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IMPACTS OF PSYCHOTROPIC PHARMACEUTICALS ON HYBRID STRIPED BASS: ALTERED PREDATION BEHAVIOR AS A FUNCTION OF CHANGES IN BRAIN CHEMISTRY

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IMPACTS OF PSYCHOTROPIC PHARMACEUTICALS ON HYBRID STRIPED BASS: ALTERED PREDATION BEHAVIOR AS A FUNCTION OF CHANGES IN BRAIN CHEMISTRY

A Dissertation
Presented to
the Graduate School of
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In Partial Fulfillment
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Doctor of Philosophy
Environmental Toxicology

By
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December 2011

Accepted By
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ABSTRACT

Advances in analytical chemistry have led to the detection of low concentrations of pharmaceuticals and personal care products (PPCPs) in wastewater treatment plant effluents as well as their receiving waters. Antidepressants are routinely found among these contaminants, but have been shown to be relatively non-toxic at environmentally measured concentrations using traditional toxicity testing techniques. The neurochemical mode of action of antidepressants warrants investigation of the effects these chemicals may have on fish behavior due to the highly conserved nature of neurotransmitter transporter targets. Using a predator prey bioassay designed in our laboratory, previous studies has shown that the antidepressant fluoxetine (Prozac®) causes significant effects on hybrid striped bass ability to capture prey. Increased time to capture prey was also correlated with decreasing brain serotonin concentrations. The goal of my dissertation was to expand our knowledge on the effects of antidepressant contaminants on the predation behavior and brain chemistry of hybrid striped bass (bass). Bass were exposed to the antidepressant fluoxetine for 27 days to determine if exposure duration has an effect on the behavioral toxicity of this chemical. We found that fluoxetine was only toxic at the same threshold found in our previous six day exposure studies and that longer term exposures did not decrease effective concentrations. Bass were exposed to the antidepressant venlafaxine (Effexor®) for six days followed by a six day recovery period at concentrations of 50, 250, and 500 µg/L.
Concentrations of 250 and 500 µg/L had a significant effect on the time to capture prey 1-3 by day six while the 50 µg/L treatment only had a significant effect on bass ability to capture prey 3. Bass were able to recover their ability to capture prey 1 and 2 but time to capture prey 3 remained elevated for all treatments after six days of depuration. Venlafaxine had a significant effect on brain serotonin concentrations, which decreased in a dose dependent manner on day three and reached a basal level for all treatments on day six. Results indicated that venlafaxine may affect appetite at low concentrations and locomotor activity at high concentrations. Using the concentrations that caused a 15% decrease in brain serotonin concentrations from our previous studies with individual exposures to fluoxetine and venlafaxine, a simple mixture exposure was performed with 1 toxic unit representing 15 µg/L of fluoxetine and 25 µg/L of venlafaxine. Concentrations of 1, 2, and 4 toxic units caused a significant increase in the time to capture prey 2 and 3 by day six. Brain serotonin concentrations reached a basal level for all mixture treatments by day three and remained decreased through day six. There was a strong exponential correlation between brain serotonin concentrations and time to capture prey 1 and 2 on day six. When comparing mixture results to the results of individual antidepressant exposures, the data indicated low concentration mixtures may act in an additive manner causing increased time to capture prey and decreased brain serotonin concentrations at half the respective concentrations of each of the individual compounds. But this effect did not increase for the higher mixture exposure
concentrations. Overall, the results of my dissertation indicate that antidepressants, even when present in low concentration mixtures, may present a significant risk aquatic organism behavior. Because effects on behavior can impact the fitness of an organism both directly and indirectly, it has implications for the population level. Though behavior is not typically used as an endpoint for risk assessment of aquatic contaminants, the behavioral mode of action of antidepressants and results of this study may warrant their inclusion.
DEDICATION

I dedicate this work to my parents, Joseph Hopkin Bisesi, Sr and Judith Ann Bisesi. I could not have come this far without the guidance, opportunities, and love they have given me throughout the years.
ACKNOWLEDGMENTS

I would like to thank my Ph.D. advisor, Dr. Stephen J. Klaine for giving me the opportunity to pursue a degree and conduct research in his laboratory. Under his direction I have gained a tremendous amount of knowledge that will be applied to career in environmental toxicology. I am forever grateful for my experiences and wouldn’t have changed them for anything. I would also like to thank the other members of my committee Dr. Cindy Lee, Dr. Heiko Schoenfuss, Dr. William Baldwin and Dr. Thomas Schwedler for their patience and guidance throughout my graduate career.

My PhD research would not have been completed without the assistance of Ron Gossett and Norm Ellis. Ron kept the aquatic research laboratory at the cherry farm up and running to ensure I always had a working facility for my fish behavior research. This involved a constant battle with equipment and weather that often required Ron to go out of his way, working nights and weekends, to ensure things ran smoothly. Norm rebuilt the HPLC that I used for my brain analyses and provided his knowledge of analytical techniques whenever I asked. Ron and Norm are truly selfless people whose value cannot be overstated and I cannot thank them enough.

Dr. Peter van den Hurk never hesitated to allow me to use any piece of his laboratory equipment. Without his equipment I would not have been able to measure my antidepressants, or prepare my brain samples.
The rest of the faculty of the environmental toxicology program as well as the staff of the department of biological sciences and the institute of environmental toxicology at Clemson University all deserve special thanks for their assistance with my research, teaching of coursework, and help with administrative issues.

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Sarah Robinson spent countless hours including evenings and weekends helping me in the lab. She also provided love and support throughout my PhD and I don’t know if I could have done it without her.

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My mother, Judy, father, Joe, and three sisters, Melanie, Hunter, and Aubree have all stood behind me while I pursued my degrees and I cannot thank them enough for believing in me. They have given me everything they could without hesitation and I hope that one day I can return the favor.

Finally, I would like to thank my good friends Alberto Bustamante and Brandon Sprague for providing with me with some of the best memories I have during my time at Clemson. Good friends are invaluable and I have two of the best.
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CHAPTER ONE: LITERATURE REVIEW

Pharmaceuticals and Personal Care Products in the Environment

In the past fifteen years advances in analytical chemistry have allowed for detection of low concentrations of contaminants in the environment. Among the contaminants regularly found in aquatic ecosystems are pharmaceuticals and personal care products (PPCPs). Both over the counter and prescription pharmaceuticals from many different classes, including analgesics, antidepressants, hormones, and antibiotics have been found in wastewater treatment plant (WWTP) effluent and their respective receiving waters [1]. In addition to pharmaceuticals, detected compounds include those typically found in products that are used on a daily basis for personal care. These include antimicrobials found in hand soap, such as triclosan, and fragrances used in perfumes, colognes, and soaps. Concentrations of PPCP’s typically found in WWTP effluent and receiving water vary by compound but can range from the low nanogram per liter (ng/L) to the low microgram per liter (µg/L) level [1-6]. In addition to PPCP’s some researchers have found detectable concentrations of illicit drugs in wastewater effluents that can be indicative of drug usage trends in large cities [7-10].

Sources of PPCPs in the Environment

The source of PPCPs in the environment can be traced to identifiable inputs such as WWTP effluent (point sources) or can be from unknown origins such as agricultural runoff (non-point source). Perhaps the largest input is
through regular use in humans. Studies have shown that up to 90% of some pharmaceuticals taken into the body do not reach their target sites and are excreted in the parent form [11]. Other pharmaceuticals can be metabolized in the body and excreted into our sewage systems as both active and inactive conjugates [12]. In addition to direct human use, unused PPCPs are often disposed of in toilets adding a significant quantity of these compounds to our sewage [13]. Once they reach WWTPs, conjugated pharmaceuticals can be de-conjugated by microorganisms during treatment resulting in parent compounds in final treated wastewater effluents [14]. Whether metabolized or un-metabolized, used or un-used, human pharmaceutical use introduces a complex mixture of PPCP’s into waste streams that ultimately end up in our wastewater treatment plants (WWTP’s).

However, most modern WWTP’s are not equipped to remove all pharmaceuticals found in waste water. Some PPCPs are resistant to biodegradation in typical activated sludge sewage treatment plants [15]. Partitioning to biosolids during this process can give the impression of “removal” from final treated effluent, but since they are not degraded they may contribute to non-point source contamination through runoff when WWTP biosolids are recycled as agricultural fertilizers [16]. A number of tertiary treatments have shown significant increases in PPCP removal efficiency, but the cost of such systems has hindered their widespread implementation [17]. Therefore, PPCP’s
have the potential to make their way into the environment through WWTP effluent [18].

In addition to WWTP effluent as a point source of PPCP’s, non-point sources may play a significant role in the introduction of PPCP’s in the environment. Leachate from PPCP’s disposed of in landfills has been shown to be a non-point source for contamination of groundwater and receiving streams [19]. Application of biosolids from WWTP’s to fields as fertilizers can act as a source for PPCP runoff into streams [16]. Veterinary pharmaceuticals applied dermally can be washed off in rain events and flushed into receiving streams. Antibiotics and hormones that are often added to feed or injected into livestock can be excreted in their waste, which can eventually wash into receiving streams. Finally, direct application of pharmaceuticals into aquaculture water provides an additional route for non-point source pollution [20].

**Antidepressants in the Environment**

Among the most commonly detected classes of PPCPs are the antidepressants. Some of the antidepressants detected in environmental matrices include fluoxetine (Prozac®), venlafaxine (Effexor®), bupropion (Wellbutrin®), sertraline (Zoloft®), paroxetine (Paxil®), citalopram (Celexa®), fluvoxamine (Luvox®) and duloxetine (Cymbalta®). Concentrations of these compounds found in aquatic matrices are typically found at ng/L concentrations with some reports of µg/L levels [1, 2, 6].
The relatively short half-life of most antidepressants, usually on the order of days [21], may decrease concern for their acute and chronic effects in the environment. However, researchers have suggested that the constant addition of new chemical through WWTP effluent provides a consistent exposure sometimes referred to as “pseudo-persistent exposure”. Antidepressants are one of the most commonly prescribed pharmaceutical classes [22].

Of particular interest are the antidepressants fluoxetine and venlafaxine (physical/chemical properties in table 1.1, structures figure 1.1). Since the widespread detection of pharmaceuticals began in the late 90’s, fluoxetine has been among the most ubiquitous PPCPs found in environmental matrices. Some concentrations that have been measured include 5.5 ng/L in a Las Vegas, NV WWTP effluent [23], 12 ng/L in an unspecified US stream [1], and 18.7 ng/L at a sewage treatment plant outfall in Oslo, Norway [6]. The highest reported value in the literature was found in the Little River, Ontario, Canada, at 99 ng/L [4].

Venlafaxine has not been examined as often as fluoxetine but has been found at much higher concentrations. Some concentrations reported in the literature include 672 ng/L downstream of a WWTP in Boulder Creek, CO, and 690 ng/L downstream of a WWTP in Four Mile Creek, IA [24]. Venlafaxine has been detected in metropolitan rivers of Madrid, Spain, at median concentrations of 57 ng/L and 300 ng/L in WWTP effluent [25, 26]. A study reporting contributions of antidepressants in WWTP effluent to receiving streams reported venlafaxine concentrations as high as 808 ng/L in treated effluent and 901 ng/L
downstream of the WWTP in the Grand River, Ontario, Canada [27]. The highest reported values in the literature were found in wastewater effluent with concentrations >2 µg/L [2]. Unpublished data have shown concentrations an order of magnitude higher than values reported in the literature (Dr. Melissa M. Schultz, College of Wooster, Personal Correspondence, 2010).

**Examining the Toxicity of Antidepressants Using Traditional Methods**

**Acute Toxicity**

Risk assessment of any chemical must begin with basic toxicity testing to find the range in which a chemical is toxic. Acute mortality testing is often the first step in this process [28]. Therefore, many researchers have examined the acute toxicity of antidepressants to aquatic animals.

Antidepressants such as fluoxetine, sertraline, and their metabolites have been shown to accumulate in the muscle, liver and brain tissue of *Ictalurus punctatus* (channel catfish), *Pomoxis nigromaculatus* (black crappie), and *Lepomis macrochirus* (bluegill) [29]. A number of antidepressants including fluoxetine, sertraline, paroxetine, bupropion, and citalopram, were measured in the brains of white suckers (*Catostomus commersonii*) in streams with measureable concentrations of antidepressants [24].

Of the antidepressants, fluoxetine (Prozac®) has been the most studied as it is a commonly prescribed antidepressant that has been consistently detected in the aquatic environment. The 48 hour acute toxicity of fluoxetine in three commonly used toxicity test species (*Pimephales promelas, Ceriodaphnia dubia,*)
*Daphnia magna* was similar with LC$_{50}$ values (median lethal concentration) between 234 and 820 µg/L [30]. The 7 day LC$_{50}$ for the western mosquito fish, *G. affinis*, was 546 µg/L [31].

Studies examining the fate of antidepressants in the environment show that the lipophilic nature of some of these chemicals causes them to partition to sediment [22, 32]. As a result researchers have examined the toxicity of fluoxetine to sediment dwelling organisms. Fluoxetine was shown to have much higher LC$_{50}$ values (15.2 mg/kg) for *C. tentans*, an emergent insect, than waterborne organisms. The other sediment dwelling organism tested (*H. azteca*) did not show a toxic response at the highest levels tested (43 mg/kg) [33]. Overall it appears that sediment dwelling organisms may be more resistant to acute toxicity of pharmaceuticals. This could also be due to increased binding of fluoxetine to organic carbon in the sediment, decreasing bioavailability.

The acute toxicity of venlafaxine has not been examined in the literature using traditional toxicity testing methods, however 21 day exposures of adult male fathead minnows to venlafaxine at environmentally relevant concentrations (305 and 1104 ng/L) caused ~40% mortality[34].

**Chronic Toxicity**

Though acute effects of antidepressants have been shown for both waterborne and sediment dwelling organisms, the concentrations used in these studies are orders of magnitude higher than those measured in WWTP effluent and their receiving waters. As a result of the low concentrations and long
exposure periods likely in the environment, researchers have also examined the chronic effects of these chemicals on aquatic organisms.

Fluoxetine did not cause any decreases in egg production, hatching success, or rate of fertilization at the highest concentration tested (5 µg/L) in Japanese medaka, *Oryzias latipes* [35]. In the presence of low concentrations of fluoxetine (71 µg/L) the western mosquito fish showed delayed development of sexual morphology [31]. Sertraline, an antidepressant with a similar mode of action to fluoxetine, caused decreased fecundity in *C. dubia* at 45 µg/L [36].

Chronic studies have also been performed for sediment dwelling organisms. Twenty eight day fluoxetine exposure at 0.94 mg/kg caused increased reproduction in the oligochaete, *Lumbriculus variegatus*, but 56 day fluoxetine exposures at 0.81 mg/kg caused decreases in reproduction in the mud snail, *Potamopyrgus antipodarum* [32].

With the exception of a single study [34], the overall consensus of the literature is that antidepressants do not cause acute and chronic effects at environmentally relevant concentrations. Antidepressants are designed to alter behavior in humans through modulation of brain neurotransmitters [37]. The same neurotransmitters and biochemical pathways also exist in fish, therefore examination of the behavioral effects of antidepressants in fish warrants investigation [38].
**Antidepressant Mode of Action**

Environmental contamination with antidepressants causes reason for concern due to the modes of action of the two antidepressants mentioned above, fluoxetine and venlafaxine. The most commonly prescribed antidepressants are designed to alter behavior in humans through blocking the reuptake of serotonin, norepinephrine, and dopamine (structures in figure 1.2), monoamines thought to be responsible for modulating behavior. There are many classes of these compounds including the selective serotonin reuptake inhibitors (SSRIs) which include fluoxetine (Prozac®), and sertraline (Zoloft®), the serotonin and norepinephrine reuptake inhibitors (SNRIs) which include venlafaxine (Effexor®), and the dopamine and norepinephrine reuptake inhibitors (DNRIs) which include bupropion (Wellbutrin®) [2]. The two chemicals that were focused on in the current study, fluoxetine and venlafaxine, work through blocking the reuptake of serotonin (SSRI) and both serotonin and norepinephrine (SNRI), respectively.

Neurotransmitters are responsible for transmitting electrochemical signals between neurons throughout the body. Monoamines such as serotonin, norepinephrine, and dopamine are neurotransmitters that are released from synapses of the axon terminal of neurons. These neurotransmitters are released into the synaptic cleft between the axon of the upstream neuron and the dendrite of downstream neuron where they bind to the receptors. Activation of receptors on post-synaptic neurons opens ligand gated ion channels, which regulate the action potential, and therefore, the signal transmission in neurons. Once signals
have been transmitted, neurotransmitters release from dendritic receptors and are either broken down by monoamine oxidases or are recycled into the synapses of the upstream neuron. Recycling is accomplished through reuptake transporters in the cell membrane of the upstream neuron [39].

Fluoxetine is known as a selective serotonin reuptake inhibitor (SSRI). It functions by blocking the serotonin reuptake transporter (5-HTT or SERT) on the synapse of the upstream neuron [37, 38]. This leaves serotonin in the synaptic cleft for longer, resulting in continued signal transduction. It is thought that decreased levels of serotonin, as well as other neurotransmitters, released during neuronal signal transduction is the leading cause of many depressive disorders. Administration of SSRI antidepressants allows for prolonged signal transduction, which is thought to decrease symptoms of many depressive disorders [37].

Because the role of individual neurotransmitters in psychological disorders is not completely understood, some researchers believe that increasing signal transduction through multiple neurotransmitters may increase the effectiveness of antidepressants. Venlafaxine is known as a serotonin and norepinephrine reuptake inhibitor (SNRI). It simultaneously blocks the reuptake transporters of both serotonin and norepinephrine. It is also thought to mildly inhibit the reuptake of dopamine [40].

It is important to note that upon initial administration of SSRI and SNRI antidepressants brain monoamine concentrations can actually decrease.
Mammalian literature suggests that when SSRI and SNRI antidepressants are bound to monoamine reuptake transporters, un-recycled monoamines can activate negative feedback loops that are responsible for maintenance of neuronal monoamine concentrations. This is accomplished through binding of autoreceptors on the axon terminal of the pre-synaptic neuron. Binding of these autoreceptors inhibits additional release of monoamines into the synaptic cleft. This feedback inhibition mechanism is thought to initially decrease monoamine release in turn decreasing overall monoamine concentrations. Over time the autoreceptors become desensitized and allow further release of monoamines, permitting the therapeutic function of the chemical. This process can take 2-4 weeks to run its course [41].

**The Role of Neurotransmitters in Behavior**

**Monoamines in Humans**

It has long been understood that neurotransmitters, especially monoamines, play a major role in modulation of behavior, as well as many physiological functions in humans. Serotonin is thought to play a role in mood, behavior, appetite, and cerebral circulation. Dopamine has been implicated in control of motor function as well as emotional reward. Norepinephrine is thought to be involved in behavioral arousal, as well as playing a major role in fight or flight response. While individual monoamines have been implicated in many behaviors and physiological processes, it is more likely that multiple neurotransmitters interact to control behavior in humans [42].
Monoamines in Fish

Monoamine signal transduction is thought to be highly conserved across kingdoms and phyla, including fish. Serotonin (5-hydroxytryptamine or 5-HT) has been implicated in partially or fully controlling a number of physiological functions in fish. These include respiration, heart rate, stress response, immune response, reproduction and behavior [38]. The relationship between 5-HT and behavior has been examined extensively.

Behaviors thought to be controlled by 5-HT include locomotion, aggression, feeding, migration, and social hierarchies. The general consensus is that an increase in brain serotonin levels causes a decrease in overall activity. Increased serotonin levels have been correlated with decreased spontaneous locomotor activity [43]. Intracerebroventricular injections of 5-HT and 5-HT receptor agonists have resulted in decreased aggression and feeding [44, 45]. Differences in brain 5-HT over the lifespan of fish have been hypothesized to influence migration and smolt transformation [46]. Finally, increased 5-HT levels post aggressive encounter have been implicated in maintaining subordinate behavior in the loser of the encounter. 5-HT levels dropped immediately in fish that won an aggressive encounter, which may help them maintain their dominance [47].

Though there seems to be a general trend toward increasing 5-HT causing decreased aggression, feeding, and dominance, a few studies have found the opposite effect. Decreased 5-HT levels resulting from exposure to
contaminants have caused increased time to capture prey and decreased appetite in fish [48, 49].

While measurement of 5-HT levels has been used for correlating behavior and brain biochemistry, some have suggested that ratios of 5-hydroxyindoleacetic acid/5-hydroxytryptamine (5-HIAA/5-HT) may be better predictors of behavior. 5-HIAA is a metabolite of 5-HT and the ratio of 5-HIAA/5-HT can give an indication of how serotonin levels are changing in the brain. 5-HIAA/5-HT ratios have been shown to increase during food deprivation, and in subordinate fish when encountering dominant fish [49-51].

Interaction between 5-HT and other neurotransmitters also presents interesting questions. Corticotropin releasing factor (CRF) has been shown to partially or fully reverse the inhibitory effects of serotonin on feeding behavior [45]. Gamma-aminobutyric acid (GABA) and dopamine may also have interactions with serotonin [52].

Norepinephrine and epinephrine have been implicated in many of the same physiological functions as serotonin. These functions include, stress response [53], feeding [54], locomotor activity [55], social hierarchies [56], and migration [57]. But the body of literature is much smaller for norepinephrine and epinephrine, which may be because the release of these neurotransmitters is often controlled by serotonin [38]. Therefore the study of serotonin may be more important. It may also be due to the difficulty in measurement of these neurotransmitters.
The literature suggests that dopamine acts in the opposite manner of serotonin, with increasing dopamine causing increased aggression, locomotor activity, and social dominance. The addition of dopamine agonists to water with tilapia increases locomotor activity while the addition of dopamine antagonists decreases locomotor activity [58]. Dopamine is also thought to play a role in cognitive learning in fish. Zebrafish exposed to nicotine, a known dopamine agonist, showed increased choice accuracy performance after a training period. Increased accuracy was also accompanied by increased levels of DOPAC (dihydroxyphenylacetic acid), the primary metabolite of dopamine, indicating increases in dopamine levels [59].

Research has shown that dopamine plays a major role in aggression and social dominance. It may act in a bimodal fashion in the brain with different regions showing changing patterns of dopaminergic activity under different scenarios. Following aggressive social interactions dopamine was shown to decrease in the hippocampus of rainbow trout while increasing in the hypothalamus and subpallium [60]. Artic charr injected with L-dopa (precursor to dopamine) have shown dominance over untreated males when grouped together which was correlated with a dose dependent increase in both dopamine and DOPAC in fish brains [61]. Researchers have detected increased levels of homovanillic acid, another dopamine metabolite, in dominant artic charr when compared to their subordinate counterparts [62]. The general consensus seems
to be that dopamine appears to “act in a stimulatory manner on aggressive or competitive behavior in fish [43].”

It is important to note that while the body of literature for monoamines and their effects on behavior is rather large, much of it is highly speculative. On one hand there are many studies in which monoamine concentrations are measured and correlated with changes in behavior. But the cause of this change in monoamine levels is not always clear. For example, in the environmental toxicology literature, contaminants such as PCB’s, lead and mercury have been implicated in changing serotonin levels, and behavior [63, 64]. But the multiple modes of action of these environmental contaminants complicate causal determination between alteration in 5-HT concentrations and behavioral changes. On the other hand there are studies that expose fish to monoamine modulators (parent monoamines, antidepressants, receptor agonist/antagonists), but do not measure the resulting 5-HT changes [45, 65]. It is assumed that the modulators will work as intended to, but a few studies have shown that may not be the case [44, 48]. Therefore, it is important to ensure that the modulator used is specific enough to ensure it is causing the behavioral effect. This must be confirmed by measuring levels of the modulators target molecule to ensure it is working as expected.

**Ecological Relevance of Behavior**

An organism’s ecological fitness can be considered its ability to successfully reproduce and contribute its genetic traits to the next generation
The importance of behavior in ecological fitness can be illustrated using the notion of scope for growth.

Scope for growth is a concept that suggests that organisms have a finite amount of energy that can be expended on biological processes required for survival. These can include basic physiological processes such as breathing, and digestion as well as outward functions required for energy collection, such as feeding. Excess energy should theoretically be funneled into processes that may not be required for individual survival but are necessary for survival of a species. These include growth and reproduction. Basic behaviors such as feeding and locomotion are necessary to provide sufficient energy for individual survival in fish. Some species of fish also have complex reproductive behaviors that are performed during mate attraction. Therefore, contaminants that affect behavior can directly decrease an organism’s scope for growth through decreasing energy uptake, as well as indirectly, by affecting behaviors that may be necessary for reproduction. Reproductive behavioral effects may be sex specific as male fish allocate more of their excess energy into reproductive behaviors and secondary sex characteristics while females divert excess energy into egg production [67]. Either route can affect an organism’s contribution to the next generation, which can ultimately cause effects to the population as a whole [68-70].

**Behavioral Endpoints in Toxicity Testing**

Concentrations of antidepressants required to cause acute and chronic effects (especially mortality) in aquatic organisms are typically much higher than
concentrations found in environmental matrices [1, 23]. Therefore, researchers have begun using non-traditional endpoints that may be more sensitive indicators of antidepressant toxicity. Behavioral endpoints are among the tools being utilized to examine these trace contaminants [48, 71, 72].

Animal behavior has been studied for many years but is underutilized in toxicity testing. Behavioral testing can be problematic because animal behavior is often relatively complex, exhibits high variability, and testing behavior requires more time than traditional acute and chronic toxicity testing. But when designed and performed correctly, behavioral endpoints can be more sensitive than traditional endpoints and can be extrapolated to the population level [73].

The use of behavioral assays in toxicity testing began during the early 80s with studies using behavior to assess the chronic toxicity of chemicals. A study examining the use of behavioral endpoints as indicators of mode of action suggested a number of endpoints that could be used in toxicity testing included schooling behavior, swimming performance, and reproductive behavior [74]. Their analysis indicated that schooling behavior seems to be the most sensitive indicator of chronic toxicity. The use of swimming capacity and swimming activity as a measurement of sublethal toxicity in fishes has been examined. Swimming capacity is a measure of a fish’s orientation and ability to swim against flow. A number of variables can be measured in an attempt to quantitate swimming activity including of the frequency and duration of movements, speed and distance traveled during movements, frequency and angles of turns, position in
the water column and form and pattern of swimming. It was found that swimming capacity is very inconsistent and often coincides with mortality. Swimming activity on the other hand is much more consistent in identifying chronic toxicity, and is also more sensitive to lower concentrations of toxicants [75]. Pesticides have been shown to increase optomotor response (movement towards reference points within a fish’s field of vision) as a result of an artificial cue. But pesticide exposure also decreased swimming capacity [76]. Because swimming and schooling behaviors can be variable and hard to interpret, the use of high speed video and tracking software has been suggested as methods for standardization of these behaviors [73].

Foraging behavior has also been suggested as a possible indicator of chronic toxicity. Studies have focused on the ability of prey to avoid capture or the reduction of feeding success either by loss of appetite or predation ability. There do seem to be apparent problems with using these behaviors to quantify chronic toxicity as they often involve qualitative observations that are not easy to quantify [77]. The use of high speed video has allowed for quantifiable changes in predator avoidance to be recorded. Exposure to estrone as embryos can cause decreases in startle response in fathead minnows. Exposure to 17β-estradiol for 12 days post hatch also adversely effected fish startle responses [78]. It has been suggested that using time to eat as well multiple foraging models can be used to quantify changes in predation behavior as an indicator of sublethal toxicity [48, 77, 79].
Reproductive behavior has also been used as a behavioral endpoint in toxicity testing. The courtship and reproductive behaviors of many fish species including the three spine stickleback, fathead minnow, mollies, and guppies, have been described in the literature. Deviations from known behaviors have been suggested as a potential sublethal toxicity endpoint [80]. Male fathead minnow reproductive behavior is a popular endpoint in behavioral toxicity testing. Larval exposures to lead caused significant decreases in nest guarding and maintenance ability once the fish reach sexual maturity [81]. Exposure to ethinylestradiol has been shown to cause decreased aggression and ultimately subordination to unexposed male fathead minnows [82]. Exposure to nonylphenol can either increase or decrease male fathead minnow nest guarding aggression depending on dose [83].

**Antidepressant Behavioral Toxicity Testing**

Antidepressants were designed to alter behavior in humans through modulation of neurotransmitters in the brain. It has been shown that the same neurotransmitters exist in fish and also influence behavior in fish [43, 44, 57, 84, 85]. Therefore, it is reasonable to believe that low level exposure to antidepressants could also cause behavioral alterations in fish.

Though the effect of antidepressants on fish behavior has been relatively understudied, some aspects have been examined. Fathead minnow embryos and larvae exposed to the SNRI venlafaxine showed increased startle response latency periods. Embryonic fathead minnows exposed to environmentally
relevant concentrations of the SSRI fluoxetine exhibited decreased escape velocity after a stimulus, while larval exposures to bupropion, a DNRI, also decreased escape velocity. Exposures to environmentally relevant doses of fluoxetine and venlafaxine caused reduced total escape response, a combination of latency and escape velocity. Finally, exposure of fathead minnow embryos and larvae to mixtures of fluoxetine, venlafaxine, sertraline, and bupropion caused decreased escape velocity as well as reduced total escape response in exposed larvae [72].

Adult male fathead minnows exposed to environmentally relevant concentrations of the antidepressants sertraline, fluoxetine, bupropion, and venlafaxine did not exhibit changes in nest guarding behavior when challenged with an unexposed male. Mixtures of these compounds also exhibited no effect [34].

Gulf toadfish, *Opsanus beta*, injected with fluoxetine showed increased plasma serotonin levels which coincided with increased aggressive behaviors from dominant individual fish when interacting with other individuals [86]. *Gammarus pulex* exposed to environmentally relevant concentrations of fluoxetine (10-100 ng/L) exhibited decreased activity when compared to controls. No effects were seen at levels in the part per billion range [71].

A predator prey bioassay that was previously designed in our lab has been used to determine the effect of antidepressants on fish brain biochemistry and predatory behavior. The predator (hybrid striped bass) was challenged with
a contaminant over a period of time. Periodically (every three days) during the challenge period the predator was given the opportunity to feed on minnows. The amount of time to capture each minnow was quantified and compared among treatments to determine if the contaminant had a significant effect on predation behavior [48, 79].

When bass were exposed to fluoxetine (Prozac), a SSRI, for six days, they exhibited increased time to capture prey. This increase was correlated with decreased brain serotonin levels. However it is important to note that concentrations necessary to cause this effect (~35 µg/L) were 20 times less than reported 48hrLC\textsubscript{50} values but three orders of magnitude higher than measured environmental concentrations [48]. Bass were able to recover, and resume normal feeding behavior after a six day depuration period [48]. Longer exposures (27 days) of bass to fluoxetine at environmentally relevant concentrations of 0.1 µg/L to 10 µg/L caused no change in predation behavior or brain chemistry [87].

**Dissertation Goals**

Building from previous work completed in our laboratory using the predatory bioassay described above, the goal of this dissertation was two-fold. First, to increase understanding of the roles neurotransmitters play in controlling behavior in fish. By using antidepressants as neurotransmitter modulators I hoped to correlate feeding behavior with changes in brain chemistry. Second, to determine if antidepressants with different modes of action found in aquatic
environments can cause effects on fish predation behavior and brain chemistry, whether exposed individually, or in mixtures. I hypothesized that exposure to fluoxetine, venlafaxine, and mixtures of the two will decrease brain neurotransmitters which will result in increased time to capture prey. I tested this hypothesis through the following objectives:

1. Determine the threshold of effect in hybrid striped bass predation behavior during long term fluoxetine exposure.

2. Determine the effects of the SNRI antidepressant venlafaxine on the brain biochemistry and predation behavior of hybrid striped bass

3. Determine the effects of mixtures of fluoxetine and venlafaxine on brain biochemistry and predation behavior in hybrid striped bass.
Fluoxetine                                       Venlafaxine

Figure 1.1: Structures of the antidepressants fluoxetine and venlafaxine
Figure 1.2 Structures of the neurotransmitters serotonin, norepinephrine, and dopamine
### Table 1.1: Physical-chemical properties of fluoxetine and venlafaxine

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight (g/mol)</th>
<th>Empirical formula</th>
<th>log $K_{ow}$</th>
<th>Solubility in water (mg/L)</th>
<th>Vapor pressure (mm Hg)</th>
<th>Henry's law constant (atm m$^3$/mol)</th>
<th>pKa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluoxetine</td>
<td>309.33[88]</td>
<td>$C_{17}H_{18}F_3NO$[88]</td>
<td>4.05[32]</td>
<td>38[32]</td>
<td>$8.9 \times 10^{-7}$[32]</td>
<td>$8.9 \times 10^{-8}$[89]</td>
<td>10.06[30]</td>
</tr>
<tr>
<td>Venlafaxine</td>
<td>277.4[88]</td>
<td>$C_{17}H_{27}NO_2$[88]</td>
<td>3.28[90]</td>
<td>270[90]</td>
<td>$4.92 \times 10^{-7}$[91]</td>
<td>$2.04 \times 10^{-11}$[90]</td>
<td>9.4[92]</td>
</tr>
</tbody>
</table>

Note: Brackets accompanying values are references for each number.
References


CHAPTER TWO:

THRESHOLD OF EFFECT FOR 27 DAY FLUOXETINE EXPOSURE ON HYBRID STRIPED BASS PREDATION BEHAVIOR

Introduction

In recent years fluoxetine, an antidepressant marketed under the trade name of fluoxetine, has become an environmental concern due to its detection in aquatic matrices [1, 2]. Concentrations as high as 99 ng/L have been detected in wastewater effluent [3].

Traditional acute and chronic toxicity tests have shown that exposure to fluoxetine at environmentally relevant concentrations does not result in mortality. The 48 hr. LC$_{50}$ for larval fathead minnows (*Pimephales promelas*) was 705 µg/L and 820 µg/L for *Daphnia magna* [4]. But the unique mode of action of fluoxetine suggests that traditional toxicity tests may not be the most appropriate method to measure toxicity.

Fluoxetine belongs to the selective serotonin reuptake inhibitor (SSRI) class of antidepressants, which works through inhibition of serotonin recycling during neuronal signal transmission [5, 6]. The serotonergic system is thought to be highly conserved across animal phyla and kingdoms, including fish [6]. Serotonin has been implicated in the control of numerous behaviors in fish including locomotion [7], aggression [8], feeding [9], migration [10], and social
hierarchies [11]. Modulation of serotonin by the SSRI antidepressant fluoxetine may impact a number of behaviors but examination of behavioral effects in fish is fairly limited.

Fluoxetine has been implicated in reduced escape response, a measure of predator avoidance, in larval fathead minnows [12]. Gulf toadfish injected with fluoxetine exhibited increased aggression, which coincided with increased plasma serotonin levels [13]. Intraperitoneal injections of fluoxetine have been shown to cause decreased aggression in bluehead wrasse (Thalassoma bifasciatum) [14].

Using an assay designed in our laboratory, the effect of fluoxetine on fish predatory behavior has been examined. Hybrid striped bass exposed to fluoxetine for six days took longer to capture their prey when compared to controls. The increase in time to capture prey was correlated with decreased serotonin levels in the brains of exposed bass [15]. This result is contradictory to the expected effect of fluoxetine, which is designed to increase levels of serotonin in the brain.

In mammalian literature, it has been shown that initial administration of SSRI antidepressants can cause decreased brain serotonin levels. One possible explanation for this is that serotonin acts as its own feedback inhibitor. Autoreceptors (5-HT1A) on presynaptic neurons bind serotonin in the synaptic cleft. When binding sites are saturated, further release of serotonin ceases.
Normally, serotonin left in the synaptic cleft is either broken down or taken back into the pre-synaptic neuron to be re-used. But because re-uptake is blocked by the SSRI antidepressant, serotonin remains in the cleft where it can continue to bind autoreceptors and prevent the further release of serotonin. It is believed that over time these autoreceptors become desensitized to excess serotonin in the cleft and allow serotonin to once again be released. But this process can take 2 to 4 weeks to complete, which may explain the decreased serotonin levels after 6 day exposures [16].

To test if this phenomenon exists in fish, hybrid striped bass were exposed to fluoxetine for a period of 27 days [17]. Because the lowest concentration tested in the initial study (23 µg/L) [15] was approximately two orders of magnitude higher than concentrations measured in waste water treatment plant (WWTP) effluent [18], concentrations closer to the WWTP effluent studies were used. Results from the 27 day indicated that exposures to 0.1, 1, and 10 µg/L for 27 days did not have a significant effect on hybrid striped bass predatory behavior. There was also no effect on brain serotonin levels in the exposed bass [17].

Short term (six day) exposures to fluoxetine caused increased time to capture prey at concentrations as low as 35 µg/L [15] while 27 day exposures at concentrations up to 10 µg/L [17] did not cause any effects. The difference in behavioral toxicity between these studies raises questions about whether
increased exposure duration can increase the effective concentration of fluoxetine. Therefore, the first objective of my dissertation research was to determine what the threshold of effect was for long term fluoxetine exposure. I hypothesized that 27 day exposures to fluoxetine (10-40 µg/L) will cause an initial dose dependent increase in time to capture prey. Though brain biochemistry was not measured in the current study, I hypothesized that as autoreceptors become desensitized to the presence of fluoxetine in synaptic clefts, I would see a recovery of ability to capture prey.

**Materials and Methods**

**Test Chemicals**

Fluoxetine hydrochloride was generously donated by Fermion (Finland). HPLC grade methanol, acetone, acetonitrile, glacial acetic acid, and triethylamine were purchased from Fisher Scientific (Pittsburgh, PA, USA). Trace metal grade concentrated hydrochloric acid was purchased from Spectrum Chemicals (Gardena, CA, USA). Water used for analytical procedures was ultra-purified using a Milli-Q Super-Q Filtration system (Millipore™, Billerica, MA, USA) with a measured resistivity of 18.2 MΩ·cm.

**Fish**

Hybrid striped bass (*Morone saxatilis x M. chrysops*) were generously donated from Southland Fisheries (Columbia, SC, USA) as fingerlings. Fish
were held in 450 L circular holding tanks in the cherry farm aquatic research lab at Clemson University (Clemson, SC, USA). Holding tanks were maintained as flow through systems, constantly supplied with fresh water from Lake Hartwell (Clemson, SC, USA). Before reaching holding systems, water was filtered through a gravel bed, and sterilized with UV radiation. Water temperature was maintained between 22 and 25°C using a mixture of ambient and heated (via inline heater) or chilled water (via inline chiller), depending on incoming water temperature. Water was constantly aerated with air stones and agitators (Boatcycle Inc., Henderson, TX, USA). During holding, bass were fed a commercial diet (Finfish Silver 4.5 mm slow sink) purchased from Zeigler Bros, Inc. (Gardners, PA, USA).

Fathead minnows were purchased from Anderson Minnow Farm (Lonoke, AR, USA). Minnows were held in 100 L holding tanks using the same water as described above. During holding times, minnows were fed a commercial diet (Tetramin® Tropical Flakes) purchased from Dr’s Foster and Smith, Inc. (Rhinelander WI, USA).

Bass Group Training

Because bass were grown on a pelleted feed, conditioning to live diet was required before the initiation of behavioral assays. Hybrid striped bass (mass: 174.54g ± 45.91, length: 212.97mm ± 20.68) were randomly removed from holding tanks and placed into a separate 300 L rectangular holding tank for group
training. Water conditions in the group holding tank were the same as holding tanks. Bass were starved for three days prior to the start of group training. On day zero of training, bass were fed four minnows/bass in the group training tank. Bass were fed an additional four minnows/bass on day three and day six.

**Experimental Design**

Hybrid striped bass were exposed to fluoxetine in a static exposure scenario with renewals on days 6, 12, 18, and 24. Exposures took place in 113 liter aquaria measuring 92.1 x 32.4 x 40 cm purchased from Deep Sea Aquatics (Garland, TX, USA). Volumes of 80 and 40 liters were measured and marked on each tank for measurement of exposure volume and half renewal volume, respectively. Each tank had a 1.9 cm PVC vertical standpipe drilled into the front panel for control of water volume when maintained as a flow through.

Water for exposure tanks was taken from Lake Hartwell (Clemson, SC, USA) (pH = 6.28 ± 0.17, hardness 24 mg/L as CaCO₃, alkalinity 10 mg/L as CaCO₃). Before entering the laboratory, water was filtered through a gravel bed and UV sterilized. Temperature was maintained via a mixing valve (M & M Control Services, Grayslake, IL, USA) combining ambient water with either chilled (via inline chiller) or heated (via inline water heater) water, depending on the temperature of lake water. Water was then passed through a multi-resin filtration system (Water and Power Technologies, Columbia, SC, USA) for
additional cleanup and dispensed into tanks. Each tank was constantly aerated via air stones and covered with two square grated covers.

Hybrid striped bass were removed from the group training tank and placed into individual aquaria (one bass/tank). Bass were allowed to acclimate to the aquaria for nine days. During the acclimation period, bass were fed four fathead minnows (~4 cm long) on days three and six. The time to capture each minnow was recorded for selection of fish for exposures.

Three days after the final individual training (day zero of exposure) the first feeding event was quantified before the addition of fluoxetine. Before each feeding event, half of the tank cover and the air stones were removed from each tank and fish were allowed five minutes to adjust. Four fathead minnows (~4 cm long) were dropped into tanks, and bass were allowed 25 minutes to consume all minnows. The time bass took to capture each minnow was recorded. Any minnows not consumed during the 25 minute feeding period were removed, and a time to capture of 1500 seconds (25 minutes) was assigned for each unconsumed minnow. Air stones and covers were replaced following the feeding event. Only bass that consumed at least three minnows during the day 0 feeding event, and exhibited comparable feeding during the previous individual training days were selected for inclusion in the test. Following the feeding event, incoming water was shut off and all tanks were set to the final test volume of 80 L. Tanks were then randomized, assigned treatments (one bass per replicate
tanks/five replicates per treatment), and spiked with appropriate volumes of fluoxetine to reach nominal concentrations. Concentrations tested were 0, 10, 20, 30, and 40 µg/L.

Additional feeding events took place on days 3, 6, 9, 12, 15, 18, 21, 24, and 27. Water quality (pH, D.O. temperature) was measured during each feeding event using a Fisher Scientific Accumet AP84 pH/dissolved oxygen meter (Fisher Scientific, Pittsburgh, PA, USA). During the exposure, five replicate bass were removed from each treatment after each feeding event and euthanized for collection of brain tissue. Because the number of exposure tanks was limited, four tests were conducted in series (27, 18, 12, and 6 days) to produce sufficient replicates for tissue collection.

*Fluoxetine Exposures*

Stock solutions of fluoxetine were prepared by dissolving fluoxetine HCl in methanol. Concentrations of stock solutions were selected to ensure <0.1 mg/L methanol in exposure tanks, the ASTM recommendation for methanol as a carrier solvent (ASTM E1241-92). Spiking solutions were prepared by adding equivalent volumes of fluoxetine stock to 1 liter of milli-Q water. Equivalent volumes of the spiking solutions were added to tanks to reach nominal concentrations. Total methanol concentrations for the highest treatment were calculated and control tanks were spiked with equivalent amounts to ensure there was no carrier solvent toxicity. Because of the low concentration of
fluoxetine in exposure tanks, spiking solutions were measured to confirm concentrations. One liter of spiking solution was sufficient for spiking tanks and extraction for measurement on HPLC with a fluorescence detector. Nominal concentrations selected for the test were 0, 10, 20, 30, and 40 µg/L. Stock and spiking solutions were prepared daily.

The half-life of fluoxetine was previously determined to be approximately three days in our system [17]. To maintain relatively constant concentrations throughout the exposure, tanks were re-spiked on days 3, 9, 15, and 21 with equivalent volumes of spiking solutions to reach nominal concentrations. To prevent buildup of nitrogenous waste products, tanks were renewed on days 6, 12, 18, and 24. Renewals were performed following the feeding event of the day by removing half the volume from the tank (~40L) and replacing with fresh water. Tanks were re-spiked with appropriate volumes of spiking solution taking into account the three day half-life and the volume removed from the tank.

**Fluoxetine Analysis**

The low concentrations of fluoxetine made it impractical to measure fluoxetine concentrations in individual exposure tanks. Therefore, concentrations of spiking solutions were measured to confirm concentrations. Measured volumes of spiking solutions were adjusted to a pH of approximately three with concentrated hydrochloric acid. Samples were extracted on 6 ml C-18 solid phase extraction cartridges with a bed weight of 500 mg (HyperSep C18 SPE
cartridge, Thermo Fisher Scientific, Pittsburgh, PA, USA). Prior to extraction, cartridges were equilibrated with 6 ml of acetone, 6 ml methanol, and 6 ml milli-q water. Samples were loaded onto the cartridges and allowed to dry under vacuum. Cartridges were stored at -20°C until elution and analysis.

Cartridges were eluted with methanol/1% acetic acid and stored in sample vials for HPLC analysis. The HPLC consisted of a Waters 1525 Breeze HPLC Pump with a Water 717 Plus auto sampler and a Waters 2475 multi wavelength fluorescence detector (Waters, Milford, MA, USA). The mobile phase consisted of 40 parts HPLC grade acetonitrile: 60 parts milli-Q water, pH adjusted to 3.0 with glacial acetic acid: 4 parts triethylamine. The mobile phase was filtered through a 0.2 µm nylon filter and degassed using a sonication bath. The flow rate was set at 1 ml/min and a 40 µl injection volume was used. A Varian Polaris C-18A reverse phase analytical column (250 mm long, 4.6 mm I.D.) was used to achieve chromatographic separation. The fluorescence detector was set at excitation wavelength 270nm and emission wavelength 300nm. Run times were approximately 6 minutes per sample.

Data Analysis

Time to capture prey was analyzed using Statistical Analysis Software 9.2 (SAS, Cary, NC, USA). A general linear means for mixture distributions (PROC GLIMMIX) procedure was used to perform a two factor ANOVA utilizing treatment and day as independent variables and tank as a random variable. The
time to capture prey data were non-normally distributed with non-homogenous variances. The GLIMMIX procedure uses an analysis matrix that accounts for non-normal distribution and non-homogenous variances in data points. Least squared means was used to perform multiple pairwise comparisons of data and differentiate statistical differences across and within treatment, day, and treatment by day interactions.

**Results**

*Water Quality Measurements*

Water quality measurements were averaged (mean ± standard deviation) for the entire test. pH, dissolved oxygen, and temperature were 6.16 ± 0.54, 8.18 mg/L ± 3.54, and 20.75°C ± 1.29, respectively.

*Fluoxetine Concentrations*

Measured fluoxetine spiking stock concentrations were used to calculate treatment concentrations. The average concentration (mean ± standard deviation) over the entire test for 10, 20, 30, and 40 µg/L treatments were 9.95 ± 1.50, 18.59 ± 3.51, 27.42 ± 3.19, and 41.17 ± 5.51 µg/L, respectively.

*Behavioral Assays*

Complete numerical results from behavioral assays are presented in Table A-1 of the appendix. The times for hybrid striped bass to capture their first prey throughout 27 day exposures to fluoxetine are presented in Figure 2.1. There
was a statistically significant increase (p<0.05) in the time to capture prey 1 for the bass exposed to 40 µg/L fluoxetine from day six through day 27 with the exception of day 21. There was a statistically significant increase in time to capture prey one for the 30 µg/L treatments on day six and day 12. There was a statistically significant increase in time to capture prey one for the 20 µg/L treatment on days 12, 24, and 27. Because the only treatment that showed a consistent significant increase in time to capture prey one was the 40 µg/L treatments, Figure 2.2 shows time to capture prey for the 0 and 40 µg/L treatments with ± 1 standard error.

The time for hybrid striped bass exposed to fluoxetine for 27 days to capture their second prey is presented in Figure 2.3. There was a statistically significant increase for the 40 µg/L treatments on days nine through 27 when compared to controls, with the exception of day 18. Because there were no other significant increases for any of the other treatments, the time to capture prey two for the controls and 40 µg/L treatments alone are presented in Figure 2.4 with ± 1 standard error.

The time for hybrid striped bass exposed to fluoxetine for 27 days to capture their third prey are presented in Figure 2.5. There was a statistically significant increase for the 40 µg/L treatment starting on day three through day 27 when compared to controls. The 30 µg/L treatment showed significant increases on days six and twelve. The 20 µg/L treatment showed significant
increases days 21 and 27. Once again, because the 40 µg/L treatment was the only concentration that showed consistent significant increases over controls, the time to capture prey three for controls and 40 µg/L treatments alone are presented in Figure 2.6 with ± 1 standard error.

The time to capture prey 4 data are not presented graphically as they were highly variable for both treatments and controls. Therefore, there were not statistically significant treatments when compared to controls.

In general, the data indicate that only the 40 µg/L treatment had a significant effect on hybrid striped bass predatory behavior. Though there were a few significant data points for the other treatments on various days for various prey, there was no trend for these treatments. Significance in these treatments could usually be attributed to high variability for that day that would usually return to control levels by the following observation period. The 40 µg/L treatments showed significant increases in time to capture prey starting on day three (prey three) and continuing to day 27 (prey 1, 2, and 3). For the 40 µg/L treatment, the time to capture prey one and two was not significant on day 21 and day 18, respectively. While these data points could indicate acclimation to fluoxetine, the data for the observations following these data points were significantly different, indicating that this is probably not the case. These data points are still elevated over the controls, and increased variability in either the controls or 40 µg/L treatments on these days probably led to their being not significant.
Qualitative Behavioral Observations

In addition to our quantitative behavioral measurements we also noted a number of qualitative behavioral changes in the 40 µg/L treatments. These observations were not compared between treatments as they were not scored quantitatively. Some of the observations included spitting out prey fish, trouble swallowing prey, maintaining a vertical position in the water column, erratic swimming, swimming with dorsal surface out of the water, and regurgitation of digested fish.

Discussion

Use of selective serotonin reuptake inhibitors, like fluoxetine, is increasing on a daily basis [19]. As a result, researchers have found detectable concentrations of these chemicals in the environment [1-3, 18, 20]. The pseudo-persistent nature of these chemicals in the environment warrants investigation of the risk they pose to aquatic organisms [21]. Though SSRIs do not appear to be acutely and chronically toxic at environmentally measured concentrations, traditional toxicity tests may not capture the subtle effects these chemicals may have on behavior [4, 22-24]. The mode of action of these chemicals may directly affect behavior through modulation of serotonin in fish [12]. Serotonin has previously been implicated in a number of behaviors in fish, and behavior plays an important role in the success or failure of populations of fish [8, 9, 11, 25].
A previous study [15] using the same assay as the current study showed that short term exposures to fluoxetine (six days) at concentrations of 23 µg/L caused increased time to capture prey in hybrid striped bass, a measure of predation behavior. Time to capture prey also increased in a dose dependent manner with increasing fluoxetine concentrations (51 and 100 µg/L). Gaworecki and Klaine 2008 [15] used concentrations that were two orders of magnitude higher than environmentally measured concentrations. Long term (27 day) exposure to fluoxetine at environmentally relevant concentrations (0.1-10 µg/L) did not have an effect on hybrid striped bass predation behavior [17].

The aim of the current study was to determine the threshold of effect for long term fluoxetine exposure. Though fluoxetine does not have an effect on predation behavior at environmentally relevant concentrations, it is important to know the threshold of effect for risk assessments.

Results from this study indicated that only the 40 µg/L (41.17 ± 5.51 µg/L measured) treatment caused consistently significant increases in time to capture prey in hybrid striped bass. While some of the other treatments caused significant increases on random days for random prey, there was no consistent trend for these treatments. Variability in response to antidepressants in mammals is well documented with differences in cellular receptor expression levels being hypothesized as the culprit [26]. Therefore, it is possible that at concentrations lower than 40 µg/L fluoxetine are unable to overcome the
individual variability of response in hybrid striped bass, which is why we saw various significant data points throughout exposures.

It is also possible that fluoxetine’s effect on appetite may have played a role in the inconsistent effect on time to capture prey for lower concentrations seen in this study. Examination of the time to capture prey one and three for the 30 µg/L treatment shows a significant increase when compared to controls on day six followed by a decrease back to control levels on day nine. A similar trend is seen on day 12 and 15. This could indicate that consumption of prey on day three in addition to fluoxetine’s effect on serotonin may have satiated the appetite of 30 µg/L treated fish. Therefore, there was a significant increase in the time capture prey on day six. Fish were hungry again by day nine but satiated on day 12 so time to capture prey was significantly increased when compared to controls. SSRI antidepressants like fluoxetine have been suggested as appetite suppressants in mammals [27]. Serotonin has also been shown to be involved in gut motility in teleost fishes [28]. Therefore, this sigmoidal response may be due to appetite modulation by fluoxetine through serotonin.

Time to capture prey four data were not presented graphically because there were no significant differences between treatments and controls for this prey. Consumption of a fourth prey was highly variable for all treatments and controls indicating that three prey was enough to satiate some bass during exposure periods which may be a limitation in our experimental design.
Results from the current study correlate well with 6 day exposure concentration data previously reported from our laboratory [15]. Similar nominal concentrations, 40 µg/L in the current study, 35 µg/L in the six day study, caused a comparable effect on time to capture prey in hybrid striped bass. The onset of effects was also similar, because effects on time to capture prey one was not seen until day six of exposure, while effects on second and third prey were seen as early as day three. This may indicate that fluoxetine may have an initial effect on appetite with longer term exposure having effects on motor function. Serotonin has been implicated in controlling appetite [9] and locomotion [7] in fish. In addition to quantitative behavioral measurements there were a number of qualitative behavior observations at our highest concentration tested that coincided with findings in our previous studies including maintaining a vertical position in the water column and swimming with dorsal surfaces out of the water [15]. Similar effects were noted in mosquitofish (Gambusia affinis) exposed to fluoxetine including changing position in the water column (associating closely with the surface), and swimming on their sides [22]. These qualitative behavioral observations are important because they are behaviors that could result in increased predation susceptibility in the environment, something that would not be captured in traditional mortality based toxicity assays.

The dose dependent manner by which fluoxetine caused increasing effects on hybrid striped bass feeding behavior in our previous study [15] led us to hypothesize that we may see a dose dependent response to fluoxetine
exposure from 10-40 µg/L. But the results show that only the 40 µg/L treatments caused a consistent increase in time to capture prey. This indicates that there is a threshold of effect around 35 – 40 µg/L fluoxetine on the predation behavior of hybrid striped bass regardless of exposure duration. A study examining the behavioral effects of diazinon, an organophosphorus pesticide that acts on the neurotransmitter acetylcholine, showed a significant increase in time to capture prey in hybrid striped bass at 64 µg/L and 101 µg/L. This increase corresponded with a threshold decrease in brain acetylcholine esterase activity. But there was not a dose dependent increase in time to capture prey in Gaworecki et al. 2009 [29] once the threshold was reached. This is unlike our short and long term fluoxetine results, which indicate that effects will continue to increase in a dose dependent manner once the effective threshold is reached [15].

While the current study cannot be considered a chronic test by traditional standards, it does constitute a longer term exposure than most studies conducted with fluoxetine. Studies that have examined the chronic effects of fluoxetine in fish have found that acute exposure to fluoxetine may be sufficient to determine the effective concentration. The acute toxicity of fluoxetine to the western mosquito fish was 546 µg/L. But chronic exposure of the same fish at lower concentrations 0.05-5 µg/L did not produce any quantifiable effects [22]. Japanese medaka exposed to fluoxetine for four weeks did not show any effects on egg production, hatching success, or rate of fertilization at the highest concentration tested (5 µg/L) [30]. The results of the current study also indicate
the 6 day exposures to fluoxetine are sufficient to determine its effective concentration, and that longer term exposures do not produce toxicity at lower concentrations.

Results from our previous 6 day fluoxetine study indicated a dose dependent decrease in brain serotonin level in fish exposed to fluoxetine. This decrease was correlated with increased time to capture prey [15]. The expected therapeutic effect of SSRIs like fluoxetine is increased serotonin levels. While this presents an apparent contradiction, investigation of mammalian literature reveals that delayed onset of therapeutic effects of SSRIs may be due to autoreceptor modulation of serotonin levels. The binding and deactivation of serotonin reuptake transporters (5-HTT) by SSRIs cause increased residence of serotonin in synaptic cleft. But autoreceptors on pre-synaptic axon terminal can regulate the further release of serotonin from the axon. The increased residence time of serotonin in the synaptic cleft results in binding to autoreceptors halting the release of additional serotonin. This can result in a decrease in serotonin levels upon initial administration of SSRIs. Continuous administration of SSRIs can result in the eventual desensitizing of autoreceptors, causing an increase in serotonin release. This process can take up to four weeks to complete [16]. Therefore, the dose dependent decrease in brain serotonin levels seen during 6 day exposures in our previous study can be expected.
We hypothesized that a four week exposure may cause serotonin levels to eventually increase due to autoreceptors becoming desensitized to fluoxetine exposures. If serotonin levels did increase we may expect a recovery of ability to capture prey as serotonin levels reach equilibrium or increase over controls. Results of the current study do not indicate fish were able to recover the ability to capture prey over long term exposure. We did find that there was not a significant difference in the time to capture prey one on day 21 and time to capture prey two on day 18, which may indicate potential recovery. But time to capture prey levels were significantly increased on the following observation period in both instances. This may indicate that time to capture prey not being significant on days 21 and 18 for prey one and two, respectively may have been be due to high individual variability on these days. Unfortunately, brain serotonin levels were not measured during the current study, therefore we can only speculate about how serotonin is behaving in the brain.

When comparing the results of the current study to the effects of fluoxetine on other behaviors, we find that our predation bioassay is slightly less sensitive when compared to smaller organisms. Exposure of embryonic fathead minnows to environmentally relevant concentrations of fluoxetine (100 ng/L) caused decreased escape velocity and total escape response [12]. *Gammarus pulex* exposed to the same concentrations exhibited decreased activity when compared to controls [31]. But exposure of adult fathead minnows to environmentally relevant concentrations of fluoxetine does not cause an effect on
male reproductive behavior [21]. While the concentration required to cause behavioral effects in our assay may be higher than those required to cause behavioral effects in prey species like minnows, they are still two orders of magnitude lower than LC$_{50}$ values from traditional toxicity tests. It is also important to understand what levels cause effects in larger predators. Use of larger organisms also allows for more tissue for biochemical and molecular analyses.

**Conclusions**

Only the 40 µg/L treatment caused consistently significant effects on predation behavior during 27 day exposures to fluoxetine. These results are consistent with our previous findings that 35 µg/L causes increased time to capture prey during six day exposures [15]. This indicates that 35-40 µg/L fluoxetine is the threshold of effect for predation behavior in this species, regardless of exposure duration. Finally, results show that six day exposures may be sufficient for testing the effects of antidepressants on predation behavior.
Figure 2.1: Time to capture prey 1 for 27 day fluoxetine exposures.

Time to capture prey one for hybrid striped bass exposed to fluoxetine for 27 days. Treatments were 0, 10, 20, 30 and 40 µg/L. Asterisks represent statistical significance when compared to control.
Figure 2.2: Time to capture prey 1 for control and 40 µg/L treatments only.

Time to capture prey one for hybrid striped bass exposed to fluoxetine for 27 days. Only control and 40 µg/L treatments are displayed as the 40 µg/L treatment was the only concentration to show consistently significant increases in time to capture prey one. Asterisks represent significant increases when compared to controls. Error bars represent ±1 standard error.
Figure 2.3: Time to capture prey 2 for 27 day fluoxetine exposures.

Time to capture prey two for hybrid striped bass exposed to fluoxetine for 27 days. Treatments were 0, 10, 20, 30 and 40 µg/L. Asterisks represent statistical significance when compared to control.
Figure 2.4: Time to capture prey 2 for control and 40 µg/L treatments only.

Time to capture prey two for hybrid striped bass exposed to fluoxetine for 27 days. Only control and 40 µg/L treatments are displayed as the 40 µg/L treatment was the only concentration to show consistent significant increases in time to capture prey two. Asterisks represent significant increases when compared to controls. Error bars represent ±1 standard error.
Figure 2.5: Time to capture prey 3 for 27 day fluoxetine exposures.

Time to capture prey three for hybrid striped bass exposed to fluoxetine for 27 days. Treatments were 0, 10, 20, 30 and 40 µg/L. Asterisks represent statistical significance when compared to control.
Figure 2.6: Time to capture prey 3 for control and 40 µg/L treatments only.

Time to capture prey three for hybrid striped bass exposed to fluoxetine for 27 days. Only control and 40 µg/L treatments are displayed as the 40 µg/L treatment was the only concentration to show consistent significant increases in time to capture prey three. Asterisks represent significant increases when compared to controls. Error bars represent ±1 standard error.
References


INTRODUCTION

Venlafaxine is a serotonin and norepinephrine reuptake inhibitor that is marketed under the trade name Effexor®. In the past five years detection of venlafaxine in the environment has become quite frequent. Concentrations reported in the literature included 672 ng/L downstream of a wastewater treatment plant (WWTP) in Boulder Creek, CO, 690 ng/L downstream of a WWTP in Four Mile Creek, IA [1], 57 ng/L in metropolitan rivers and 300 ng/L in WWTP effluent in Madrid Spain [2, 3]. Concentrations as high as 808 ng/L and 901 ng/L were found in the treated effluent and downstream, respectively, of a WWTP in the Grand River, Ontario, Canada [4]. The highest reported values of >2 µg/L were found in WWTP effluent from a metropolitan wastewater treatment plant in St. Paul, MN [5]. Unpublished data have shown concentrations an order of magnitude higher than values reported in the literature (Dr. Melissa M. Schultz, College of Wooster, Personal Correspondence, 2010).

The mode of action of venlafaxine works through blocking serotonin (SERT) and norepinephrine (NET) reuptake transporters on the axons of presynaptic neurons. The ultimate goal of this mode of action is increasing neurotransmitters in the synapses of the brain. This results in increased nerve
impulses which have been shown to have a positive effect on depressive disorders [6, 7]. These monoaminergic biochemical pathways are highly conserved in fish [6] and have been implicated in a number of behaviors including feeding [8], locomotion [9], and aggression [10].

While venlafaxine is marketed as a serotonin and norepinephrine reuptake inhibitor, it has a much higher affinity for SERT; its binding affinity for NET is relatively low compared to other antidepressants designed specifically to effect norepinephrine reuptake. The inhibition constant ($K_i$) of an antidepressant is a measure of the concentration required to displace 50% of the natural ligand of the receptor. For venlafaxine, one study found the $K_i$ for NET was 1644 nmol/L compared to its $K_i$ for SERT, which was 102 nmol/L. In the same study, for the antidepressant, fluoxetine, a well-known SSRI, the $K_i$ for inhibition of SERT was 20 nmol/L and 2186 for NET [11]. Therefore, venlafaxine may not be as effective in modulating serotonin as fluoxetine but its increased modulation of norepinephrine may result in different effects. It is important to note that these inhibition constants were for human SERT and NET and that similar constants for fish have not been developed for these chemicals.

While concentrations of venlafaxine found in aquatic matrices have been orders of magnitude higher than SSRI antidepressants, its toxicity to aquatic organisms has not been examined extensively. Exposure to environmentally relevant concentrations of venlafaxine (305 and 1104 ng/L) did not have an effect
on male fathead minnow (Fimphales promelas) reproductive behavior. However, there was significant reduction in survival of exposed males, with ~60%, and ~75% survival in 305 ng/L and 1104 ng/L exposed, respectively [12]. Exposures of embryonic fathead minnows to 500 ng/L venlafaxine caused increased latency, time before initiation of escape response, and decreased total escape response to stimuli after hatching. Fathead minnows exposed to 5000 ng/L of venlafaxine for 12 days after hatching exhibited increased latency and decreased escape responses to stimuli [13].

The objective of the current study was to determine the effect of the antidepressant venlafaxine on the predation behavior and brain chemistry of hybrid striped bass using a predator prey bioassay designed in our laboratory [14]. Previous research using this assay showed that six day exposures to fluoxetine caused increased time to capture prey at concentrations as low as 35 µg/L. This decrease in time to capture prey was correlated with a decrease in brain serotonin levels in bass [8]. Additional research showed that exposure durations greater than six days did not increase behavioral toxicity in bass [15]. Therefore, we chose a six day exposure scenario with a six day recovery period. This objective was completed to test the following hypotheses:

1. Bass exposed to venlafaxine will have decreased brain serotonin and norepinephrine levels.
2. Bass will recover brain serotonin and norepinephrine levels after a depuration period.

3. Decreased brain neurotransmitter levels will cause increased time to capture prey, and recovered brain neurotransmitter levels will result in recovery of feeding efficiency.

Materials and Methods

Test Chemicals

Venlafaxine hydrochloride (LKT laboratories), sodium hydroxide, monochloroacetic acid, HPLC grade methanol, acetone, acetonitrile, glacial acetic acid, perchloric acid, tetrahydrofuran, and triethylamine were purchased from Fisher Scientific (Pittsburgh, PA, USA). Trace metal grade concentrated hydrochloric acid was purchased from Spectrum Chemicals (Gardena, CA, USA). MS-222 (Tricaine-S) was purchased from Western Chemical (Ferndale, WA, USA). Serotonin creatinine sulfate complex, dopamine HCl, norepinephrine HCl, 3,4-dihydroxybenzylamine (DHBA), 5-hydroxyindoleactic acid (5-HIAA), sodium octyl sulfate, and disodium EDTA dihydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used for analytical procedures was ultra-purified using a Milli-Q Super-Q Filtration system (Millipore™, Billerica, MA, USA) with a measured resistivity of 18.2 MΩ·cm.
Fish

Hybrid striped bass (Morone saxatilis x M. chrysops) were generously donated from Southland Fisheries (Columbia, SC, USA) as fingerlings. Fish were held in 450 L circular holding tanks in the cherry farm aquatic research lab at Clemson University (Clemson, SC, USA). Holding tanks were maintained as flow through systems, constantly supplied with fresh water from Lake Hartwell (Clemson, SC, USA). Before reaching the holding systems, water was filtered through a gravel bed, and sterilized with UV radiation. Water temperature was maintained between 22 and 25°C using a mixture of ambient and heated (via inline heater) or chilled water (via inline chiller), depending on incoming water temperature. Water was constantly aerated with air stones and agitators (Boatcycle Inc, Henderson, TX, USA). During holding, bass were fed a commercial diet (Finfish Silver 4.5 mm slow sink) purchased from Zeigler Bros, Inc. (Gardners, PA, USA).

Fathead minnows were purchased from Anderson Minnow Farm (Lonoke, AR, USA). Minnows were held in 100 L holding tanks for using the same water as described above. During holding, minnows were fed a commercial diet (Tetramin® Tropical Flakes) purchased from Dr’s Foster and Smith, Inc. (Rhinelander WI, USA).
**Bass Group Training**

Because bass were grown on a pelleted feed, conditioning to live diet was required before the initiation of behavioral assays. Hybrid striped bass (mass: 249.68g ± 80.09, length: 232.81mm ± 25.63) were randomly removed from holding tanks and placed into a separate 300 L rectangular holding tank for group training. Water conditions in this tank were the same as the holding tanks. Bass were starved for three days prior to the start of group training. On day zero of training, bass were fed four minnows/bass in the group training tank. Bass were fed an additional four minnows/bass on day three and day six.

**Experimental Design**

Hybrid striped bass were exposed to venlafaxine in a static exposure scenario for six days followed by a six day recovery period. Exposures took place in 113 liter aquaria measuring 92.1 x 32.4 x 40 cm purchased from Deep Sea Aquatics (Garland, TX, USA). Volumes of 80 and 40 liters were measured and marked on each tank for measurement of exposure volume and half renewal volume, respectively. Each tank had a 1.9 cm PVC vertical standpipe drilled into the front panel for control of water volume when maintained as a flow through.

Water for exposure tanks was taken from Lake Hartwell (Clemson, SC, USA) (pH = 6.28 ± 0.17, hardness 24 mg/L as CaCO₃, alkalinity 10 mg/L as CaCO₃). Before entering the laboratory, water was filtered through a gravel bed and UV sterilized. Temperature was maintained via a mixing valve (M & M
Control Services, Grayslake, IL, USA) combining ambient water with either chilled (via inline chiller) or heated (via inline water heater) water, depending on temperature of lake water. Water was then passed through a multi-resin filtration system (Water and Power Technologies, Columbia, SC, USA) for additional cleanup and dispensed into tanks. Each tank was constantly aerated via air stones and covered with two square grated covers.

Hybrid striped bass were removed from the group training tank and placed into individual aquaria (one bass/tank). Bass were allowed to acclimate to the aquaria for nine days. During the acclimation period, bass were fed four fathead minnows (approximately four cm long) on days three and six. The time to capture each minnow was recorded for selection of fish for exposures.

Three days after the final individual training (day zero of exposure) the first feeding event was quantified before the addition of venlafaxine. Before each feeding event, half of the tank cover and the air stones were removed from each tank and fish were allowed five minutes to adjust. Four fathead minnows (approximately four cm long) were dropped into tanks, and bass were allowed 25 minutes to consume all minnows. The time bass took to capture each minnow was recorded. Any minnows not consumed during the 25 minute feeding period were removed, and a time to capture of 1500 seconds (25 minutes) was assigned for each unconsumed minnow. Air stones and covers were replaced following the feeding event. Only bass that consumed at least three minnows
during the day zero feeding event, and exhibited comparable feeding during the previous individual training days were selected for inclusion in the test. Following the feeding event, incoming water was shut off and all tanks were set to the final test volume of 80 L. Tanks were then randomized, assigned treatments (one bass per replicate tank, five tanks per treatment), and spiked with appropriate volumes of venlafaxine to reach nominal concentrations. Concentrations tested were 0, 50, 250, and 500 µg/L.

Additional feeding events took place on days three and six. Following the feeding event on day 6 fresh water flows were turned on to flush the venlafaxine from the tanks. Flow rates were set at 0.22 liters per minute, resulting in a hydraulic retention time of ~2.7 hours in our system. This allowed for ~8.8 turnovers in a 24 hour period. Additional feeding events took place on days nine and twelve to measure behavioral recovery after contaminant removal.

Water quality (pH, D.O. temperature) was measured during each feeding event using a YSI 556 multi-parameter instrument (Yellow Springs Instruments, Yellow Springs, OH, USA). During the exposure and recovery period, five replicate bass were removed from each treatment after each feeding event and euthanized for collection of brain tissue. Because the number of exposure tanks was limited, four tests were conducted in series (12, 9, 6, and 3 days) to produce sufficient replicates for tissue collection.
Venlafaxine Exposures

Stock solutions of venlafaxine were prepared by dissolving venlafaxine HCl in methanol. Concentrations of stock solutions were selected to ensure <0.1 mg/L methanol in exposure tanks, the ASTM recommendation for methanol as a carrier solvent (ASTM E1241-92). Equivalent volumes of the stock solutions were added to tanks to reach nominal concentrations. Total methanol concentrations for the highest treatment were calculated and control tanks were spiked with equivalent amounts to ensure there was no carrier solvent toxicity. After a two hour equilibration period, water samples were extracted from each tank to measure venlafaxine concentrations via HPLC with a fluorescence detector. Nominal concentrations selected for the test were 0, 50, 250, 500 μg/L. Stock solutions were prepared daily.

Stability of Venlafaxine

The stability of venlafaxine in our system was measured to determine if re-spiking was necessary to maintain constant exposure concentrations. Prior to the initiation of behavioral studies, a six day exposure was performed, using the same experimental setup as described above using three replicate bass/treatment. Water samples were extracted from each tank daily and measured to determine venlafaxine concentrations.
**Venlafaxine Analysis**

The concentration of venlafaxine in each exposure tank was measured on day zero and day six of exposures. One tank for each treatment was also sampled on day seven to ensure removal of venlafaxine from the tanks. Water samples from tanks were adjusted to a pH of approximately three with concentrated hydrochloric acid. Samples were extracted on 6 ml C-18 solid phase extraction cartridges with a bed weight of 500 mg (HyperSep C18 SPE cartridge, Thermo Fisher Scientific, Pittsburgh, PA, USA). Prior to extraction, cartridges were equilibrated with 6 ml of acetone, 6 ml methanol, 6 ml Milli-Q water in order. Samples were then loaded onto the cartridges and they were allowed to dry under vacuum. Cartridges were stored at -20°C until elution and analysis.

Cartridges were eluted with methanol/1% acetic acid and stored in sample vials for HPLC analysis. The HPLC consisted of a Waters 1525 Breeze HPLC Pump with a Water 717 Plus auto sampler and a Waters 2475 multi wavelength fluorescence detector (Waters, Milford, MA, USA). The mobile phase consisted of 40 parts HPLC grade acetonitrile: 60 parts Milli-Q water, pH adjusted to 3.0 with glacial acetic acid: 4 parts triethylamine. The mobile phase was filtered through a 0.2 µm nylon filter and degassed using a sonication bath. The flow rate was set at 1 ml/min and a 20 µl injection volume was used. A Varian Polaris C-18A reverse phase analytical column (250 mm long, 4.6 mm
I.D.) was used to achieve chromatographic separation. The fluorescence detector was set at excitation wavelength 275nm and emission wavelength 305nm. Run times were approximately 5 minutes per sample.

**Brain Tissue Preparation**

Bass were euthanized after each feeding event by immersion in MS-222 until opercular movement ceased. Brains were quickly removed and stored at -80°C until processing. For monoamine analysis brains were weighed and 1 mL of 0.1N perchloric acid was added to the sample container. Samples were sonicated at 20% amplitude for 10 seconds with a Branson Sonifier (model S-450) probe sonicator (Emerson Electric Co, Danbury, CT, USA). Samples were then centrifuged at 25,000 RPM (57,000 G) for 25 minutes at 4°C to remove cellular debris. Supernatant was removed and placed in a 1.5 mL microcentrifuge tube and centrifuged again at 17,000G for 15 minutes to ensure complete removal of debris. A 200 µL aliquot was taken from the supernatant and spiked with 50 ppb DHBA (internal standard). Samples were stored at -80°C until analysis. Another 5 µL aliquot was taken from the supernatant and diluted in 20 µL of 0.1N perchloric acid for protein concentration analysis.

**Protein Concentration Analysis**

Brain protein concentrations were measured using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Aliquots from brain extracts were diluted 1:4 in
0.1N perchloric acid before analysis. Brain monoamine concentrations were normalized to protein concentrations.

**Monoamine Analysis**

Brain samples were analyzed for serotonin (5-HT), dopamine (DA), norepinephrine (NE), 5-HIAA, and DHBA via HPLC with an electrochemical detector using a method modified from Lin and Pivorun [16]. The chromatographic system consisted of a Rainin Rabbit HPX HPLC pump, with a Bioanalytical Systems LC-4C amperometric detector. Chromatographic separation was achieved with an ODS-2 Hypersil 250mm x 4.6 mm C18 reverse phase analytical column (Fisher Scientific, Pittsburgh, PA). Aliquots of 50 µL were injected into a 20 µL sample loop of a rotary injection valve. The flow rate was set to 1.0 mL/min and the electrode potential was maintained at +0.8 volts vs. Ag/AgCl. Sample run times were approximately 27 minutes.

The mobile phase consisted of 14.2 g monochloroacetic acid, 4.7 g sodium hydroxide, 10.0 mg disodium EDTA, 150 mg sodium octyl sulfate dissolved in 934 mL of Milli-Q water with 30 mL of methanol and 36 mL of tetrahydrofuran. The mobile phase was filtered through a 0.45 µm nylon filter (Millipore™, Billerica, MA, USA) and degassed via sonication bath before use.
Data Analysis

Time to capture prey was analyzed using Statistical Analysis Software 9.2 (SAS, Cary, NC, USA). A general linear means for mixture distributions (PROC GLIMMIX) procedure was used to perform a two factor ANOVA utilizing treatment and day as independent variables and tank as a random variable. The time to capture prey data were non-normally distributed with non-homogenous variances. The GLIMMIX procedure uses an analysis matrix that accounts for non-normal distribution and non-homogenous variances in data points. Least squared means was used to perform multiple pairwise comparisons of data and differentiate statistical differences across and within treatment, day, and treatment by day interactions.

Brain monoamine data also exhibited non-homogenous variances; therefore the general linear means for mixture distributions was used to perform a two factor ANOVA utilizing treatment and day as independent variables and tanks as a random variable. Least squared means was used to perform multiple pairwise comparisons of data and differentiate statistical differences across and within treatment, day, and treatment by day interactions.
Results

Water Quality Measurements

Water quality measurements were averaged (mean ± standard deviation) for the entire test. pH, dissolved oxygen, and temperature were 6.91 ± 0.33, 8.46 mg/L ± 0.63, and 22.76°C ± 1.75, respectively.

Venlafaxine Stability

Results for day zero and day six of the venlafaxine stability study are presented in Figure 3.1. Concentrations of venlafaxine remained stable over a six day exposure period. Therefore, tanks were not re-spiked during behavioral exposures and concentrations were only measured on days zero and six.

Venlafaxine Concentrations

The average concentration (mean ± standard deviation) over the entire test for 50, 250, and 500 µg/L treatments were 35.68 ± 6.75, 198.67 ± 22.21, and 465.39 ± 43.68 µg/L, respectively.

Behavioral Assays

Complete numerical results from behavioral assays are presented in Table A-2 of the appendix. After three days of exposure to 250 µg/L venlafaxine there was a statistically significant increase in time to capture prey one when compared to controls (Figure 3.2). By day six the time to capture prey for both
the 250 and 500 µg/L treatments were significantly increased over controls. After three days of depuration, on day nine only the 250 µg/L treatments remained significantly increased. Finally on day twelve (six days of depuration) all treatments returned to control levels.

The time to capture prey two is presented in Figure 3.3. The 250 and 500 µg/L treatments were significantly increased when compared to controls on day three and day six. Following a three day recovery period (day nine), only the 250 µg/L treatment was significantly increased over controls. Though the 250 and 500 µg/L treatments seem elevated over controls on day twelve (six days of depuration) there was no significant difference between these treatments and the controls.

The time to capture prey three for hybrid striped bass exposed to venlafaxine is presented in Figure 3.4. The 250 and 500 µg/L treatments were significantly increased over controls by day three. By day six all three venlafaxine treatments were significantly increased. The 50, 250, and 500 µg/L treatments remained elevated over controls after three and six days of depuration (days nine and twelve, respectively).

The time to capture prey four data are not presented graphically as they were highly variable for both treatments and controls. Therefore, there were no statistically significant increases in time to capture prey in treatments when compared to controls.
In general the 250 and 500 µg/L treatments caused significant increases in time to capture prey one, two, and three with the exception of day three, when only the 250 µg/L treatment was significant. While these treatments were able to recover their ability to capture prey one and two after removal of venlafaxine, their time to capture prey three was still significantly increased over controls after six days of depuration. The lowest concentration tested, 50 µg/L, did not cause any significant increase in time to capture prey one and two on any day. But by day six the time to capture prey three was significantly increased when compared to controls. The time to capture prey three for the 50 µg/L treatment remained significantly elevated after six days of depuration.

Qualitative Behavioral Observations

In addition to our quantitative behavioral measurements we also noted a number a qualitative behavioral changes in the 250 and 500 µg/L treatments. These observations were not compared among treatments as they were not scored quantitatively. Some of the observations included spitting out prey fish, trouble swallowing prey, maintaining a vertical position in the water column, erratic swimming, swimming with dorsal surface out of the water, and gulping for air at the surface.

Brain Chemistry

Brain concentrations of norepinephrine, dopamine, 5-HT and 5-HIAA are presented graphically in Figures 3.5, 3.6, 3.7, and 3.8 respectively (values for 5-
HT and 5-HIAA are presented in the appendix Table A-3). There were no significant effects on brain norepinephrine and dopamine levels throughout the exposure and recovery period. There was a significant reduction in brain serotonin concentrations when compared to controls on day 3 for the highest concentration, 500 µg/L. On day six all three venlafaxine concentrations were associated with statistically significant decreases in brain serotonin levels. Though an effect was seen for all three venlafaxine concentrations, the corresponding serotonin concentrations were not decreased in a dose dependent manner. Serotonin concentrations for the 50, 250, and 500 µg/L concentrations were similar and were not statistically different from each other. On day nine (recovery period) only the 500 µg/L treatment remained decreased when compared to controls and all brain serotonin levels had returned to control levels by day twelve.

There were also changes in 5-HIAA (primary metabolite of serotonin) levels in the brains of exposed hybrid striped bass. By day three all three exposure concentrations caused significant decreases in 5-HIAA levels. These levels remained significantly decreased through day nine. By day twelve, 5-HIAA levels had all returned to control levels with the exception of the highest treatment. 5-HIAA/5-HT ratio can be an indicator of how serotonin is changing over time and results are presented in Figure 3.9. There were no significant effects on brain 5-HIAA/5-HT ratio with the exception of the 500 µg/L treatment on day six, which was significantly decreased compared to controls and the other
treatments. This is due to 5-HT concentrations leveling out by day six while 5-HIAA levels continued to decrease.

Because serotonin was the only measured neurotransmitter that was significantly affected by venlafaxine, brain serotonin levels as a function of venlafaxine concentration are presented in Figure 3.10. On day three of venlafaxine exposure brain serotonin concentrations decreased in a linear dose dependent manner. But by day six of exposure serotonin concentrations in the brains had reached a basal level for all venlafaxine treatments, indicating a saturation of the effect of venlafaxine.

Behavior/Brain Chemistry Effects

The correlation between brain serotonin concentrations and the time to capture prey for hybrid striped bass exposed to venlafaxine are presented in Figure 3.11. There was an exponential increase in the time to capture prey one and three as a function of brain serotonin levels. This was due to the similar approximate concentration of brain serotonin levels on days six, but increasing time to capture prey with increasing venlafaxine concentrations.

Discussion

The ever increasing use of antidepressants has resulted in their presence in our waste streams and ultimately our aquatic ecosystems [5, 17-21]. But the effects of antidepressants on aquatic organisms are understudied.
Antidepressants have been shown to be relatively non-toxic at environmentally relevant concentrations using traditional toxicity testing methods [22, 23]. However, their mode of action and intended use warrants investigation of their behavioral effects. The aim of the current study was to understand how aquatic exposure to the antidepressant venlafaxine affects the brain chemistry and predation behavior of hybrid striped bass.

The results of the current study showed that hybrid striped bass exposed to venlafaxine for six days at concentrations of 50, 250, and 500 µg/L had significant changes in brain chemistry and their ability to capture fathead minnows was significantly reduced. The behavioral results indicate that venlafaxine may have effects on both locomotor activity as well as appetite. At a low exposure concentration (50 µg/L) bass were able to efficiently capture their first two fish in times comparable to controls but had significant increases in time to capture their third prey. This may indicate that venlafaxine may act as an appetite suppressant at low concentrations while not having an inhibitory effect on locomotor activity. Antidepressants that effect serotonin have been shown to suppress appetite in mammals [24]. Higher concentration exposures resulted in increased time to capture prey one and two on day six. While this could indicate further suppression of appetite and not necessarily an effect on locomotor activity, several missed attempts were also observed at these concentrations indicating it could be a combination of the two effects. Serotonin has also been implicated in controlling locomotor activity in fish [10].
Brain chemistry data showed that venlafaxine caused significant decreases in brain serotonin concentrations. These results are counterintuitive as the expected therapeutic effect of venlafaxine is increased brain serotonin and norepinephrine concentrations. But the mammalian literature has shown that short term exposure to antidepressants may initially cause decreased brain serotonin levels [25]. When SSRI and SNRI antidepressants are bound to monoamine reuptake transporters, un-recycled monoamines can activate negative feedback loops that are responsible for maintenance of neuronal monoamine concentrations. This is accomplished through binding of autoreceptors on the axon terminal of the pre-synaptic neuron. Binding of these autoreceptors inhibits additional release of monoamines into the synaptic cleft. This feedback inhibition mechanism is thought to initially decrease monoamine release in turn decreasing overall monoamine concentrations. Over time the auto receptors may become desensitized and allow further release of monoamines, permitting the therapeutic function of the chemical. This process may take 2-4 weeks to run its course [25]. Initial decreases in serotonin concentrations, potentially due to this phenomenon, were also observed when hybrid striped bass were exposed to fluoxetine for six days [8] as well as in Betta splendens injected with fluoxetine for 14 days [26].

The primary metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA) is often measured with serotonin as it can give an indication of how serotonin is changing in the brain. Administration of serotonin modulating antidepressants
might be expected to cause increases in 5-HIAA level due to the decrease in reuptake of serotonin and increased metabolism by monoamine oxidases. Stress has also been shown to increase 5-HIAA concentrations in the brains of artic charr [27]. But in the current study 5-HIAA levels mirrored decreasing serotonin levels up to day six. These data are consistent with the decreased serotonin concentrations in the current study and decreases in 5-HIAA concentrations during SSRI antidepressants have also been reported in hybrid striped bass [8] and Betta splendens [26] as well as rats exposed to the SSRI antidepressant citalopram [28].

Even after three days of depuration, 5-HIAA levels decreased to their lowest measured level. These results may indicate that though serotonin concentrations in the 50 and 250 µg/L returned to control levels after three days of depuration, 5-HIAA levels were still significantly decreased. We hypothesized that the brain was suppressing the metabolism of serotonin in an attempt to regain serotonin homeostasis.

Results from the current study did not show any significant effects on brain norepinephrine concentrations even though venlafaxine is known to interact with both the serotonin and norepinephrine transporter. This may be due to the large difference in the affinities of venlafaxine for the respective serotonin and norepinephrine reuptake transporters (SERT and NET). While receptor affinity data for this chemical are not available for fish, results in mammalian systems
have shown that venlafaxine has a high affinity for the SERT (Kᵢ = 102 nmol/L) and a relatively low affinity for the NET (Kᵢ = 1644 nmol/L) [11]. Therefore, it would take an order of magnitude higher concentration of venlafaxine to cause a comparable effect on norepinephrine to the effect of the venlafaxine on serotonin concentrations in this study.

Examination of correlations between venlafaxine concentrations and brain chemistry reveal that venlafaxine initially decreases serotonin in a dose dependent manner (Figure 3.10, day three data); however, over time this decrease reaches saturation resulting in apparent basal levels (Figure 3.10, day six data). Previous studies with hybrid striped bass exposed to the SSRI antidepressant fluoxetine showed a dose dependent decrease in brain serotonin concentrations as a function of increasing fluoxetine concentrations [8]. While this was the case for venlafaxine on day three, serotonin decrease was saturated on day six indicating a difference between the effects of the two antidepressants. This could potentially be related to the differences in their stability in our exposure system. Fluoxetine had a previously determined half-life of three days in our exposure system while venlafaxine concentrations were stable over six day exposures [8]. Therefore, fluoxetine had to be re-spiked every three days during exposures to maintain relatively constant exposure concentrations. In spite of this, the fluoxetine concentrations changes by as much as 50 % between day zero and day three and between day three and day six. We hypothesized that fluctuations in exposure may have resulted in less fluoxetine being absorbed
and ultimately reaching the brain while venlafaxine may have had more time to interact with its targets due to its increased stability.

When examining this hypothesis in the context of aquatic toxicology literature, fluctuating exposure concentrations have been shown to be less toxic than continuous exposures to peak episode concentrations for non-bioaccumulative contaminants such as acids and some metals [29]. But the toxicity of fluctuating exposures to more lipophilic contaminants can be dependent on body burden, half-life in the animal, and reversibility of the toxic mechanism [30]. Antidepressants like fluoxetine have been shown to bioaccumulate in the tissues of fish [1, 31], and have been shown to have a relatively long biological half-life (9.4 ± 1.1 days) in Japanese medaka (Oryzias latipes) [32]. But the toxic effects of low concentration exposures to venlafaxine have been shown to be fairly reversible in the current study, as well as our previous study with fluoxetine [8]. Without the measurement of body burdens in the current study, the differences in these factors make it hard to determine if fluctuating concentrations of fluoxetine were the cause of a lower effect on serotonin concentrations when compared to venlafaxine.

Correlations between brain serotonin levels and behavior were not quite as clear because brain serotonin concentrations were approximately the same for all exposure concentrations on day six but there were differences in behavioral data between treatments. Hybrid striped bass exposed to fluoxetine
exhibited a linear relationship between increasing time to capture prey and decreasing brain serotonin levels [8]. A similar relationship could not be established in the current study as all treatments caused a decrease of serotonin to basal levels by day six. We hypothesized that modulation of serotonin in tissues other than the brain may have caused the increase in time to capture prey for higher venlafaxine concentrations once brain serotonin concentrations had been depressed to a basal level. For example serotonin containing neurons have been found in enteric (gastrointestinal) nervous system of fish [33]. Serotonin has been shown to play a role in gut motility in goldfish (*Carassius auratus*), which may influence appetite [34]. Cells containing serotonin have been found in the pineal gland of fish, an organ that plays a role in a number of physiological and behavioral processes [35]. Serotonin may also influence appetite and behavior through modulation of downstream processes. Serotonin has been implicated in controlling the release of various hormones in fish including growth hormone, and gonadotropins, hormones responsible for regulating growth and reproductive function [36]. Release of cortisol, a hormone associated with stress response that is responsible for nutrient metabolism and immune suppression, has also been shown to be controlled by serotonin receptors [37-39]. Corticotropin-releasing factor, a neuropeptide implicated in controlling behavior in a number of vertebrates, including fish, may also be regulated by serotonergic systems [40].
Other laboratories investigating the effects of venlafaxine in fish have shown that venlafaxine does not always act in a dose dependent manner. Adult fathead minnows exposed to venlafaxine for 21 days had increased mortality at lower exposure concentrations when compared to higher concentrations and controls [12]. Fathead minnow embryos exposed to low concentrations of venlafaxine exhibited increased latency to escape response and decreased total escape response when compared to controls and higher levels of venlafaxine [13]. These studies indicate that over time lower doses of venlafaxine may be just as effective as or more effective than high doses. While brain chemistry was not measured in these other studies, this could be due to venlafaxine causing serotonin to decrease to its basal level.

Behavioral results from this study indicate that venlafaxine has less of an effect than fluoxetine. Fluoxetine concentrations as low as 35 µg/L (nominal) were shown to cause significant reduction in the ability of hybrid striped bass to capture prey one, two, and three [8] while the lowest concentration tested in the current study (50 µg/L nominal) only caused a significant effect on the time to capture prey three. Fluoxetine caused a 49% reduction in brain serotonin levels at the highest level tested (150 µg/L nominal, day nine) [41]. The current study also had a maximum reduction of 49% of serotonin but the concentration required to cause this effect was 500 µg/L (day three). While all exposure concentrations eventually reached a basal threshold on day six, the maximum percent reduction of serotonin on this day was 36% (50 µg/L nominal). The
differences in the effects of these chemicals on serotonin can be explained by differences in receptor affinity because fluoxetine has a factor of five lower $K_i$ for SERT (20 nmol/L) when compared to venlafaxine (102 nmol/L) [11].

Venlafaxine has been measured in wastewater effluent at concentrations as high as 2 µg/L [5]. Venlafaxine has also been shown to cause significant effects on predator avoidance behavior at environmentally relevant concentrations [13]. While the results of the current study indicate that venlafaxine does have an effect on the brain chemistry and behavior of hybrid striped bass, it is important to note that concentrations are at the high end of environmentally measured concentrations. Since municipal wastewater effluents contain many SSRIs future research should focus on exposure to multiple antidepressants that have a similar mode of action to determine if they are additive.

**Conclusions**

Hybrid striped bass exposed to venlafaxine exhibited increased time to capture prey when compared to controls. Low concentration exposures may affect appetite while higher concentration exposures may also affect locomotor activity. Exposure to venlafaxine for three days resulted in a dose-dependent decrease in brain serotonin concentrations while six day exposure caused reduction to an apparent basal level. Unfortunately, brain serotonin concentrations were not predictive of behavioral changes. Mammalian receptor
affinity data were able to explain the difference in potency of venlafaxine and fluoxetine.
Figure 3.1: Stability of venlafaxine over 6 days.

Concentrations of venlafaxine on day 0 and day 6 of stability study. Error bars represent ±1 standard deviation.
Figure 3.2: Time to capture prey 1 for hybrid striped bass exposed to venlafaxine.

Time to capture prey 1 for hybrid striped bass exposed venlafaxine for 6 days and allowed to recover for 6 days. Treatments were 0, 50, 250, and 500 µg/L. Asterisks represent statistical significance when compared to control. Error bars represent ±1 standard error.
Figure 3.3: Time to capture prey 2 for hybrid striped bass exposed to venlafaxine.

Time to capture prey 2 for hybrid striped bass exposed to venlafaxine for 6 days and allowed to recover for 6 days. Treatments were 0, 50, 250, and 500 µg/L. Asterisks represent statistical significance when compared to control. Error bars represent ±1 standard error.
Figure 3.4: Time to capture prey 3 for hybrid striped bass exposed to venlafaxine.

Time to capture prey 3 for hybrid striped bass exposed venlafaxine for 6 days and allowed to recover for 6 days. Treatments were 0, 50, 250, and 500 µg/L. Asterisks represent statistical significance when compared to control. Error bars represent ±1 standard error.
Figure 3.5: Brain norepinephrine concentrations in hybrid striped bass exposed to venlafaxine.

Brain norepinephrine concentrations for hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Error bars represent ±1 standard deviation.
Figure 3.6: Brain dopamine concentrations in hybrid striped bass exposed to venlafaxine.

Concentrations of dopamine in the brains of hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Asterisks represent statistical significance when compared to controls. Error bars represent ±1 standard deviation.
Figure 3.7: Brain serotonin concentrations in hybrid striped bass exposed to venlafaxine.

Concentrations of serotonin in the brains of hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Asterisks represent statistical significance when compared to controls. Error bars represent ±1 standard deviation.
Figure 3.8: Brain 5-HIAA concentrations in hybrid striped bass exposed to venlafaxine.

Concentrations of 5-HIAA in the brains of hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Asterisks represent statistical significance when compared to controls. Error bars represent ±1 standard deviation.
Figure 3.9: 5-HIAA/5-HT ratios in hybrid striped bass exposed to venlafaxine.

5-HIAA/5-HT ratios in the brains of hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Asterisks represent statistical significance when compared to controls. Error bars represent ±1 standard deviation.
Figure 3.10: Brain serotonin levels as a function of measured venlafaxine concentration.

Brain serotonin concentrations as a function of measured venlafaxine concentrations on days 3 and 6. A linear equation was used to fit a curve to the data for day three. A linear equation was used to fit a curve to the data for venlafaxine treatments on day 6. Equations and correlation coefficients are displayed above and below the respective curves.

\[ y = -0.1406x + 137.49 \]
\[ R^2 = 0.9436 \]

\[ y = 0.0163x + 76.729 \]
\[ R^2 = 0.9405 \]
Figure 3.11: Time to capture prey as a function of brain serotonin levels on day 6.

Time to capture prey 1 and 2 for hybrid striped bass exposed to venlafaxine for 6 days as a function of brain serotonin concentrations.
References


CHAPTER FOUR:

THE EFFECT OF MIXTURES OF FLUOXETINE AND VENLAFAXINE ON THE 
PREDATION BEHAVIOR AND BRAIN CHEMISTRY OF HYBRID STRIPED 
BASS

Introduction

Increased use of pharmaceuticals and personal care products (PPCPs) has led to increased concentrations in municipal wastewater effluents and receiving streams [1-6]. Antidepressants are among the most frequently detected classes of pharmaceuticals. The antidepressants fluoxetine (Prozac®), venlafaxine (Effexor®), bupropion (Wellbutrin®), sertraline (Zoloft®), paroxetine (Paxil®), citalopram (Celexa®), fluvoxamine (Luvox®) and duloxetine (Cymbalta®) have all been detected in the nanograms per liter to low micrograms per liter concentrations in environmental matrices [1, 2, 6].

The relative hazard of some of these compounds to aquatic organisms has been studied. The 48-hour acute toxicity of fluoxetine in three commonly used toxicity test species (Pimephales promelas, Ceriodaphnia dubia, Daphnia magna) was similar with LC<sub>50</sub> values (median lethal concentration) between 234 and 820 µg/L [7]. The 48-hour LC<sub>50</sub> for larval fathead minnows and C. dubia exposed to sertraline was 205 (pH 7.5) and 146-202 µg/L, respectively [8, 9]. The 48-hour LC<sub>50</sub> for C. dubia exposed to paroxetine and citalopram was 734 - 1175 and 3396 - 4713 µg/L, respectively [9]. The general consensus is that
antidepressants are not toxic at environmentally relevant concentrations using traditional toxicity tests.

These antidepressants specifically target the transport of monoamine neurotransmitters into pre-synaptic axons in the brains of humans. Through this pathway monoamine concentrations are modulated in an effort to treat a number of depressive disorders [10]. Monoaminergic transport proteins and receptors in fish have been shown to share high genetic sequence identity with a number of mammal species, and therefore may be expected to exhibit similar responses to antidepressants [11, 12]. The monoamine neurotransmitters serotonin, norepinephrine, and dopamine have been implicated in stress response [13], feeding [14], locomotor activity [15], social hierarchies [16], and migration [17] in fish. The potential for antidepressant effects on brain monoamines, and subsequent behaviors has led researchers to examine the behavioral effects of antidepressants in fish.

The antidepressant fluoxetine has been shown to decrease escape velocity and total escape response after a stimulus in embryonically exposed fathead minnows (Pimephales promelas) [18]. Gulf toadfish injected with fluoxetine exhibited increased aggression, which coincided with increased plasma serotonin levels [19]. Intraperitoneal injections of fluoxetine have been shown to cause decreased aggression in bluehead wrasse (Thalassoma bifasciatum) [20]. Exposures of embryonic fathead minnows to venlafaxine
caused increased latency, the time before initiation of escape response, and decrease total escape response to stimuli after hatching. Fathead minnows exposed to venlafaxine for 12 days after hatching exhibited increased latency and decreased escape responses to stimuli [18].

Fluoxetine caused increased time to capture prey in hybrid striped bass at concentrations between 30 and 40 µg/L during 6 and 27-day exposures [21, 22]. Increased time to capture prey was correlated with decreased brain serotonin levels during six day exposures [21]. Hybrid striped bass exposed to venlafaxine exhibited increased time to capture various prey during six day exposures to 50, 250, and 500 µg/L. Brain serotonin concentrations initially decreased in a dose dependent manner after three days before reaching an apparent minimal basal level for all exposure concentrations after six day exposures [22].

While laboratory research on the effects of individual antidepressants have increased our understanding of how these contaminants may influence the behavior of aquatic organisms, environmental exposure scenarios present a different level of complexity. As environmental detection data have shown, antidepressants in environmental matrices are present in complex mixtures [2, 23]. However, few studies have quantified the effects of antidepressant mixtures on fish behavior. Further, none have related any behavioral effects to changes in brain chemistry.
Scientists have struggled with assessing the risk of mixtures of endocrine disruptors as a whole for over a decade. An EPA sponsored science advisory panel, convened in the late 1990s, recommended delaying the screening of contaminant mixtures with endocrine disrupting potential until more data was available for individual compounds [24]. One of the most troubling concepts in mixture toxicity assessment is how to address the concept of additivity. Because organisms exhibit finite responses to contaminants, you cannot expect a chemical mixture to have an infinitely additive effect with increasing number of chemicals. For example, if individual compounds with similar modes of action are expected to cause a 10% decrease in some endpoint (i.e. survival, gene expression) and you expose an organism to a mixture of 20 of these chemicals, you cannot have a 200% decrease in your endpoint [25]. Therefore, the concept of dose addition has been employed as it can account for saturation of effects so that paradoxical predictions that exceed the maximum effect are avoided. The dose addition concept states that if chemical A and chemical B act through a similar mode of action, chemical A can be replaced, in whole or in part, with an equally effective concentration of chemical B, without diminishing the combined effect of the two chemicals. The most important part of this concept is the use of equally effective concentrations as concentrations of different chemicals cannot always be expected to cause similar effects. Therefore, chemicals must me normalized to a specific effect and dosing regimes adjusted accordingly, if additivity is to be assessed [24]. Concentrations of mixtures that are normalized
to some effect are often described as toxic equivalency factors and are expressed as toxic units [26, 27].

The dose addition concept has frequently been applied to the assessment of mixture toxicity of estrogenic compounds. Normalizing concentrations of estrogenic compounds in mixtures has been achieved through the use of estradiol equivalency factors (EEQs), which are determined by normalizing the magnitude of effect for a suspected estrogenic compound to the effect of the same concentration of estradiol [28]. EEQs have been used to predict the relative contribution of individual estrogenic compounds in complex mixtures of estrogenic compounds in wastewater effluents [29, 30]. Using mathematical models based of the relative potency of estradiol, ethynylestradiol, nonylphenol, octylphenol, and bisphenol A, researchers were able to predict the additive effects of mixtures on vitellogenin induction in male fathead minnows [31]. Low concentrations of estrogenic compounds that do not elicit effects individually have also been shown to cause adverse effects when combined in mixtures [31-33]. Use of the dose addition concept has also been used to successfully to predict the additive effects of anti-androgens, and thyroid disrupting chemicals [24].

Though the prediction of mixture toxicity using the dose addition concept has been widely used for other endocrine disruptor classes, research on mixtures of pharmaceuticals, especially antidepressants, is fairly limited. A few examples
include mixtures of fluoxetine, venlafaxine, sertraline, and bupropion causing decreased escape velocity in fathead minnow exposed as embryos and larvae, as well as reducing total escape response in exposed larvae [18]. Adult male fathead minnows exposed to an environmentally relevant mixture of sertraline, fluoxetine, bupropion, and venlafaxine did not exhibit effects on nest guarding behavior when challenged with an unexposed male [34].

Based on results of our previous research on the individual behavioral and biochemical effects of fluoxetine and venlafaxine on hybrid striped bass, the goal of the current study was to determine if mixtures of these two pharmaceuticals might be additive. To achieve this goal the following hypotheses were tested:

1. Mixtures of fluoxetine and venlafaxine will have an additive effect on brain serotonin.

2. Mixtures of fluoxetine and venlafaxine will cause increased time to capture prey for bass.

3. Increased time to capture prey will correlate with changes in brain chemistry.

Materials and Methods

Test Chemicals

Fluoxetine hydrochloride was generously donated by Fermion (Finland). Venlafaxine hydrochloride (LKT laboratories), sodium hydroxide,
monochloroacetic acid, HPLC grade methanol, acetone, acetonitrile, glacial acetic acid, perchloric acid, tetrahydrofuran, and triethylamine were purchased from Fisher Scientific (Pittsburgh, PA, USA). Trace metal grade concentrated hydrochloric acid was purchased from Spectrum Chemicals (Gardena, CA, USA). MS-222 (Tricaine-S) was purchased from Western Chemical (Ferndale, WA, USA). Serotonin creatinine sulfate complex, dopamine HCl, norepinephrine HCl, 3,4-dihydroxybenzylamine (DHBA), 5-hydroxyindoleacetic acid (5-HIAA), sodium octyl sulfate, and disodium EDTA dihydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water used for analytical procedures was ultra-purified using a Milli-Q Super-Q Filtration system (Millipore™, Billerica, MA, USA) with a measured resistivity of 18.2 MΩ·cm.

Fish

Hybrid striped bass (*Morone saxatilis x M. chrysops*) were purchased from Keo Fish Farms (Keo, AR, USA) as fingerlings. Fish were held in 450 L circular holding tanks in the cherry farm aquatic research lab at Clemson University (Clemson, SC, USA). Holding tanks were maintained as flow through systems, constantly supplied with fresh water from Lake Hartwell (Clemson, SC, USA). Before reaching the holding systems, water was filtered through a gravel bed, and sterilized with UV radiation. Water temperature was maintained between 22 and 25°C using a mixture of ambient and heated (via inline heater) or chilled water (via inline chiller), depending on incoming water temperature. Water was
constantly aerated with air stones and agitators (Boatcycle Inc, Henderson, TX, USA). During holding, bass were fed a commercial diet (Finfish Silver 4.5 mm slow sink) purchased from Zeigler Bros, Inc. (Gardners, PA, USA).

Fathead minnows were purchased from Anderson Minnow Farm (Lonoke, AR, USA). Minnows were held in 100 L holding tanks using the same water as described above. During holding minnows were fed a commercial diet (Tetramin® Tropical Flakes) purchased from Foster and Smith, Inc (Rhinelander WI, USA).

Bass Group Training

Because bass were grown on a pelleted feed, conditioning to live diet was required before the initiation of behavioral assays. Hybrid striped bass (mass: 227.65g ± 47.60, length: 234.48mm ± 26.18) were randomly selected from the holding tanks and placed into a separate 300 L rectangular holding tank for group training. Water conditions in this tank were the same as the holding tanks. Bass were starved for three days prior to the start of group training. On day zero of training, bass were fed four minnows/bass in the group training tank. Bass were fed an additional four minnows/bass on day three and day six.

Experimental Design

Hybrid striped bass were exposed to mixtures of fluoxetine and venlafaxine in a static exposure scenario for six days followed by a six day
recovery period. Exposures took place in 113 liter aquaria measuring 92.1 x 32.4 x 40 cm purchased from Deep Sea Aquatics (Garland, TX, USA). Volumes of 80 and 40 liters were measured and marked on each tank for measurement of exposure volume and half renewal volume, respectively. Each tank had a 1.9 cm PVC vertical standpipe drilled into the front panel for control of water volume when maintained as a flow through system.

Water for exposure tanks was taken from Lake Hartwell (Clemson, SC, USA) (pH = 6.28 ± 0.17, hardness 24 mg/L as CaCO$_3$, alkalinity 10 mg/L as CaCO$_3$). Before entering the laboratory, water was filtered through a gravel bed and UV sterilized. Temperature was maintained via a mixing valve (M & M Control Services, Grayslake, IL, USA) combining ambient water with either chilled (via inline chiller) or heated (via inline water heater) water, depending on the temperature of lake water. Water was passed through a multi-resin filtration system (Water and Power Technologies, Columbia, SC, USA) for additional cleanup and dispensed into tanks. Each tank was constantly aerated via air stones and covered with two square grated covers.

Hybrid striped bass were removed from the group training tank and placed into individual aquaria (one bass/tank). Bass were allowed to acclimate to the aquaria for nine days. During the acclimation period, bass were fed four fathead minnows (approximately four cm long) on days three and six. The time to capture each minnow was recorded for selection of fish for exposures.
Three days after the final individual training (day zero of exposure) the first feeding event was quantified before the addition of antidepressant mixtures. Before each feeding event, half of the tank cover and the air stones were removed from each tank and fish were allowed five minutes to adjust. Four fathead minnows (approximately four cm long) were dropped into tanks, and bass were allowed 25 minutes to consume all minnows. The time bass took to capture each minnow was recorded. Any minnows not consumed during the 25 minute feeding period were removed, and a time to capture of 1500 seconds (25 minutes) was assigned for each unconsumed minnow. Air stones and covers were replaced following the feeding event. Only bass that consumed at least three minnows during the day zero feeding event, and exhibited comparable feeding during the previous individual training days were selected for inclusion in the test. Following the day zero feeding event, incoming water was shut off and all tanks were set to the final test volume of 80 L. Tanks were then randomized, assigned treatments (five bass/treatment/test), and spiked with appropriate volumes of venlafaxine to reach nominal concentrations.

Concentrations were selected based on the lowest concentration that caused significant effects during individual fluoxetine and venlafaxine behavioral tests. A toxic unit approach was taken when determining mixture concentrations. Concentrations of 30 µg/L for fluoxetine and 50 µg/L for venlafaxine were selected as one toxic unit (TU) for the individual compounds as they both represent an approximate 15% decrease in brain serotonin concentrations by
day three based on the results of our previous studies with individual exposures to these compounds (see table 4.1 and [21]). Using this approach, the following treatments were tested: control, 1 TU (15 µg/L fluoxetine; 25 µg/L venlafaxine), two TU (30 µg/L fluoxetine; 50 µg/L venlafaxine), and four TU (60 µg/L fluoxetine; 100 µg/L venlafaxine) were tested. Assuming additivity, expected decrease in brain serotonin concentrations on day three for treatments of one, two, and four toxic units would be 15, 30, and 60%, respectively.

Additional feeding events took place on days three and six of exposure. Following the feeding event on day six, fresh water flows were turned on to flush the antidepressants from the tanks. Flow rates were set at 0.22 liters per minute, resulting in a hydraulic retention time of ~2.7 hours in our system. This allowed for ~8.8 turnovers in a 24 hour period. Additional feeding events took place on days nine and twelve to measure behavioral recovery after exposure ceased.

Water quality (pH, D.O. temperature) was measured during each feeding event using a YSI 556 multi-parameter instrument (Yellow Springs Instruments, Yellow Springs, OH, USA). During the exposure and recovery period, five replicate bass were removed from each treatment after each feeding event and euthanized for collection of brain tissue. Because the number of exposure tanks was limited, four tests were conducted in series (12, 9, 6, and 3 days) to produce sufficient replicates for tissue collection.
Antidepressant Mixture Exposures

Stock solutions of fluoxetine and venlafaxine were prepared by dissolving fluoxetine HCl and venlafaxine HCl in methanol. Concentrations of stock solutions were selected to ensure <0.1 mg/L methanol in exposure tanks, the ASTM recommendation for methanol as a carrier solvent (ASTM E1241-92). Equivalent volumes of the stock solutions were added to tanks to reach nominal concentrations. Total methanol concentrations for the highest treatment were calculated and control tanks were spiked with equivalent amounts to ensure there was no carrier solvent toxicity. After a two hour equilibration period, water samples were extracted from each tank to measure antidepressant concentrations via HPLC with a fluorescence detector. Stock solutions were prepared daily. Previous studies showed that fluoxetine has a half-life in our system of approximately three days. Therefore, tanks were re-spiked with half the concentration of fluoxetine on day three of the exposures to maintain nominal concentrations [21]. Venlafaxine was shown to be stable in our system over six day periods [22].

Antidepressant Analysis

The concentrations of fluoxetine and venlafaxine in each exposure tank were measured on day zero. One tank for each treatment was also measured on day three and day six to confirm concentrations. Water samples from tanks were adjusted to a pH of approximately three with concentrated hydrochloric acid.
Samples were extracted on 6 ml C-18 solid phase extraction cartridges with a bed weight of 500 mg (HyperSep C18 SPE cartridge, Thermo Fisher Scientific, Pittsburgh, PA, USA). Prior to extraction, cartridges were equilibrated with 6 ml of acetone, 6 ml methanol, 6 ml Milli-Q water in order. Samples were then loaded and the cartridges were allowed to dry under vacuum. Cartridges were stored at -20°C until elution and analysis.

Cartridges were eluted with methanol/1% acetic acid and stored in sample vials for HPLC analysis. The HPLC consisted of a Water 1525 Breeze HPLC Pump with a Water 717 Plus auto sampler and a Waters 2475 multi wavelength fluorescence detector (Waters, Milford, MA, USA). The mobile phase consisted of 40 parts HPLC grade acetonitrile: 60 parts Milli-Q water, pH adjusted to 3.0 with glacial acetic acid: 4 parts triethylamine. The mobile phase was filtered through a 0.2 µm nylon filter and degassed using a sonication bath. The flow rate was set at 1 ml/min and a 40 µl injection volume was used. A Varian Polaris C-18A reverse phase analytical column (250 mm long, 4.6 mm I.D.) was used to achieve chromatographic separation. The fluorescence detector was set at excitation wavelength 270nm and emission wavelength 300nm. Run times were approximately 12 minutes per sample.

**Brain Tissue Preparation**

Bass were euthanized after each feeding event by immersion in MS-222 until opercular movement ceased. Brains were quickly removed and stored at -
80°C until processing. For monoamine analysis via HPLC brains were weighed and 1 mL of 0.1N perchloric acid was added to the sample container. Samples were sonicated at 20% amplitude for 10 seconds with a Branson Sonifier (model S-450) probe sonicator (Emerson Electric Co, Danbury, CT, USA). Samples were then centrifuged at 25,000 RPM (57,000 G) for 25 minutes at 4°C to remove cellular debris. Supernatant was removed and placed in a 1.5 mL microcentrifuge tube and centrifuged again at 17,000G for 15 minutes to ensure complete removal of debris. A 200 µL aliquot was taken from the supernatant and spiked with 50 ppb DHBA (internal standard). Samples were stored at -80°C until analysis. Another 5 µL aliquot was taken from the supernatant and diluted in 20 µL of 0.1N perchloric acid for protein concentration analysis.

Protein Concentration Analysis

Brain protein concentrations were measured using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Aliquots from brain extracts were diluted 1:4 in 0.1N perchloric acid before analysis. Brain monoamine concentrations were normalized to protein concentrations.

Monoamine Analysis

Brain samples were analyzed for serotonin (5-HT), dopamine (DA), norepinephrine (NE), 5-HIAA, and DHBA via HPLC with an electrochemical detector using a method modified from Lin and Pivorun [35]. The chromatographic system consisted of a Rainin Rabbit HPX HPLC pump, with a
Bioanalytical Systems LC-4C amperometric detector. Chromatographic separation was achieved with an ODS-2 Hypersil 250mm x 4.6 mm C18 reverse phase analytical column (Fisher Scientific, Pittsburgh, PA). Aliquots of 50 µL were injected into a 20 µL sample loop of a rotary injection valve. The flow rate was set to 1.0 mL/min and the electrode potential was maintained at +0.8 volts vs. Ag/AgCl. Sample run times were approximately 29 minutes.

The mobile phase consisted of 14.2 g monochloroacetic acid, 4.7 g sodium hydroxide, 10.0 mg disodium EDTA, 150 mg sodium octyl sulfate dissolved in 967 mL of Milli-Q water with 15 mL of methanol and 18 mL of tetrahydrofuran. The mobile phase was filtered through a 0.45 µm nylon filter (Millipore™, Billerica, MA, USA) and degassed via sonication bath before use.

**Data Analysis**

Time to capture prey was analyzed using Statistical Analysis Software 9.2 (SAS, Cary, NC, USA). A general linear means for mixture distributions (PROC GLIMMIX) procedure was used to perform a two factor ANOVA utilizing treatment and day as independent variables and tank as a random variable. The time to capture prey data were non-normally distributed with non-homogenous variances. The GLIMMIX procedure uses an analysis matrix that accounts for non-normal distribution and non-homogenous variances in data points. Least squared means was used to perform multiple pairwise comparisons of data and
differentiate statistical differences across and within treatment, day, and treatment by day interactions.

Brain monoamine data also exhibited non-homogenous variances, therefore the general linear means for mixture distributions was used to perform a two factor ANOVA utilizing treatment and day as independent variables and tanks as a random variable. Least squared means was used to perform multiple pairwise comparisons of data and differentiate statistical differences across and within treatment, day, and treatment by day interactions.

**Results**

**Water Quality Measurements**

Water quality measurements were averaged for the entire test. pH, dissolved oxygen, and temperature were (mean ± standard deviation) 6.51 ± 0.35, 8.27 mg/L ± 0.48, and 24.39°C ± 1.04, respectively.

**Antidepressant Concentrations**

The average concentration (mean ± standard deviation) over the entire test for one, two, and four TU treatments were 10.81 ± 1.36, 23.84 ± 2.63, and 55.47 ± 8.72 µg/L, respectively for fluoxetine and 15.50 ± 2.02, 32.43 ± 3.21, and 74.30 ± 11.32 µg/L, respectively for venlafaxine.
Behavioral Assays

Complete numerical results from behavioral assay are presented in the appendix Table A-4. The time for hybrid striped bass to capture their first prey is presented in Figure 4.1. There was a statistically significant increase \((p<0.05)\) in the time to capture prey one for the highest treatment when compared to controls on day three. By day six of exposure the one and four toxic unit mixtures showed a significant increase in time to capture prey. Though the two toxic unit mixture appear to be increased the value was not statistically significant with a \(p\) value of 0.06. The one and two toxic unit treatments were able to recover to control levels by day nine (day three of depuration) and stayed at control levels through day twelve (sixth day of depuration). The four toxic unit treatment remained elevated over controls even after a six day depuration period.

The time to capture prey two is presented in Figure 4.2. The two and four toxic unit mixtures caused a significant increase in the time to capture prey two by day three of exposure. By day six of exposure all three treatments caused a significant increase in the time to capture prey two. By day nine (three days of depuration) the one and two toxic unit treatments had returned to control levels while the four toxic unit treatment remained significantly increased over controls. On day twelve the one toxic unit treatment became significantly elevated over controls while the two and four toxic unit treatments were not significant. It is important to note that the four toxic unit treatment appeared elevated over
controls but was not statistically significant on day twelve. This may be due to low replication and high variability on this day.

The time to capture prey three for hybrid striped bass exposed to venlafaxine for six days is presented in Figure 4.3. All toxic unit mixtures were significantly increased over controls on day three and six of exposure. The two and four toxic unit mixtures remained significantly elevated over controls after three days of depuration (day nine). After six days of depuration (day twelve) the one toxic unit was once again significantly elevated over controls while the four toxic unit was no longer significant. Once again appears that the four toxic unit treatments are elevated over controls but they were not significant due to low replication and high variability.

The time to capture prey four data are not presented graphically as they were highly variable for both treatments and controls. Therefore, there were no statistically significant increases in time to capture prey in treatments when compared to controls.

Qualitative Behavioral Observations

Similar to previous studies with venlafaxine and fluoxetine, a number of qualitative behavioral traits were observed in the highest treatment (four toxic unit mixture). Some of the observations included spitting out prey fish, trouble swallowing prey, maintaining a vertical position in the water column, erratic
swimming, swimming with dorsal surface out of the water, and gulping for air at the surface.

_Brain Chemistry_

Concentrations of norepinephrine and dopamine in the brains of bass exposed to mixtures of fluoxetine and venlafaxine for six days are presented in Figures 4.4 and 4.5, respectively. There were no dose-dependent trends for either of these neurotransmitters for the exposure or recovery period.

Brain serotonin concentrations were significantly decreased by day three of exposure (Figure 4.6). Serotonin concentrations remained at this apparent basal level through the end of exposures on day six. Following three days of depuration (day nine) the one toxic unit mixture treatment returned to control levels while the two and four toxic unit mixtures remained significantly decreased when compared to controls. After six days of depuration (day twelve) all treatments had returned to control levels.

The primary metabolite of serotonin, 5-HIAA, followed the same trends as serotonin in the brains of exposed bass (Figure 4.7). Metabolite concentrations reached a significantly decreased basal level by day three of exposure and remained decreased through the end of exposure on day six. After three days of depuration the one toxic unit mixture treatment returned to control levels while the two and four toxic unit mixtures remained significantly decreased. By the
sixth day of depuration all treatments had returned to control levels. (Numerical results for 5-HT and 5-HIAA are presented in the appendix Table A-5)

The 5-HIAA/5-HT ratio for hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine is presented in Figure 4.8. There were no significant changes in this ratio for the exposure or recovery periods.

**Brain Chemistry/Behavior Relationship**

Because serotonin was the only neurotransmitter measured to show a significant trend over the exposure and recovery periods, relationships between mixture concentrations, behavioral results, and serotonin concentrations were examined. There was a linear relationship among toxic unit treatments and brain serotonin concentrations as seen in Figure 4.9. This was not surprising as brain serotonin concentrations quickly reached a basal level for all exposure scenarios by the third day of exposure. There was an exponential relationship between brain serotonin concentrations and the time to capture prey on day 6 (Figure 4.10). Overall, the data indicate that mixtures of the antidepressants fluoxetine and venlafaxine cause an exponential decrease in brain serotonin concentrations which coincides with increased time to capture prey one and two on day six.

**Mixtures vs. Individual Compounds**

Predictions for 1:1 additivity for percent serotonin decrease were generated from the percent decrease in serotonin concentrations from controls
caused by one toxic unit (23 µg/L fluoxetine [21], 35 µg/L venlafaxine [22]) for each individual antidepressant. For example, the one toxic unit mixture had half the concentration of one toxic unit of each individual antidepressant (15 µg/L fluoxetine, 25 µg/L). Therefore, we predicted that if fluoxetine and venlafaxine were strictly additive, the percent serotonin decrease for the one toxic unit mixture would be the sum of half the percent serotonin decrease of one toxic unit from the individual antidepressant exposures. The percent decrease in brain serotonin concentrations from mixtures were then compared to predicted values. If predicted values fell within the standard deviation of the mean for mixtures, that specific mixture was considered additive. If the predicted values were higher than the standard deviation of the mean the mixture was considered less than additive. The data (Table 4.1) showed that on day three the percent decrease in brain serotonin was additive for the one and two toxic unit mixtures but less than additive for the four toxic unit mixture. On day six only the one toxic unit mixture was shown to be additive.

Time to capture prey one, two, and three from one toxic unit of each of the individual antidepressant was used to predict effects of antidepressant mixtures on hybrid striped bass predation behavior (Table 4.2). Calculations were made in similar fashion to brain serotonin predictions with the one toxic unit mixture receiving half the effect of one toxic unit of each individual compound. If predicted time to capture prey was within the standard error of the mean for each mixture that mixture was considered to act in an additive manner. On day three,
all three mixture treatments showed additive effects on the time to capture prey one while none of the mixture treatments caused additive effects for prey two and three. On day six only the one toxic unit treatment caused additive effect, but this effect was seen for prey one, two, and three.

Overall the data showed that low concentration (one toxic unit) mixtures act in a predictively additive manner on brain serotonin concentrations and predation behavior. Higher concentration mixtures (two, four toxic units) did not have the predicted effect on brain chemistry due to brain serotonin concentrations reaching an apparent basal threshold level. These mixtures also did not have an additive effect on time to capture prey.

**Discussion**

A number of studies have shown that antidepressants are present in complex mixtures in the environment [1-6]. But characterization of the toxicity of these mixtures is difficult because the contribution of individual compounds is largely unknown. Also, interactions between chemicals *in vivo* are unpredictable in most cases. The objective of the current study was to examine a simple mixture of antidepressants whose effects have previously been characterized individually.

Hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine exhibited similar effects on predation behavior to those previously reported for the individual compounds. In general there was a dose dependent increase in
time to capture prey one, two, and three on the third day with more treatments becoming significant with each additional prey. But on day six, the time to capture prey one for the two toxic unit treatment was no longer significant when compared to controls. The p-value was close to significant (0.06) and insignificance was probably due to high variability for this treatment on this day. This is further evidenced by all treatments causing significant increases in time to capture prey two and three on day six.

During the recovery period, the one and two toxic unit mixture treatments were able to recover by day nine but the one toxic unit treatments were increased over controls again on day twelve. The highest treatment (four toxic units) remained significantly increased when compared to controls throughout the recovery period. Insignificance on day twelve for prey two and three was due to high variability and low replication on these days. The recovery results were similar to recovery periods for individual exposures to fluoxetine and venlafaxine. Bass exposed to 100 µg/L fluoxetine for six days were unable to recovery their ability to capture prey after a six day recovery period [21]. Similarly, bass exposed to venlafaxine at concentrations of 465 µg/L for six days were also unable to recover their ability to capture prey after a six day recovery period.

Venlafaxine is designed to inhibit the transport of both norepinephrine and serotonin but there was only one day and one treatment when norepinephrine differed significantly from controls (Figure 4.4). Our previous study that exposed
bass to higher concentrations of venlafaxine showed similar lack of effect on norepinephrine [22]. While venlafaxine is marketed as a serotonin and norepinephrine reuptake inhibitor, it has an order of magnitude lower affinity for the norepinephrine transporter when compared to the serotonin transporter in mammalian systems [36]. This translates into needing an order of magnitude higher concentration of venlafaxine to have a similar effect on norepinephrine as seen on serotonin. Therefore, it was not surprising that there was no effect on norepinephrine in this study.

Serotonin concentrations reached a basal level for all mixture exposure treatments by day three and remained decreased through day six (Figure 4.6). When comparing mixture exposure serotonin concentrations to individual exposure serotonin concentrations there was an interesting difference between day three and six. Our previous work showed that fluoxetine caused decreases in serotonin levels in a dose dependent manner [21]. Venlafaxine eventually caused serotonin to decrease to a threshold level regardless of treatment, but serotonin initially decreased in a dose dependent manner [22]. Results from the current study showed that serotonin concentrations reached an apparent basal level by day three marking a much faster decrease than the previous study (Figure 4.6). This immediate decrease to basal threshold levels for serotonin correlated well with the increased time to capture prey discussed above (Figure 4.10). Selective serotonin reuptake inhibitor antidepressants have been reported to decrease brain serotonin concentrations in hybrid striped bass [21], betta
To our knowledge, our previous work with venlafaxine [22] and the current study are the only examples that demonstrate antidepressants causing serotonin to reach basal threshold levels.

Analysis of the additive effect of antidepressant mixtures revealed that low concentration mixtures caused additive decreases in brain serotonin concentrations on day three and day six (Table 4.1). But higher concentrations did not cause an additive effect. The same phenomenon was seen when comparing the time to capture prey to predictive additivity based on individual exposure effects (Table 4.2). Low concentration mixtures caused additive effects on the time to capture all prey by day six. These data indicate that once serotonin reaches its apparent basal threshold level, the capacity for effects increasing in an additive manner is diminished. There is also evidence in the literature of lower concentrations of antidepressants and antidepressant mixtures having significant effect when higher concentrations have no effect. Embryonic exposures to low concentrations of venlafaxine (500 ng/L) caused significant effects on escape latency behavior and total escape response while higher exposure concentrations did not cause any effects. After larval exposures to low concentrations of antidepressant mixtures, significant effects were seen on escape velocity and total escape response while higher concentration mixtures had no effect [18]. Low concentration exposure to venlafaxine caused significant mortality when compared to controls in exposed adult fathead minnows while higher concentrations caused less of an effect [34].
While we did not see additive effects for the two higher mixture exposures, we saw effects on brain serotonin and time to capture prey extend into the recovery period. Therefore, it appears that although brain serotonin concentrations did not decrease any further with higher mixture exposures, these exposures caused longer lasting effects even after exposure ceased. Serotonin has been implicated in controlling a number of physiological functions in fish both directly and indirectly [39]. Direct control can be achieved through modulation of serotonin in the brain as well as other organs that play a role in appetite and behavior. Serotonin containing neurons have been found in the enteric (gastrointestinal) nervous system of fish [40] and play a role in gut motility, which can influence appetite [41]. Serotonin containing cells have also been found in the pineal gland of fish, an organ that plays a role in behavioral and physiological functions [42]. Serotonin can indirectly affect downstream processes by controlling the release of hormones and peptides such as gonadotropin, growth hormone [43], cortisol [44-46], and corticotropin-releasing factor [47]. Therefore, it is possible that serotonin returned to control levels by day twelve in the brains of fish for the highest mixture treatment, but an unknown downstream response in other organs and hormones regulated by serotonin, may have continued to increase time to capture prey after depuration.

Though research on the effects of pharmaceutical mixtures is fairly limited, comparison of our results to the body of research on the toxicity of mixtures of other endocrine disruptors reveals that the results of this study are consistent
with previously shown additivity concepts. Normalizing mixture exposure concentrations to specific effect level was effective in predicting the additive effects of antidepressants in the current study. The same method has been successfully used to examine the effects of mixtures of polychlorinated biphenyls (PCBs), polychlorinated dibenzodioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) [27]. The development of estradiol equivalency factors has also been used to predict the additive effects of estrogenic mixtures in fish [31]. The same method has also been successfully employed for anti-androgens and thyroid-disrupting compounds [24]. The current study was also able to exemplify one of the underlying theories of the dose addition concept, which is that high concentration mixtures will not cause consistently increasing effects on a measured endpoint due to saturation of effects on molecular targets [24].

The similarities in the additivity of antidepressants in the current study and additivity of mixtures of other endocrine disrupting compounds in the literature, suggests that antidepressant mixtures present a significant risk to our environment. The current study showed that low concentrations of antidepressants act in an additive manner on fish brain chemistry and behavior. Therefore, pursuit of a more robust mixture exposure scenario may reveal that antidepressant mixtures at currently measured concentrations can produce additive effects that pose a significant risk to aquatic organisms. Development of a serotonin depression index of antidepressants may also assist in the prediction
of the effects of antidepressant mixtures found in the receiving waters of waste water treatment plant effluent.

Behavior plays a role in a number of daily activities that aquatic organisms must perform to survive. Effects on behavior can have a direct impact on organism fitness through disruption of feeding or predator avoidance. Reproduction in aquatic organisms often involves complex mating behaviors or displays that must be performed for individuals to contribute their genes to the next generation. Disruption of these behaviors can also indirectly impact an entire population in a negative way. Therefore, it is important to understand how contaminants that may not directly affect survival can impact sensitive behavioral traits. The results of the current study indicate that low concentration antidepressant mixtures have an additive effect on brain chemistry and behavior at an accelerated rate when compared to individual compounds. Therefore, these compounds present a significant risk and need to be further investigated.

Conclusions

Overall results from the current study indicate that antidepressant mixtures cause an immediate decrease in brain serotonin concentrations to an apparent basal level. Decreases in brain serotonin in turn caused an exponential increase in time to capture prey. Examination of brain serotonin concentrations and time to capture prey data for organisms exposed to antidepressant mixtures compared to those organisms exposed only to individual antidepressants
suggest that low concentration mixtures act in a predictively additive manner. Additive effects were unable to be predicted for higher concentrations mixtures, which could be expected due to the saturation of serotonin depression in the brains of exposed fish. The antidepressant mixtures in the current study acted in a similar manner to mixtures of other endocrine disrupting compounds suggesting that antidepressant mixtures present a significant risk to aquatic organisms.
Figure 4.1: Time to capture prey 1 for hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine.

Time to capture prey 1 for hybrid striped bass exposed mixtures of fluoxetine and venlafaxine for 6 days and allowed to recover for 6 days. Treatments were 0, 1, 2, 4 toxic units. Asterisks represent statistical significance when compared to control. Error bars represent ±1 standard error.
Figure 4.2: Time to capture prey 2 for hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine.

Time to capture prey 2 for hybrid striped bass exposed mixtures of fluoxetine and venlafaxine for 6 days and allowed to recover for 6 days. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control. Error bars represent ±1 standard error.
Figure 4.3: Time to capture prey 3 for hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine.

Time to capture prey 3 for hybrid striped bass exposed mixtures of fluoxetine and venlafaxine for 6 days and allowed to recover for 6 days. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control. Error bars represent ±1 standard error.
Figure 4.4: Brain norepinephrine concentrations in hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine for 6 days.

Brain norepinephrine concentrations for hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control on that day. Error bars represent ±1 standard deviation.
Figure 4.5: Brain dopamine concentrations in hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine for 6 days.

Brain dopamine concentrations for hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control on that day. Error bars represent ±1 standard deviation.
Figure 4.6: Brain serotonin concentrations in hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine for 6 days.

Brain serotonin concentrations for hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control on that day. Error bars represent ±1 standard deviation.
Figure 4.7: Brain 5-HIAA concentrations in hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine for 6 days.

Brain 5-HIAA concentrations for hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control on that day. Error bars represent ±1 standard deviation.
Figure 4.8: Brain 5-HIAA/5-HT ratios in hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine for 6 days.

Brain 5-HIAA/5-HT ratios for hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Treatments were 0, 1, 2, and 4 toxic units. Asterisks represent statistical significance when compared to control on that day. Error bars represent ±1 standard deviation.
Figure 4.9: Brain serotonin concentrations as a function of mixture concentrations.

Brain serotonin concentrations as a function of mixture concentrations in toxic units on days 3 and 6. Curves were fit using a logarithmic equations displayed below the curve for day 3 and above the curve for day 6. Correlation coefficients are displayed for the respective equations.

\[
y = 3.3762x + 92.558 \\
R^2 = 0.892
\]

\[
y = -1.2216x + 109.3 \\
R^2 = 0.2601
\]
Figure 4.10: Time to capture prey as a function of brain serotonin levels on day 6.

Time to capture prey 1 and 2 for hybrid striped bass exposed to venlafaxine for 6 days as a function of brain serotonin concentrations. Curves were fit using exponential equations. Respective equations and correlation coefficients are displayed above and below curves.
Table 4.1: Additivity analysis based on 1:1 additivity predicted from % serotonin decreases as a result of individual antidepressant exposures

<table>
<thead>
<tr>
<th>Day</th>
<th>Treatment (Toxic Units)</th>
<th>Fluoxetine (% decrease)</th>
<th>Venlafaxine (% decrease)</th>
<th>Mixtures (mean ± SD)</th>
<th>Predicted 1:1 Additivity</th>
<th>Additive?</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>13.57</td>
<td>15.28</td>
<td>34.37 ± 16.21</td>
<td>14.42</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td></td>
<td>29.67 ± 9.60</td>
<td>28.85</td>
<td>Additive</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td></td>
<td></td>
<td>26.87 ± 14.54</td>
<td>57.69 &lt; Additive</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>13.52</td>
<td>36.74</td>
<td>25.95 ± 10.16</td>
<td>25.13</td>
<td>Additive</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>22.65 ± 10.34</td>
<td>50.26 &lt; Additive</td>
<td></td>
</tr>
<tr>
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<td>4</td>
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<td></td>
<td>27.69 ± 8.62</td>
<td>100.52 &lt; Additive</td>
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</tbody>
</table>

Percent decrease in serotonin when compared to controls data from individual exposures to fluoxetine [21] and venlafaxine [22] are used to predict 1:1 additivity of antidepressant mixtures. Serotonin data for fluoxetine were from 23.2 ± 6.6 µg/L (mean ± SD) exposures and 35.68 ± 6.75 µg/L exposures for venlafaxine. Mixtures were determined to be additive if the predicted additivity fell within the standard error of the mean for each mixture.
Table 4.2: Additivity analysis based on 1:1 additivity of time to capture prey data from individual antidepressant exposures

<table>
<thead>
<tr>
<th>Day</th>
<th>Prey (seconds)</th>
<th>Fluoxetine (seconds)</th>
<th>Venlafaxine (seconds)</th>
<th>1 Toxic Unit mean ± SE (seconds)</th>
<th>Predicted 1:1 Additivity for 1 toxic unit</th>
<th>Additive?</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>50.79</td>
<td>175.43</td>
<td>99.9 ± 74.94</td>
<td>113.11</td>
<td>additive</td>
</tr>
<tr>
<td>2</td>
<td>414</td>
<td>340</td>
<td></td>
<td>182 ± 101.262</td>
<td>377</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>3</td>
<td>772</td>
<td>714.57</td>
<td></td>
<td>549.45 ± 160.15</td>
<td>743.285</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>1</td>
<td>397</td>
<td>135.56</td>
<td></td>
<td>410 ± 160.69</td>
<td>266.28</td>
<td>additive</td>
</tr>
<tr>
<td>2</td>
<td>575.25</td>
<td>501.83</td>
<td></td>
<td>616.07 ± 192.95</td>
<td>538.54</td>
<td>additive</td>
</tr>
<tr>
<td>3</td>
<td>1256.92</td>
<td>942.94</td>
<td></td>
<td>866.87 ± 184.02</td>
<td>1099.93</td>
<td>additive</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Prey (seconds)</th>
<th>Fluoxetine (seconds)</th>
<th>Venlafaxine (seconds)</th>
<th>2 Toxic Unit mean ± SE (seconds)</th>
<th>Predicted 1:1 Additivity for 2 toxic unit</th>
<th>Additive?</th>
</tr>
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<tbody>
<tr>
<td>3</td>
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<td>50.79</td>
<td>175.43</td>
<td>232.5 ± 102.70</td>
<td>226.22</td>
<td>additive</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>414</td>
<td>340</td>
<td>475.59 ± 137.90</td>
<td>754</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>3</td>
<td>772</td>
<td>714.57</td>
<td></td>
<td>661.59 ± 143.77</td>
<td>1486.57</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>1</td>
<td>397</td>
<td>135.56</td>
<td></td>
<td>294.13 ± 140.38</td>
<td>532.56</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>2</td>
<td>575.25</td>
<td>501.83</td>
<td></td>
<td>428.29 ± 150.76</td>
<td>1077.08</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>3</td>
<td>1256.92</td>
<td>942.94</td>
<td></td>
<td>723.29 ± 169.16</td>
<td>1500</td>
<td>&lt; additive</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Day</th>
<th>Prey (seconds)</th>
<th>Fluoxetine (seconds)</th>
<th>Venlafaxine (seconds)</th>
<th>4 Toxic Unit mean ± SE (seconds)</th>
<th>Predicted 1:1 Additivity for 4 toxic unit</th>
<th>Additive?</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>50.79</td>
<td>175.43</td>
<td>588.18 ± 151.97</td>
<td>452.44</td>
<td>additive</td>
</tr>
<tr>
<td>2</td>
<td>414</td>
<td>340</td>
<td></td>
<td>646.45 ± 144.72</td>
<td>1500</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>3</td>
<td>772</td>
<td>714.57</td>
<td></td>
<td>1054.2 ± 134.31</td>
<td>1500</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>397</td>
<td>135.56</td>
<td>638.41 ± 180.46</td>
<td>1065.12</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>2</td>
<td>575.25</td>
<td>501.83</td>
<td></td>
<td>821.24 ± 180.27</td>
<td>1500</td>
<td>&lt; additive</td>
</tr>
<tr>
<td>3</td>
<td>1256.92</td>
<td>942.94</td>
<td></td>
<td>1191.4 ± 132.98</td>
<td>1500</td>
<td>&lt; additive</td>
</tr>
</tbody>
</table>
Time to capture prey data from individual exposures to fluoxetine [21] and venlafaxine [22] are used to predict 1:1 additivity of antidepressant mixtures. Time to capture prey data for fluoxetine are from $23.2 \pm 6.6 \, \mu g/L$ (mean ± SD) exposures and $35.68 \pm 6.75 \, \mu g/L$ exposures for venlafaxine. 1, 2, and 4 toxic unit mixtures are compared in panels A, B, and C respectively. Mixtures were determined to be additive if the predicted additivity fell within the standard error of the mean for each mixture.
References


CHAPTER FIVE:
OVERALL CONCLUSIONS

Effects of long term exposure to fluoxetine on hybrid striped bass behavior

1. Long term exposure to fluoxetine did not decrease the toxic threshold of effect when compared to short term exposures.
2. Six day exposures are sufficient to examine the behavioral toxicity of fluoxetine

Effects of venlafaxine on the brain chemistry and behavior of hybrid striped bass

1. Venlafaxine caused an initial dose dependent decrease in brain serotonin concentrations of exposed hybrid striped bass and eventually caused a depression of serotonin to an apparent basal threshold level for all treatments.
2. Venlafaxine increased time to capture all prey for hybrid striped bass exposed at concentrations of 250 and 500 µg/L. The lowest concentration tested, 50 µg/L, only increased the time to capture prey 3.
3. At low concentrations venlafaxine appears to affect appetite while higher concentrations may also affect locomotor function
4. Brain serotonin concentrations were not predictive of changes in predation behavior
5. The differential behavioral toxicity of fluoxetine and venlafaxine can be explained by the higher affinity of fluoxetine for the serotonin transporter when compared to venlafaxine.

**Effects of mixtures of fluoxetine and venlafaxine on brain chemistry and behavior of hybrid striped bass**

1. Antidepressant mixtures caused a decrease in brain serotonin concentrations to an apparent basal threshold level for all treatments by day 3.

2. Time for bass to capture their prey was significantly affected by all antidepressant mixtures by day 6.

3. Low concentration mixtures acted in an additive manner on brain serotonin concentrations and time to capture prey while higher concentrations were less than additive.

4. The lack of additivity in higher concentration mixture treatments may be due to saturation of the antidepressant effects on serotonin transporters.

Examination of the behavioral effects of antidepressants is an ecologically relevant endpoint due to the unique mode of action of these chemicals. The results of my dissertation showed that use of this endpoint can provide insight into the potential sublethal effects of antidepressants that may not be captured during traditional toxicity testing. Our laboratory has now shown that two different antidepressants, fluoxetine and venlafaxine, cause significant effects on
the brain chemistry and behavior of hybrid striped bass. Low concentration mixtures of these two compounds were also shown to have additive effects on these endpoints. Therefore, the presence of low concentration mixtures of antidepressants in the environment may cause significant effects on fish behavior. Behavior can both directly and indirectly affect fish fitness and ultimately population health. As a result, inclusion of behavioral endpoints may be essential for the assessment of risk that antidepressants pose to aquatic organisms to ensure adequate protection of populations.
Table A-1: Time for hybrid striped bass exposed to fluoxetine to capture their prey

<table>
<thead>
<tr>
<th>Prey</th>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
<th>Day 15</th>
<th>Day 18</th>
<th>Day 21</th>
<th>Day 24</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>±SE</td>
<td>Mean</td>
<td>±SE</td>
<td>Mean</td>
<td>±SE</td>
<td>Mean</td>
<td>±SE</td>
<td>Mean</td>
</tr>
<tr>
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<td>Control</td>
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<td>2.24</td>
<td>8.21</td>
<td>3.93</td>
<td>29.16</td>
<td>18.11</td>
<td>11.71</td>
<td>17.11</td>
<td>29.64</td>
</tr>
<tr>
<td></td>
<td>10 µg/L</td>
<td>8.32</td>
<td>2.34</td>
<td>55.26</td>
<td>36.06</td>
<td>106.26</td>
<td>123.07</td>
<td>129.36</td>
<td>106.12</td>
<td>248.00</td>
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<tr>
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<td>20 µg/L</td>
<td>4.35</td>
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<td>125.53</td>
<td>89.28</td>
<td>129.85</td>
<td>114.26</td>
<td>443.46</td>
<td>187.77</td>
<td>71.50</td>
</tr>
<tr>
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<td>30 µg/L</td>
<td>5.37</td>
<td>1.39</td>
<td>250.63</td>
<td>127.62</td>
<td>318.16</td>
<td>122.11</td>
<td>11.45</td>
<td>14.60</td>
<td>387.86</td>
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<tr>
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<td>40 µg/L</td>
<td>3.25</td>
<td>0.85</td>
<td>47.81</td>
<td>19.93</td>
<td>338.31</td>
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<td>237.00</td>
<td>498.38</td>
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<td>Control</td>
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<td>5.02</td>
<td>61.26</td>
<td>45.68</td>
<td>119.53</td>
<td>81.24</td>
<td>75.14</td>
<td>41.15</td>
<td>105.80</td>
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<td>21.68</td>
<td>5.28</td>
<td>219.32</td>
<td>108.86</td>
<td>107.57</td>
<td>217.93</td>
<td>227.50</td>
<td>119.18</td>
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<td>1.86</td>
<td>284.24</td>
<td>140.82</td>
<td>353.59</td>
<td>140.20</td>
<td>593.77</td>
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<td>3.72</td>
<td>274.24</td>
<td>125.90</td>
<td>559.05</td>
<td>155.39</td>
<td>527.64</td>
<td>160.87</td>
<td>543.25</td>
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<tr>
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<td>40 µg/L</td>
<td>12.63</td>
<td>3.45</td>
<td>122.81</td>
<td>44.84</td>
<td>612.88</td>
<td>168.99</td>
<td>773.64</td>
<td>246.74</td>
<td>765.00</td>
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<td>117.53</td>
<td>77.47</td>
<td>331.58</td>
<td>142.28</td>
<td>20.14</td>
<td>115.43</td>
<td>319.71</td>
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<td>127.97</td>
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<td>141.26</td>
<td>408.36</td>
<td>160.87</td>
<td>621.79</td>
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<td>4.28</td>
<td>482.47</td>
<td>165.07</td>
<td>649.47</td>
<td>178.23</td>
<td>619.46</td>
<td>201.37</td>
<td>699.77</td>
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<td>30 µg/L</td>
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<td>7.92</td>
<td>513.68</td>
<td>158.82</td>
<td>923.74</td>
<td>159.21</td>
<td>712.79</td>
<td>178.80</td>
<td>833.29</td>
</tr>
<tr>
<td></td>
<td>40 µg/L</td>
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<td>27.22</td>
<td>629.31</td>
<td>177.44</td>
<td>1004.06</td>
<td>169.56</td>
<td>1007.00</td>
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<td>30 µg/L</td>
<td>65.21</td>
<td>18.15</td>
<td>846.74</td>
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<td>1304.26</td>
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</table>
Time for hybrid striped bass to capture four consecutive prey during exposure to fluoxetine for 27 days. Values are presented as means ± standard error. Data are presented graphically in figure 2.1, 2.2, and 2.3. Concentrations of 10, 20, 30, and 40 µg/L were measured at (mean ± SD) 9.949 ± 1.495, 18.59 ± 3.513, 27.42 ± 3.186, and 41.17 ± 5.506 µg/L, respectively.
### Table A-2: Time for hybrid striped bass exposed to venlafaxine to capture their prey

<table>
<thead>
<tr>
<th>Prey</th>
<th>Treatment</th>
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<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
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<tbody>
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<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
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<td>7.60 4.55</td>
<td>19.40 13.47</td>
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<tr>
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<td>50 µg/L</td>
<td>7.74 1.95</td>
<td>175.43 87.84</td>
<td>135.56 82.87</td>
<td>337.80 195.91</td>
<td>6.80 2.11</td>
</tr>
<tr>
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<td>250 µg/L</td>
<td>8.39 3.05</td>
<td>602.91 143.08</td>
<td>931.06 159.25</td>
<td>532.89 242.52</td>
<td>163.80 157.56</td>
</tr>
<tr>
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<td>500 µg/L</td>
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<td>336.95 133.33</td>
<td>833.86 177.61</td>
<td>397.38 240.73</td>
<td>116.20 107.97</td>
</tr>
<tr>
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<td>Control</td>
<td>17.17 5.57</td>
<td>230.70 98.06</td>
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<td>155.20 149.43</td>
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<tr>
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<td>50 µg/L</td>
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<td>25.35 10.77</td>
<td>817.39 129.89</td>
<td>1299.33 109.89</td>
<td>728.11 246.75</td>
<td>589.20 330.89</td>
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<tr>
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<td>500 µg/L</td>
<td>14.47 3.58</td>
<td>754.21 157.47</td>
<td>1083.36 161.46</td>
<td>427.00 235.07</td>
<td>571.80 333.13</td>
</tr>
<tr>
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<td>Control</td>
<td>52.43 16.92</td>
<td>402.00 127.93</td>
<td>372.94 134.87</td>
<td>310.70 198.24</td>
<td>213.00 177.60</td>
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<tr>
<td></td>
<td>50 µg/L</td>
<td>70.09 29.66</td>
<td>714.57 148.06</td>
<td>942.94 154.87</td>
<td>902.70 241.30</td>
<td>912.40 359.87</td>
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<tr>
<td></td>
<td>250 µg/L</td>
<td>25.35 10.77</td>
<td>817.39 129.89</td>
<td>1299.33 109.89</td>
<td>728.11 246.75</td>
<td>589.20 330.89</td>
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<td>14.47 3.58</td>
<td>754.21 157.47</td>
<td>1083.36 161.46</td>
<td>427.00 235.07</td>
<td>571.80 333.13</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>328.30 103.96</td>
<td>615.70 143.27</td>
<td>670.50 162.09</td>
<td>528.80 213.77</td>
<td>485.00 269.64</td>
</tr>
<tr>
<td></td>
<td>50 µg/L</td>
<td>395.04 121.32</td>
<td>1005.35 133.84</td>
<td>1236.67 104.62</td>
<td>924.60 235.58</td>
<td>916.20 357.52</td>
</tr>
<tr>
<td></td>
<td>250 µg/L</td>
<td>355.22 106.40</td>
<td>1283.96 105.62</td>
<td>1435.89 64.11</td>
<td>1223.89 184.39</td>
<td>1033.60 289.89</td>
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<tr>
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<td>500 µg/L</td>
<td>244.68 88.84</td>
<td>1225.74 119.29</td>
<td>1500.00 0.00</td>
<td>1193.88 203.16</td>
<td>959.80 332.30</td>
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</tbody>
</table>

Time for hybrid striped bass to capture four consecutive prey during 6-day exposures to venlafaxine followed by a 6 day recovery period. Values are presented as means ± standard error. Data are presented graphically in figure 3.2, 3.3, and 3.4. Concentrations of 50, 250, and 500 µg/L were measured at (mean ± SD) 35.68 ± 6.75, 198.67 ± 22.21, and 465.39 ± 43.68 µg/L, respectively.
<table>
<thead>
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<th>Prey</th>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
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<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
</tr>
<tr>
<td>5-HT</td>
<td>Control</td>
<td>166.81 26.31</td>
<td>144.22 24.98</td>
<td>123.24 23.40</td>
<td>130.60 28.74</td>
<td>150.24 37.95</td>
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<tr>
<td></td>
<td>50 µg/L</td>
<td>166.81 26.31</td>
<td>122.65 41.68</td>
<td>77.94 23.40</td>
<td>88.96 11.71</td>
<td>110.40 7.26</td>
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<td>250 µg/L</td>
<td>166.81 26.31</td>
<td>113.64 65.77</td>
<td>78.95 10.01</td>
<td>89.55 8.00</td>
<td>164.92 38.02</td>
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<td>500 µg/L</td>
<td>166.81 26.31</td>
<td>71.08 19.59</td>
<td>84.70 16.12</td>
<td>70.27 15.74</td>
<td>132.56 29.47</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>Control</td>
<td>87.37 11.83</td>
<td>82.06 10.27</td>
<td>68.63 17.29</td>
<td>50.28 9.50</td>
<td>72.93 12.29</td>
</tr>
<tr>
<td></td>
<td>50 µg/L</td>
<td>87.37 11.83</td>
<td>62.13 17.27</td>
<td>46.29 14.75</td>
<td>29.50 2.80</td>
<td>57.34 3.75</td>
</tr>
<tr>
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<td>250 µg/L</td>
<td>87.37 11.83</td>
<td>52.97 10.75</td>
<td>52.27 5.58</td>
<td>35.74 6.08</td>
<td>69.93 22.84</td>
</tr>
<tr>
<td></td>
<td>500 µg/L</td>
<td>87.37 11.83</td>
<td>48.23 12.64</td>
<td>27.90 8.11</td>
<td>26.80 2.46</td>
<td>55.00 15.92</td>
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</table>

Concentrations of 5-HT and 5-HIAA (pg/µg protein) in the brains of hybrid striped bass exposed to venlafaxine for 6 days followed by a 6 day recovery period. Values are presented as a mean ± standard deviation. Data are presented graphically in figures 3.7 and 3.8. Concentrations of 50, 250, and 500 µg/L were measured at (mean ± SD) 35.68 ± 6.75, 198.67 ± 22.21, and 465.39 ± 43.68 µg/L, respectively.
Table A-4: Time for hybrid striped bass exposed to mixtures of fluoxetine (FLX) and venlafaxine (VEN) to capture their prey

<table>
<thead>
<tr>
<th>Prey</th>
<th>Treatment</th>
<th>Day 0</th>
<th></th>
<th>Day 3</th>
<th></th>
<th>Day 6</th>
<th></th>
<th>Day 9</th>
<th></th>
<th>Day 12</th>
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</thead>
<tbody>
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<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
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<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
<td>Mean ±SE</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>5.95 ±1.6646</td>
<td>2.9 ±0.5754</td>
<td>3.6667 ±1.1939</td>
<td>6.7778 ±4.6838</td>
<td>2.5 ±0.9574</td>
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<tr>
<td></td>
<td>1 toxic unit</td>
<td>2.8 ±0.388</td>
<td>99.9 ±74.94</td>
<td>410 ±160.69</td>
<td>201.8 ±150.92</td>
<td>307 ±298.26</td>
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<tr>
<td></td>
<td>2 toxic units</td>
<td>9.45 ±4.2836</td>
<td>232.5 ±102.7</td>
<td>294.12 ±140.38</td>
<td>109 ±96.185</td>
<td>4.6 ±1.1662</td>
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<tr>
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<td>4 toxic units</td>
<td>9.27 ±3.2754</td>
<td>588.18 ±151.97</td>
<td>638.41 ±180.46</td>
<td>525.9 ±220.85</td>
<td>605.8 ±365.07</td>
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<tr>
<td>2</td>
<td>Control</td>
<td>17.75 ±6.5578</td>
<td>6.65 ±0.955</td>
<td>8.5333 ±2.3882</td>
<td>11.333 ±7.0455</td>
<td>5.5 ±1.5</td>
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<tr>
<td></td>
<td>1 toxic unit</td>
<td>6.45 ±0.6862</td>
<td>182 ±101.26</td>
<td>616.07 ±192.95</td>
<td>315 ±197.53</td>
<td>656.8 ±344.61</td>
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<tr>
<td></td>
<td>2 toxic units</td>
<td>18.90 ±8.5442</td>
<td>475.59 ±137.9</td>
<td>428.29 ±150.76</td>
<td>341.6 ±193.83</td>
<td>9.8 ±3.3226</td>
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<tr>
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<td>4 toxic units</td>
<td>32.63 ±17.587</td>
<td>646.45 ±144.72</td>
<td>821.24 ±180.26</td>
<td>563.4 ±217.56</td>
<td>621.4 ±358.79</td>
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<tr>
<td>3</td>
<td>Control</td>
<td>31.5 ±10.124</td>
<td>18.45 ±5.125</td>
<td>34.733 ±14.308</td>
<td>20.333 ±8.0691</td>
<td>9.75 ±2.3585</td>
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<td>1 toxic unit</td>
<td>13.75 ±2.8292</td>
<td>549.45 ±160.15</td>
<td>866.87 ±184.02</td>
<td>431.4 ±191.6</td>
<td>777.8 ±297.11</td>
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<td>2 toxic units</td>
<td>40.22 ±11.736</td>
<td>661.59 ±143.77</td>
<td>723.29 ±169.17</td>
<td>657.7 ±231.17</td>
<td>62 ±49.011</td>
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<td>4 toxic units</td>
<td>70.31 ±26.482</td>
<td>1054.2 ±134.31</td>
<td>1191.5 ±132.99</td>
<td>1071.4 ±218.37</td>
<td>646.2 ±349.11</td>
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<tr>
<td>4</td>
<td>Control</td>
<td>242.35 ±110.13</td>
<td>42.45 ±14.47</td>
<td>270.13 ±116.87</td>
<td>51 ±20.277</td>
<td>18.75 ±3.8595</td>
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<td>1 toxic unit</td>
<td>222.15 ±108.94</td>
<td>860.65 ±162.4</td>
<td>1110 ±172.89</td>
<td>809.7 ±206.8</td>
<td>1093.2 ±234.67</td>
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<td>2 toxic units</td>
<td>339.82 ±114.25</td>
<td>914.27 ±140.31</td>
<td>924.47 ±165.34</td>
<td>777.5 ±212.65</td>
<td>613.8 ±361.79</td>
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<td>4 toxic units</td>
<td>231.5 ±95.193</td>
<td>1298.6 ±99.131</td>
<td>1500 ±0</td>
<td>1115.8 ±195.72</td>
<td>1427.4 ±72.6</td>
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</table>
Time for hybrid striped bass to capture four consecutive prey during 6-day exposures to mixtures of fluoxetine and venlafaxine followed by a 6 day recovery period. Values are presented as means ± standard error. Data are presented graphically in figure 4.1, 4.2, and 4.3. Toxic units represent 15 µg/L FLX and 25 µg/L VEN (1 toxic unit), 30 µg/L FLX and 50 µg/L VEN (2 Toxic Units), 60 µg/L FLX and 100 µg/L VEN (4 Toxic Units).
<table>
<thead>
<tr>
<th>Prey</th>
<th>Treatment</th>
<th>Day 0</th>
<th>Day 3</th>
<th>Day 6</th>
<th>Day 9</th>
<th>Day 12</th>
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</thead>
<tbody>
<tr>
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<td>5-HT</td>
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<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
<td>Mean ±SD</td>
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<tr>
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<td>135.15 15.07</td>
<td>144.10 46.16</td>
<td>142.76 32.05</td>
<td>142.66 7.86</td>
<td>133.37 20.89</td>
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<td>135.15 15.07</td>
<td>94.58 23.36</td>
<td>105.70 14.51</td>
<td>126.42 21.21</td>
<td>117.94 21.18</td>
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<td>135.15 15.07</td>
<td>101.35 13.83</td>
<td>110.43 14.76</td>
<td>99.48 10.65</td>
<td>120.61 5.17</td>
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<td>135.15 15.07</td>
<td>105.38 20.95</td>
<td>103.23 11.53</td>
<td>106.31 14.63</td>
<td>120.70 10.38</td>
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<tr>
<td>5-HIAA</td>
<td>Control</td>
<td>55.55 8.55</td>
<td>54.96 15.92</td>
<td>55.54 7.29</td>
<td>49.61 4.37</td>
<td>42.23 4.75</td>
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<tr>
<td>1 toxic unit</td>
<td>55.55 8.55</td>
<td>33.66 9.17</td>
<td>33.11 4.30</td>
<td>39.37 7.45</td>
<td>31.23 4.91</td>
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<td>2 toxic units</td>
<td>55.55 8.55</td>
<td>36.04 8.47</td>
<td>33.45 6.24</td>
<td>29.44 4.29</td>
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<tr>
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<td>55.55 8.55</td>
<td>34.18 8.33</td>
<td>34.48 4.41</td>
<td>28.14 4.77</td>
<td>34.49 5.19</td>
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Concentrations of 5-HT and 5-HIAA (pg/µg protein) in the brains of hybrid striped bass exposed to mixtures of fluoxetine and venlafaxine for 6 days followed by a 6 day recovery period. Values are presented as a mean ± standard deviation. Data are presented graphically in figures 4.6 and 4.7. 1, 2 and 4 toxic units represent measured concentrations of 10.81 ± 1.36, 23.84 ± 2.63, and 55.47 ± 8.72 µg/L, respectively for fluoxetine and 15.50 ± 2.02, 32.43 ± 3.21, and 74.30 ± 11.32 µg/L, respectively for venlafaxine.