and sertraline only exposures to hybrid striped bass to complete the mixture comparison. Figures 4.1-4.4 compare the high exposure treatment from the mixture study to the low exposure treatments from the individual SSRI studies. Table 4.1 shows the concentrations for each treatment chosen in the comparison to confirm the similarities in the aqueous exposure concentrations. All studies followed a similar experimental setup with six days of exposure and six days of recovery.

Understanding how individual compounds interact in a mixture can help with determining environmental toxicity to non-target organisms, since mixture interactions can cause a different response than individual exposure reactions. When the components of a mixture work according to the same mode of action and do not interfere with one another they are said to act additively. When the components in a mixture create a greater than expected effect, the result is synergism. When the component's combined effect is smaller than expected the interaction is antagonistic [3]. SSRI's comparable structures

and mode of actions would support an additive interaction among members of this drug class [4].

On day 3, fluoxetine and sertraline exposed fish took longer to capture prey compared to the mixture and citalopram studies. The mixture time to capture prey was well below fluoxetine and sertraline for the first two prey, but started to reach sertraline levels for the third prey (figure 4.1). The individual SSRIs may have inhibited each other in the mixture study resulting in the decreased time to capture prey observed. On day 6 and 9, fluoxetine fish took longer to capture prey than all the other studies, and sertraline and citalopram were below the mixture study (figure 4.2 and figure 4.3). Sertraline and citalopram may have had some antagonistic effects on fluoxetine during the end of the exposure and beginning of the recovery period resulting in lowered time to capture prey for the mixture study. On day 12, the mixture treatment took longer to capture prey compared to sertraline and fluoxetine studies (figure 4.4) (no citalopram data was available for the recovery period). Fluoxetine and sertraline may have acted synergistically, with fluoxetine playing the largest role, between days 9 and 12 causing the increase in time to capture prey observed.

Despite the comparable mode of action of the investigated SSRIs, comparing the results of the mixture studies with the exposures to individual components of the mixture suggests these drugs do not act synergistically. This may be caused by differences in binding affinity for the serotonin reuptake transporter (SERT), competition for SERT in the mixture exposure, and other pharmacokinetic interactions that could cause deviations from a simple additive relationship.

Pharmacokinetic properties of SSRIs have been poorly studied in aquatic species. One study looked at some properties of fluoxetine on Japanese medaka and reported that fluoxetine has a high Volume of Distribution, with a calculated bioconcentration factor of 80, and that fluoxetine is more acutely toxic than fluvoxamine, paroxetine, and citalopram [5]. Brook trout exposed to 20% v/v wastewater effluent saw similar tissue level accumulations of citalopram, fluoxetine, and sertraline in the liver and brain [6]. Pharmacokinetics of SSRIs in humans has been well studied. Research determined that fluoxetine is completely absorbed after oral administration and has the largest volume of distribution (V_d) (14-100 L/kg) among the SSRI drugs, in part due to lysosomal trapping in tissues [7]. Fluoxetine is extensively metabolized into norfluoxetine which retains SSRI properties, and both fluoxetine and norfluoxetine have relatively long half-lives of up to four days [7]. Sertraline is completely adsorbed from the intestine by eight hours after ingestion and has a V_d exceeding 20 L/kg. Sertraline is quickly metabolized into N-desmethylsertraline which has a half-life 3x longer than the parent compound [7]. Citalopram is considered one of the safest SSRIs when examining pharmacokinetic drug interactions. Citalopram is metabolized into two primary metabolites which occur in racemic mixtures with the S+ enantiomer retaining SSRI properties, but the S-enantiomer is found at higher concentrations [7]. All these differences in absorption, distribution, metabolism and excretion of the individual SSRIs influence the eventual concentration at the receptor site, and thus influence the toxicological endpoints.

Thus, determining how pharmaceuticals interact in a mixture proofs to be challenging. This research supported a non-additive relationship between the SSRIs

fluoxetine, sertraline, and citalopram. Sertraline would likely contribute to the mixture study more than citalopram due to the lower binding affinity citalopram has for the hybrid striped bass serotonin reuptake transporter [1]. In the environment, SSRIs would be in a mixture with a variety of other pharmaceuticals. In these complex mixtures seeing effects that fall into the synergistic or antagonistic category are rare [8]. While this SSRI only comparison supports evidence of both synergistic and antagonistic effects in mixtures, in more complex environmental mixtures, only a few compounds may be the driving force in mixture interactions, while those same compounds also buffer the effects of other smaller synergistic and antagonistic interactions [8, 9]. A more in-depth experiment using wastewater effluent with known concentrations of SSRIs would give further information on how SSRIs work in a more complex mixture setting.

Conclusions

1. **When comparing the individual SSRI experiments to the mixture study, fluoxetine appears to play the largest role in the effect on time to capture prey by hybrid striped bass.**
2. **Sertraline and citalopram may have inhibited some of the toxic effects of fluoxetine during the exposure period in the mixture.**
3. **Brain serotonin levels alone may not be enough to explain the behavioral effects observed from SSRI exposure in hybrid striped bass.**
4. **The peripheral nervous system should be further investigated for changes after an exposure to SSRIs occurs, rather than just focusing on changes in brain chemistry.**

Figures and Tables

Treatment	**Actual aqueous concentrations (µg/l)**
Mixture- high trt Sweet (2015)	4-sertaline 16-fluoxetine 84-citalopram
Sertraline only- low trt	4.5
Fluoxetine only- low trt Gaworecki et al. (2008)	23.2
Citalopram only- low trt	70.8

Table 4.1: Aqueous water concentrations from treatments used in the comparison of a SSRI mixture and individual SSRI exposures.

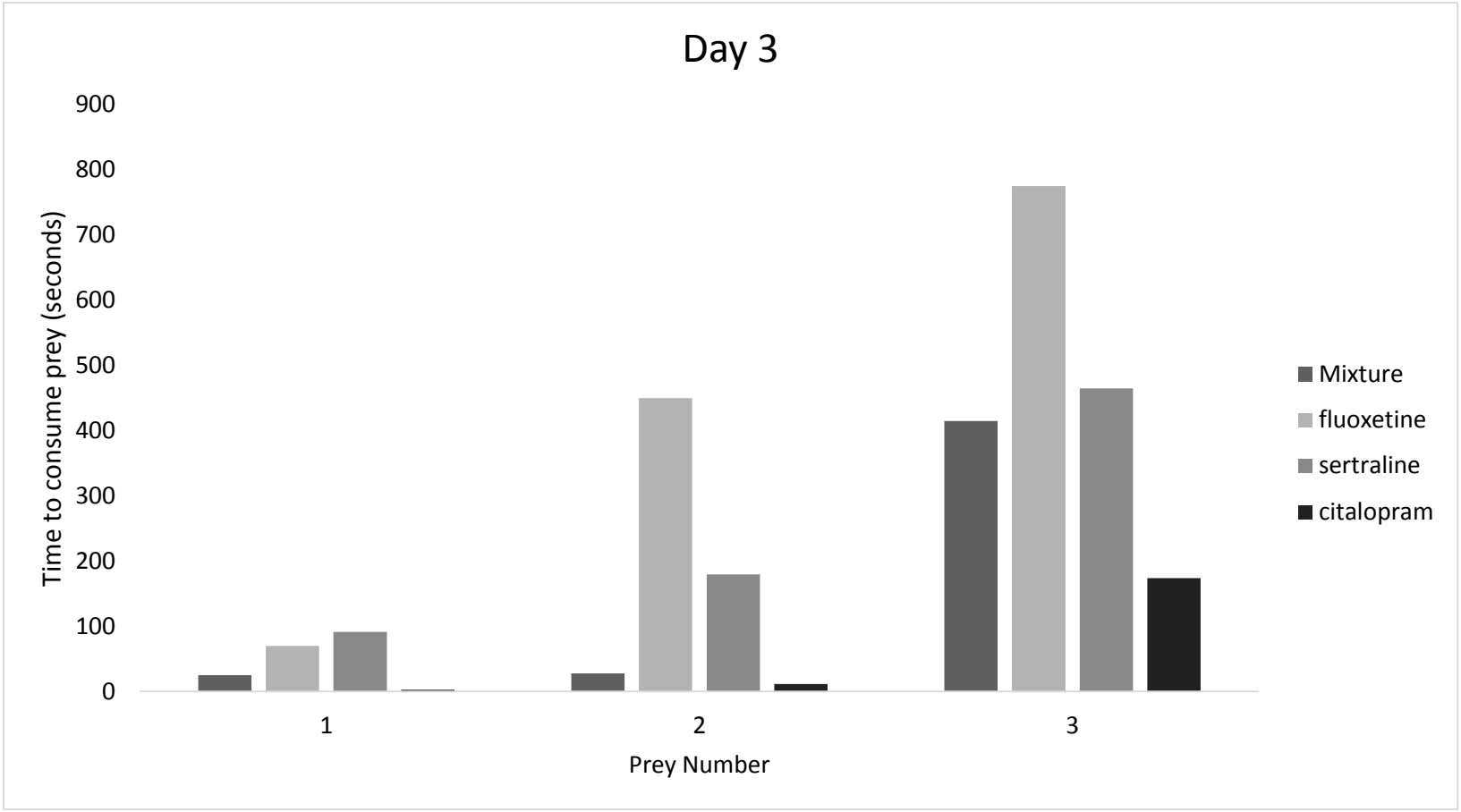

Figure 4.1: Comparing time to capture prey 1, 2, and 3 on day 3 after exposure to SSRIs individually or in a mixture. Fluoxetine only exposure time to capture prey is higher than all the other SSRI studies. Sertraline is higher than the mixture study, but the mixture study time to capture prey reaches sertraline levels by the third prey. Citalopram time to capture prey is well below the other SSRI studies.

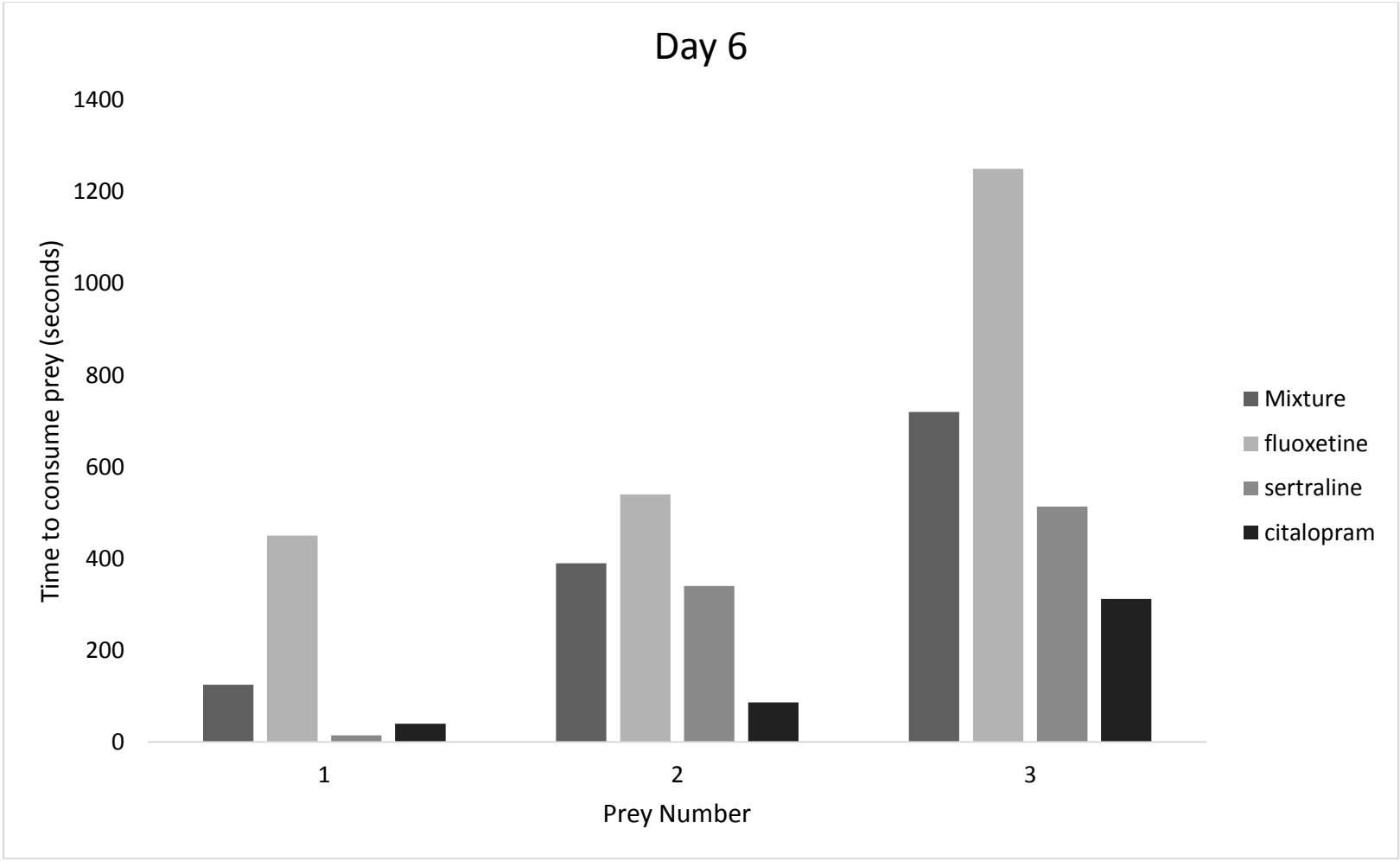

Figure 4.2: Comparing time to capture prey 1, 2, and 3 on day 6 after exposure to SSRIs individually or in a mixture. The fluoxetine study took longer to capture prey than the other SSRI studies. The sertraline study was close in time to capture the 2nd prey to the mixture study. Citalopram time to capture prey was well below the other SSRI studies.

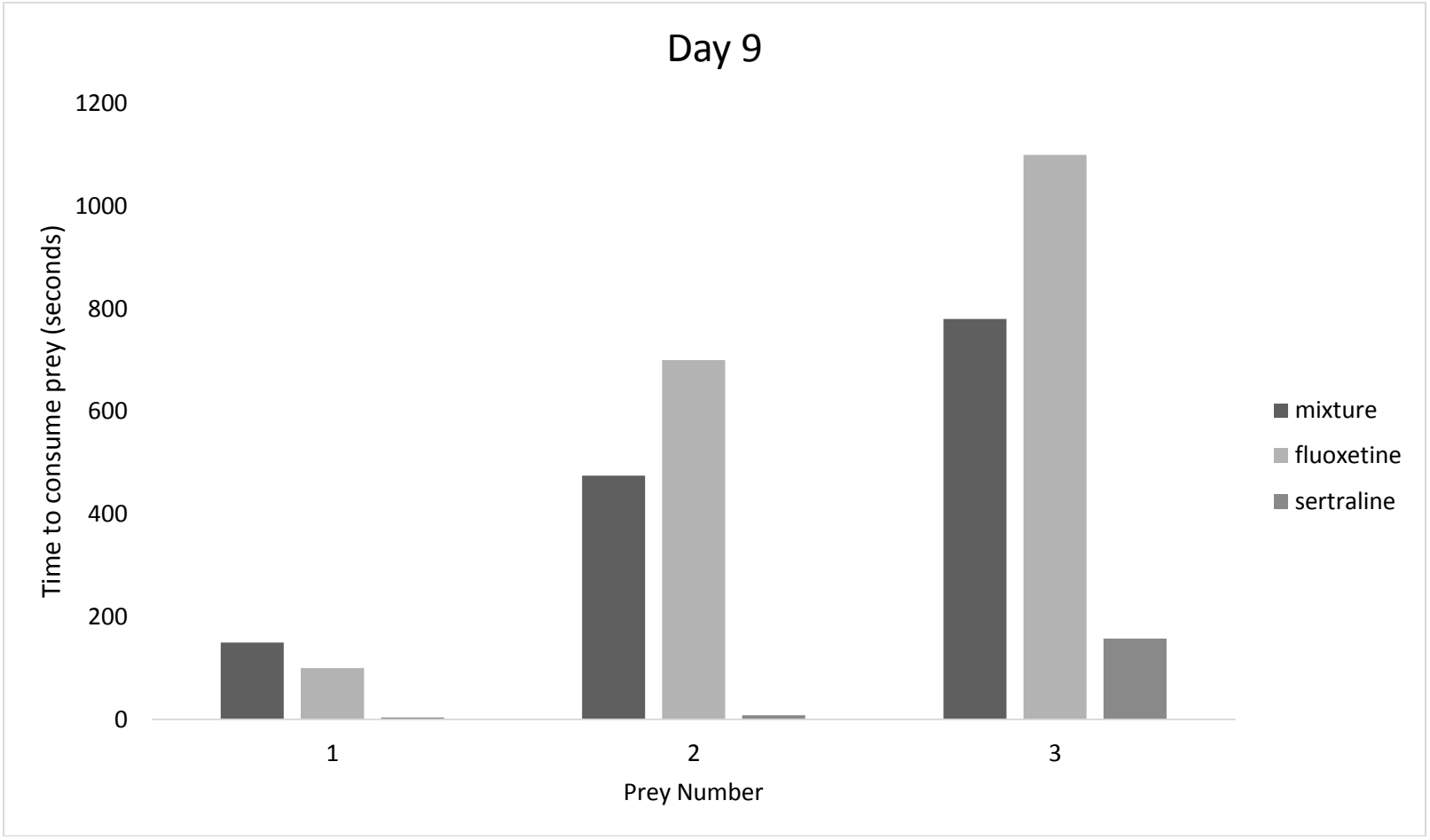

Figure 4.3: Comparing time to capture prey 1, 2, and 3 on day 9 after exposure to SSRIs individually or in a mixture. Fluoxetine took longer to capture prey compared to the mixture and sertraline studies. Citalopram was not studied to day 9, so no data was available for comparison. Sertraline was well below the mixture and fluoxetine studies.

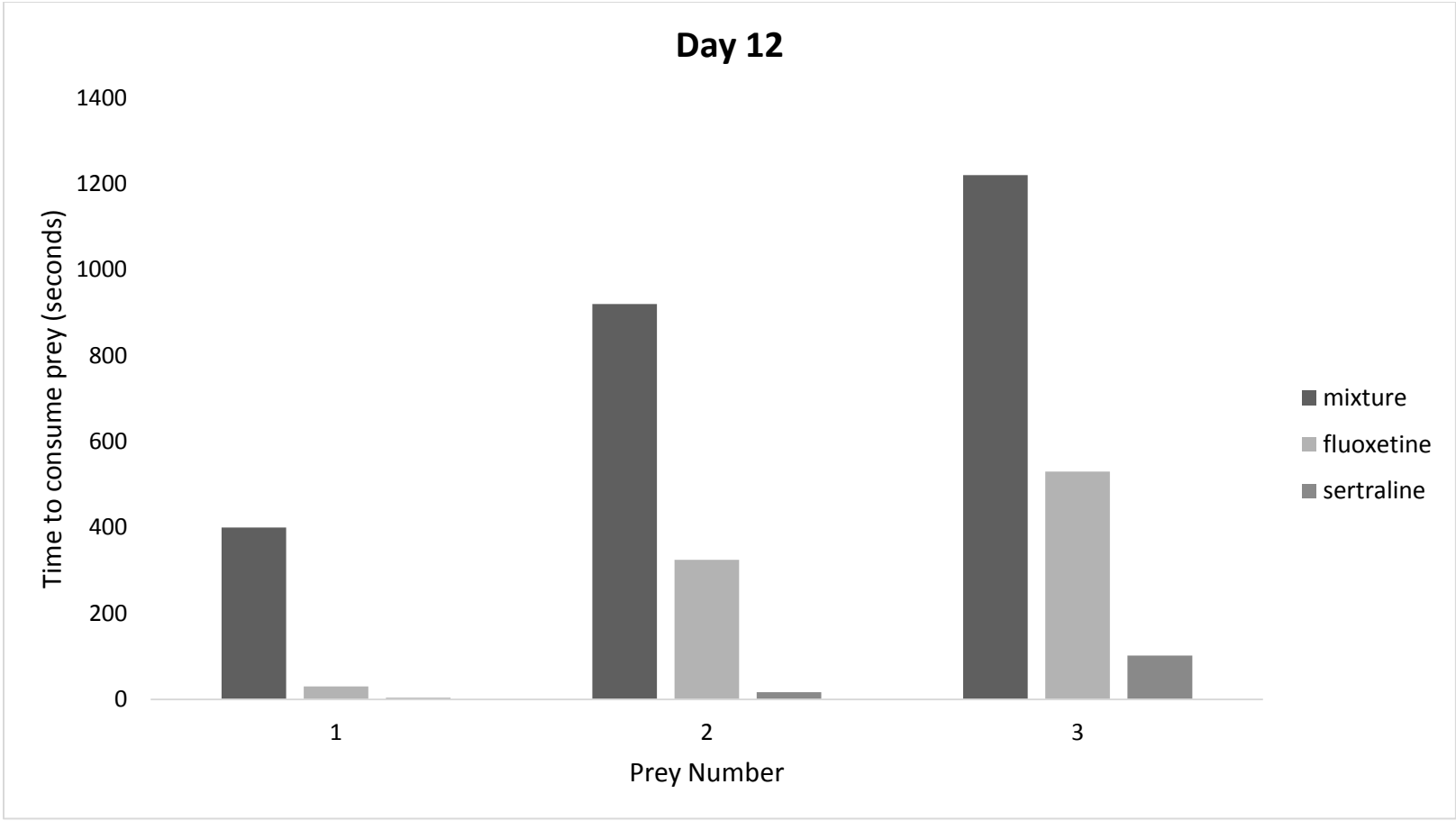

Figure 4.4: Comparing time to capture prey 1, 2, and 3 on day 12 after exposure to SSRIs individually or in a mixture. The mixture study took longer to capture prey compared to fluoxetine and sertraline studies. Fluoxetine took longer to capture prey compared to the sertraline study. Citalopram was not studied on day 12, so no data was available for comparison.

References

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