8-2008

Changes in Brain Biochemistry and Feeding Behavior in Hybrid Striped Bass Exposed to Environmental Contaminants

Kristen Gaworecki
Clemson University, kristeg@clemson.edu

CHANGES IN BRAIN BIOCHEMISTRY AND FEEDING BEHAVIOR IN HYBRID STRIPED BASS EXPOSED TO ENVIRONMENTAL CONTAMINANTS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

---

by
Kristen M. Gaworecki
August 2008

Accepted by:
Dr. Stephen J. Klaine, Committee Chair
Dr. Cindy M. Lee
Dr. Edward E. Little
Dr. Aaron P. Roberts
Dr. Thomas Schwdler

ABSTRACT

Biochemical endpoints are useful when determining contaminant exposure in aquatic organisms. However, behaviors can also be impaired by environmental contaminants. Links between changes in brain biochemistry and behavior have been made, and understanding these relationships could increase the utility of these endpoints in ecological risk assessments. The overall goal of this research was to determine relationships between an ecologically relevant fish behavior and brain biochemistry. To accomplish this goal, I developed a behavioral bioassay that quantified the time it took exposed hybrid striped bass (*Morone saxatilis x M. chrysops*) to capture unexposed prey, fathead minnows (*Pimephales promelas*). Chemically targeted brain neurotransmitters were also monitored, and the relationship with behavior was determined.

Six-day acute exposures to (1) a pesticide (diazinon) targeting acetylcholinesterase (AChE) and (2) a pharmaceutical (fluoxetine) targeting serotonin were conducted with a 6-day recovery periods. Brain biochemistry and behaviors were plotted against each other. Our results indicated that there was a threshold response between AChE activity and feeding behavior following diazinon exposure concentrations of 19.1 ± 0.7, 64.0 ± 2.0, and 101.9 ± 1.4 µg/l. By day 6, AChE activity was significantly inhibited in the low, medium, and high treatment groups by 66.3, 82.2, 86.4%, respectively. However, there were no signs of behavioral impairment in the lowest treatment group. During the 6-day recovery period, there were concentration- and duration-dependent changes in feeding behavior and AChE activity, in which time to capture prey decreased more rapidly than AChE activity increased. Following fluoxetine

exposures (23.2 ± 6.6, 51.4 ± 10.9, and 100.9 ± 18.6 µg/l), a linear response between decreased serotonin activity and increased feeding behavior was observed. However, maximum serotonin depression in the low, medium, and high treatment groups occurred on day 9 (day 3 of the recovery period) with concentrations at 23.7, 28.0, and 49.1% of controls, respectively. Our results also indicated that during the recovery period, there was a concentration- and duration-dependent increase in serotonin activity accompanied by a decrease in time to capture prey.

A 27-day chronic exposure to fluoxetine was also conducted at lower exposure concentrations (0.08 ± 0.02, 0.87 ± 0.12, 9.44 ± 0.82 µg/l) than the acute exposure. It was concluded that although fluoxetine can cause impaired serotonin levels and feeding behavior, this was not observed at more environmentally relevant concentrations over the 27 days.

ACKNOWLEDGEMENTS

I would like to thank my advisor, Stephen Klaine, for pushing me to do my best and never to give up. I would also like to thank my other committee members, Aaron P. Roberts, Thomas Schwedler, Ed Little, and Cindy M. Lee for their contributions and support in all aspects of my research throughout my work.

I would also like to thank everyone who has helped me achieve my goals, especially Ron Gossett. Every time I ever need help in this project (which was quite often) you were always right there to lend an hand and improve anything you could with my test setups and everything over at Cherry Farm. All that for the price of a brownie! I cannot thank you enough! I would also like to thank Kathy Bryant for always pointing me in the right direction and helping me whenever I asked; Norman Ellis for all his help with my analytical work; Tom Schwedler, Scott Davis, and Joe Tomasso for giving me their expert advice on fish; and Billy Bridges for his help with my statistics. Many thanks goes to all my labmates Anthony Sowers, Sarah White, Holly Zahner, Sarah Robinson, Joe Bisesi, Amber Stojak, Amanda Steffens, Neil Capper, and Aaron Edgington who all helped with my experiments. There is no way I would have been able to conduct my tests without your help. The fish thank you too because otherwise they would have starved!

Finally, I would like to thank my family for their support and encouragement from day one. And most of all, I would like thank Jeff Beckhorn for being so patient, understanding, thoughtful, and loving throughout our time here at Clemson. You helped me through the toughest times here without ever asking for anything in return.

TABLE OF CONTENTS

Page

Table of Contents (Continued) Page

Table of Contents (Continued) Page

LIST OF TABLES

LIST OF FIGURES

PREFACE

Brain biochemistry can affect an organism's behavior, the interaction between individuals, and ultimately the success of the entire population. Many environmental contaminants affect the nervous system including brain chemistry. These contaminants find their way into surface waters where they pose a risk to aquatic organisms, including fish. However, few researchers have quantified the relationship between brain chemistry and fish behavior. Such a relationship could be very useful in ecological risk assessment including making biochemical markers more ecologically relevant. While many researchers have quantified the response of biochemical or behavioral endpoints to sublethal chemical stress, few have attempted to establish a relationship between the two.

This dissertation consists of a literature review and three journal articles. The literature review discusses the connection between brain biochemistry and behavior, environmental contaminants that can act on the nervous system, and behavioral bioassays used to as tools for measuring effects of environmental stressors. The first journal article (Chapter 2) describes effects of a 6-day diazinon exposure followed by a 6-day recovery period on acetylcholinesterase activity and prey-capture in hybrid striped bass. The second journal article (Chapter 3) describes the effects of 6-day fluoxetine exposure followed by a 6-day recovery period on serotonin and prey capture in hybrid striped bass. The final journal article (Chapter 4) evaluates the effects of a longer fluoxetine exposure (27-day) on monoamine levels and prey capture in hybrid striped bass using lower concentrations than in the 6-day exposure detailed in Chapter 3.

CHAPTER 1: LITERATURE REVIEW

Connecting Brain Biochemistry and Behavior in Toxicology

In the past few decades, there has been an increase in research characterizing various biochemical endpoints. While these endpoints are easily correlated with organism exposure, they are not used as often in risk assessments because their ecological relevance is not as well defined. They have not yet been linked directly to impacts at the organism level, let alone at the population and community levels [1]. Therefore, correlating behavioral and physiological changes is an important research focus because it could enhance the prediction of population-level responses from biomarker data.

Behaviors are the result of genetic, biochemical, and physiological processes [2] that operate through the central nervous system (CNS) permitting an animal to exist in an optimal environment [3]. Changes in behaviors critical to organism survival (habitat selection, competition, predator avoidance, prey selection, and reproduction) can be induced through direct effects to the nervous system or indirect physiological alterations [2]. Therefore, it is important to know the relationship between biochemical and behavioral endpoints before we can fully determine the ecological relevance of change in either.

A major objective of the behavioral sciences is to characterize behaviors and identify the circumstances that bring it about and the consequences that change it [4]. Behavior provides a unique perspective between organisms and their environment and can be crucial for developing mechanistic causes of contaminant effects. In turn,

understanding the mechanisms responsible for these changes could serve as a valuable guide to further interpret behaviors [4,5].

Since behavior is regarded as the net output of the sensory, motor, and cognitive function in the nervous system, it can be a sensitive endpoint of chemical-induced neurotoxicity [6]. There are numerous sites of action for toxicants to affect the nervous system, so interference at any of these sites could block or alter the sequence of neural responses and inhibit or alter behavior [2]. Neurotoxicity has been defined as 'any adverse effect on the structure or function of the central and/or peripheral nervous system produced by chemical exposure', and exposure to neurotoxicants can result in sensory, motor, and cognitive dysfunction [6]. Thus, behaviors could also be impaired as a result of contaminant exposure and techniques have been derived from experimental neurology to detect and characterize such changes. However the degree of change can be dependent upon the chemical(s), concentration, and duration of exposure.

Ecological Relevance of Behavior

The effects of environmental contaminants can be studied at the biochemical and cellular levels, organismal level, and population or community level [7]. Each level of organization is important because they typically carry out different operations [4]. Many times we look at biochemical biomarkers because they are useful as early warning signs of chemical exposure [7], but it can also be assumed that changes at lower levels of organization could escalate to community level effect if left unabated [7]. Therefore, since behavior is an individual-level response having clear links to biochemistry and

population dynamics, changes in behavior could be an ecologically relevant endpoint for monitoring environmental stress.

Ecological fitness

Ecological fitness can include an organism's ability to find food and shelter, avoid predation, and reproduce. Ultimately, these behaviors are critical to the population. Behavioral responses are important for survival and ecological fitness because they are necessary for performing essential life functions like habitat selection, competition, predator avoidance, prey selection, and reproduction [2]. Unfortunately, environmental pollutants can pose serious risk to many aquatic organisms [5] altering normal behaviors, which could impair survival of an organism or the population.

Traditionally, regulatory guidelines for aquatic pollutants in natural ecosystems have been based on acute lethality tests like the 96-h LC50, but impacts on development, growth, and reproduction are also frequently studied [5]. While the development of water-quality criteria has often relied heavily on chemical concentrations causing mortality and impaired growth in laboratory exposures, this may not be the best predictor of impacts in the field when organisms are exposed to sublethal concentrations [3]. Acute tests useful for generating guidelines for preventing physiological death tend to ignore ecological death that could occur at sublethal concentrations of toxicants [5]. Therefore, behavioral indicators of toxicity may be ideal for assessing sublethal impacts of exposure [5] because many times, behavioral changes can occur before death with sublethal concentrations. While they may seem to be unharmed, organisms may be

unable to function normally [5] so by testing ecologically relevant behaviors, we can get a better idea about ecological fitness and effects at the population and community levels.

Population implications

Since environmental contaminants can elicit a wide variety of biochemical responses and adverse effects in an organism's behavior, reproduction, and development, it can be hypothesized that there may also be population level impacts in multiple species or in ecosystems [8]. While chemicals affect individual organisms, the ultimate level of concern may be the population or community level. Many times, the fate and effect of environmental toxicants has been studied with the aim of understanding how the structure and functioning of populations, communities and ecosystems are affected [9].

There are many factors to consider when assessing the impacts of an environmental contaminant. Dose, body burden, duration, and timing of exposure at critical life stages (age and development period) are all important considerations for assessing the adverse effects [8,10]. Many times effects are delayed and are not fully or obviously expressed until offspring reach maturity or middle age, even though critical exposure may have occurred during early embryonic, fetal, or neonatal life [10]. In addition, effects may be reversible or irreversible, immediate (acute) or latent (not expressed for a period of time) [8]. Therefore, toxicological effects observed within individual organisms do not necessarily all have the same potential to impact populations, nor should it be expected that effects would elicit population responses at the same exposure levels [8].

Environmental Contaminants and the Nervous System

There are a wide variety of contaminants released into the environment from residential, commercial, and industrial sources that can be toxic to people, fish, wildlife, and plants. Many of these contaminants may not be deadly at the levels found in the environment, but can possibility interact with the nervous system and cause adverse effects. Many toxic chemicals including metals, PCBs, PAHs, pesticides, and pharmaceuticals can affect either the central or peripheral nervous system [11] and cellular metabolism.

Pesticides

Classes

There are number of different types of pesticides that are available for use and have been classified as algaecides, bactericides, fungicides, herbicides, insecticides, and rodenticides, depending on the target pest. They are used for preventing, controlling, or lessening the damage caused by pests, and have mainly been used to protect crops. In this discussion, I will focus on insecticides, and more specifically, organophosphates and carbamates.

There has been a major shift in insecticide use from organochlorine (OC) compounds to organophosphorous (OP) and carbamate (CB) insecticides. The use of OCs in the U.S. began in the 1940s until the 1970s when most uses were banned or severely restricted when potential human health concerns and adverse ecological effects became apparent. In addition, OC insecticides (especially DDT) were resistant to degradation and have long environmental half-lives, causing harm long after their ban.

One reason OP and CB insecticides were favored for replacing OCs was that while they, too, are highly toxic, they are considered to have relatively short half-lives (2-4 weeks) and are readily metabolized and excreted [12, 13].

There are about 200 OPs and 50 CBs formulated into thousands of products available around the world for use in wetlands, rangelands, cultivated crops, forests, and rural and urban settings. However, 95% of OP products are used for agriculture and mosquito control [13]. OP insecticides came into wide-scale use in the US in the late 1960s and 1970s. By the late 1980s, they made up more than one third of registered pesticides [13] and accounted for approximately 65% of total insecticide use with seven of the top 11 insecticides used (in terms of mass applied) being OPs [12]. OPs widely used in the US included chloyprifos, malathion, methyl parathion, parathion, fenthion, and diazinon. However, some of these chemicals have been reviewed by regulatory agencies for environmental and public health concerns and are now classified as restricted-use pesticides in the US. While some OPs that have had most uses withdrawn or cancelled in the US, they may still be available in other countries despite their environmental concerns [13]. In addition, only eight of the 50 CBs are used for insect control on crops, forests, and rangelands and out of these eight, carbofuran, methomyl, and carbaryl account for more than 90% of the use [13].

Sources of Exposure

Pesticides are purposely introduced into the environment for many purposes including agriculture, forestry, transportation (weed control along roadsides and railways), household, and various commercial and industrial uses [12]. While

nonagricultural uses can be substantial in some areas, the majority of pesticide use (70-80%) has been for agricultural purposes [12]. Agricultural application practices include aerial spraying, near-ground spraying from a tractor, soil incorporation, chemigation, and direct application to plant foliage [12]. Except for mosquito control, nearly all OP and CB application is on terrestrial landscapes [13]. There are, however, some pesticides that have been applied directly to surface waters for controlling algae, macrophytes, insects, and fish parasites, but these applications are usually carried out by federal, state, and local government agencies, or through permits issued by these agencies [12].

In any case, it is believed that all pesticides may eventually enter an aquatic system, affecting a much larger number of species than originally intended [13]. They have been invariably detected in waters, soils, and vegetation outside the treated areas [13] as a result of applicator error, drift, runoff, or drainage induced by rain or irrigation [12]. Pesticides have been detected in every region of the United States where surface waters have been analyzed [12] and are present throughout most of the year in streams draining agricultural and urban watersheds; however, their occurrence does not necessarily cause adverse effects due to low concentrations (ng/L) detected [14,15]. In addition, OPs and CBs are comparably labile in the environment and do not bioaccumulate or biomagnify to any important degree in aquatic or terrestrial food chains [13].

Four main factors affect pesticide transport in runoff. First are rainfall intensity, duration, amount, and timing with respect to pesticide application. Second are soil texture, organic matter content, water content, and slope and topography of the field.

Third are physical and chemical properties of the pesticide including water solubility, acid/base and ionic properties, sorption properties, and persistence. Finally are agricultural management practices including pesticide formulation, application rate and placement, erosion control practices, plant residue management, use of vegetative buffer strips, and irrigation practices [12].

Pharmaceuticals

Classes

Each year the US Food and Drug Administration (FDA) approves several new drugs that are classified under different categories including estrogens for contraceptives and hormone replacement, analgesics (painkillers), non-steroidal anti-inflammatory drugs, antibiotics, anti-cancer drugs, blood-pressure medications, and antidepressants. Nicotine and caffeine are also considered to be drugs. In general, these medical substances can be divided into two groups: (a) medical substances used by humans and (b) veterinary medicines. While classes of human use drugs have been mentioned, veterinary medicines for domestic animals, poultry and livestock, and fish farms can also include antimicrobials/parasitics, hormones, non-steroidal anti-inflammatories, antidepressants, CNS agents, gastrointestinal agents, and cardiovascular agents [16].

Sources of Exposure

Drugs are similar to pesticides with regard to their contribution to water pollution, but unlike regulated pesticide disposal, drug disposal has not been regulated. Little is known about the extent of environmental occurrence, transport or ultimate fate after their intended use, yet just about every class of pharmaceutical has been identified in the

environment [17]. Sources of contamination include production facilities, hospitals, private households, veterinaries, agricultural farms (livestock, poultry, and fish), and wastewater treatment plants [18]. While the occurrence of pharmaceuticals and personal care products (PPCPs) in the environment is not a new phenomenon, it has only become more widely evident in the last decade as a result of continually improving chemical analysis methodologies with lower limits of detection for a wide array of xenobiotics [19].

PPCPs can be inadvertently released to the environment directly (disposal and wastage from external application) or indirectly (excretion, washing, and swimming). But municipal sewage (treated and untreated) is the major source for most drug classes and quantities [19]. Wastewater treatment plants (WWTPs) can process and remove most chemicals, but many pharmaceuticals are not fully removed [17, 20]. As a result, WWTP effluent has been a significant contributing source of pharmaceuticals in receiving streams. One reason is that drugs tend to be hydrophilic in order to pass through cell membranes [16]. This would require different treatment technologies for removal from wastewater than many other pollutants which tend to be somewhat hydrophobic. While effective wastewater treatment methods are being researched, reverse osmosis and granular activated carbon have been valuable in removing certain classes of pharmaceuticals from water. Unfortunately, implementing these technologies can be extremely expensive leaving drugs to flow continuously into waterways. But the fact that pharmaceuticals can be continually introduced to the aquatic environment (even at low concentrations) creates a sense of ‘persistence’ of compounds that otherwise many not be

environmentally stabile creating a chronic exposure scenario [20-22]. It has also been found that some drugs could be detected in fish tissue samples where the stream was almost entirely comprised of effluent discharge [21].

Terrestrial runoff from confined animal feeding operations, excreta from medicated pets and livestock, overflow or leakage from storage structures and wind-borne drift of agriculturally applied antimicrobials to crops have been additional sources of pharmaceutical contamination of terrestrial and aquatic environments [17, 19]. Medical substances used in fish farms have also been directly applied to receiving waters since the most convenient method of treating fish with antibiotics and chemotherapeutics is by the use of feed additives. Typically, large portions of medicated feed are not consumed, resulting in potential sediment accumulation affecting other aquatic organisms [16].

Neurotransmitters of Interest

The mammalian nervous system has over 30 substances classified as neurotransmitters identified. Neurotransmitters are chemical messengers that relay, amplify, and modulate signals between a neuron and another cell across a synapse [23]. This includes acetylcholine, amino acids (glycine and glutamate), and biogenic amines, which are products of amino acid decarboxylation (dopamine, norepinephrine, epinephrine, γ-aminobutyric acid, histamine, and serotonin).

Acetylcholine

The role of the neurotransmitter acetylcholine (ACh) in the firing of cholinergic synapses between motor neurons and skeletal muscle cells is well known. Acetylcholine

is present in somatic and autonomic motor neurons in the spinal cord and brain stem, in autonomic (parasympathetic) ganglia, and skeletal muscles [24]. It is a quaternary amine synthesized by the binding of choline to acetyl-coenzyme A by the enzyme choline acetyltransferase. Binding of ACh opens cation channels (mainly Na^+) in skeletal muscle cells, eliciting an action potential that spreads out in multiple directions to signal muscles to contract. In cardiac muscle fibers however, ACh can decrease contraction, likely due do differences in receptor structures [24].

One of the most commonly observed indicators of neural function is brain acetylcholinesterase (AChE). Acetylcholinesterase is responsible for degrading/hydrolyzing ACh to end cholinergic neural transmission and prevent ACh accumulation in and around a synapse [5, 25]. Located on post-synaptic membranes, AChE plays an important role in regulating nerve impulse transmission at cholinergic synapses. Once ACh is broken down to acetate and choline by AChE, choline is taken back up into the nerve terminal by high affinity transporter proteins [24]. This is one of the most important factors in regulating the synthesis of ACh [24]. Since ACh molecules involved in a nerve impulse must be degraded in the few milliseconds before the potential arrival of the next nerve impulse [26], AChE plays a significant role in this process. A single AChE molecule can break down 5,000 ACh molecules to choline and acetate following their release into the synapse [24].

Serotonin

Serotonin is found thorough out the body in blood platelets, mast cells, and chromaffin cells in the gut; but serotonergic neurons are found almost exclusively in a

group of nuclei near the midline of the brain stem reticular formation, called the raphe nuclei [24]. Serotonin is a neurotransmitter involved in the transmission of nerve impulses, synthesized from the amino acid, tryptophan. Tryptophan is converted to 5-hydroxytryptophan (5-HTP) by the enzyme tryptophan-5-hydroxylase. Next, 5-HTP is converted to serotonin (5-hydroxytryptamine, 5-HT) by 5-hydroxytryptophan decarboxylase [24]. While the main metabolite of 5-HT is 5-hydroxyindoleacetic acid (5-HIAA), via aldehyde dehydrogenase or monoamine oxidase, 5-HT can also be converted to melatonin by 5-hydroxyindole-*O*-methyltransferase.

Like other neurotransmitters, 5-HT is released from the presynaptic cell and can bind to receptor proteins on both the pre- and post-synaptic cell. It can change the electrical state of the cell by exciting the cell, passing along the chemical message, or inhibiting it. Neurotransmitter action can be stopped by diffusion out of the synaptic cleft or through enzymatic degradation by monoamine oxidase (MAO) or catechol-*O*-mehtyltransferase (COMT) [27]. Present in the synaptic cleft and presynaptic nerve, MAO and COMT deactivate neurotransmitters making them unrecognizable by the receptors [24]. But under normal circumstances, the principle mechanism of signal inactivation is transporter-mediated uptake of monoamines from the synapse back into the presynaptic cell where they are reprocessed [28].

Transporter proteins in the membrane of nerve terminals put an end to transmitter action and control extracellular concentrations of monoamines. The task of transporters is not to remove all traces of neurotransmitters from the extracellular fluid, but rather to regulate a baseline concentration [24]. Since secretion and elimination of serotonin is

highly regulated, there are mechanisms in place for controlling moderate fluctuations via negative feedback control [8]. Autoreceptors located on the pre-synaptic membrane can modulate transmitter release as a kind of negative feed back control [24].

Since serotonergic neurons have axons that project to many different parts of the brain, 5-HT affects several behaviors [29]. Serotonin is involved in the control of appetite, sleep, learning and memory, temperature regulation, mood, behavior (aggression and dominance), cardiovascular function, muscle contraction, endocrine regulation, and depression. It has also been implicated in the central control of regulation of circadian rhythm, cognitive ability, reproduction/sexual behavior, memory, and attention [29-32].

Mechanisms

Acetylcholinesterase Inhibitors

The principle toxicity of organochlorine and carbamate insecticides is based on nervous system disruption by inhibition of cholinesterase activity in the CNS and at the neuromuscular junctions [13]. For example, OPs interact with a hydroxyl group on AChE, which is a functional part of the enzyme. Once phosphorylated, AChE has no activity [23]. While the mode of action is similar for OP and CB pesticides, there are many differences between these classes. One difference is the faster onset of acute toxicity by CBs as a result of direct ChE inhibition, whereas most OPs must first undergo an oxidative desulfuration step for maximum potency [13].

Secondly, unlike OPs, CBs are considered to bind reversibly allowing cholinesterase to become reactivated. However, it is possible for AChE levels to recover following OP exposure, but the inhibitory effects on AChE activity lasts longer than the

original exposure [33, 34]. A rapid recovery within a few hours may follow CB exposure, but it may take 1-3 weeks for AChE recovery following a single OP exposure [13]. However, the time it takes for AChE levels to fully recover is dependent on the rate of new enzyme synthesis, species, type and concentration of OP, and overall degree of AChE inhibition [35-37]. Therefore, cumulative depression of AChE may occur and persist from repeated exposure to some OPs, but generally not with CBs.

When OPs bind to ChE, a relatively stable bond is formed preventing deactivation of acetylcholine, and thus permitting a buildup of ACh and an overstimulation of the cholinergic nervous system. If this disruption is prolonged, the system for relaying impulses across the post-synaptic membrane becomes rundown, leading to synaptic block. This causes muscle rigidity from continual stimulation and can lead to paralysis and possibly death due to respiratory failure [23, 38, 39]. Following a sublethal exposure, carbohydrate metabolism, reproduction, and behavior can also be impaired [38].

OP and CB insecticides have a broad-spectrum toxicity and the relationship between depressed AChE activity and behavior has been studied in many species ranging from invertebrates to mammals [23]. There are considerable differences across species in the degree of AChE inhibition that can be tolerated without physiological impairment, which are attributed to variations in rates of uptake, detoxification, activation, and/or excretion [25, 35, 38, 40]. The degree of AChE inhibition is also dependent upon which insecticide the organism is exposed to [34, 38, 41, 42], exposure concentration [43] and

exposure duration [44]. Organism age can also be a factor affecting the degree of AChE inhibition [45].

Overall, it appears that a 70-80% decrease in brain AChE activity can be tolerated before death occurs in fish, so care should be taken when interpreting results of AChE measurements in fish brains because this may not be the ultimate 'cause of death' [25]. This interpretation must consider the fact that the test chemical often acts on a variety of points in the endocrine and nervous systems simultaneously. Mortality may be due to inhibition of other enzymes, especially those taking part in carbohydrate and protein metabolisms, rather than just AChE inhibition [46]. Pesticides may also cause oxidative stress in an organism, leading to the generation of reactive oxygen species at levels surpassing antioxidant defenses. This could, in turn, result in harmful effects on DNA, proteins, and lipids [46]. In addition, sublethal doses of diazinon has been shown to negatively affect blood stream estradiol levels [47] and testis structure [48] of bluegill, which could potentially impair reproductive success.

Brain neurotransmitter levels and enzyme activity correlate well with behavioral states [5]. Many researchers have considered the relationship between brain AChE activity and various behaviors including swimming [42, 38, 49-51] and foraging/feeding [33, 52]. Sometimes there were linear relationships between changes in behavior and AChE, but many times there were significant decreases in AChE activity before behavior responses were noted [39]. Whether a linear relationship or a threshold response is noted, changes in behavior could eventually alter an organism's ecological fitness leaving it

more susceptible to predation, less efficient at capturing prey, or unable to successfully court or reproduce. These effects could ultimately affect the population.

Selective Serotonin Reuptake Inhibitors (SSRI)

As stated previously, serotonin is involved in controlling a number of behaviors. It has been shown that decreased levels of synaptic serotonin (5-HT) and/or norepinephrine (NE) can give rise to depression, obsessive thoughts, and a lack of impulse control. Therefore, drugs like selective serotonin reuptake inhibitors (SSRIs), selective norepinephrine reuptake inhibitors (SNRI), and selective serotonin and norepinephrine reuptake inhibitors (SSNRI) were developed to lessen these ailments [28]. Reuptake inhibitors elicit their effect of increasing levels of specific monoamines at the synapse by interacting with, and binding to, monoamine transporters in the CNS, without being transported themselves [28]. By blocking transporters in inhibiting the recapture of neurotransmitters from the synapse, extracellular 5-HT and/or NE concentrations can become elevated [53].

SSRIs have a high affinity to 5-HT uptake sites, low affinity to NE uptake sites, and even lower affinity for neurotransmitter receptors [53]. Inhibition of 5-HT reuptake transporters promotes 5-HT neurotransmission, but autoreceptor activation could signal a decrease in neurotransmission once high levels of 5-HT are recognized [54]. Therefore, although transporters are blocked immediately following administration of reuptake-inhibiting drugs, it is believed that the 2-3 week delay in noticeable therapeutic effects (increased 5-HT levels) is attributed to autoreceptor activation [55]. Many studies have shown that chronic SSRI treatment eventually leads to the functional desensitization of 5-

HT_{1A} autoreceptors on serotoninergic cells, allowing serotonergic neurotransmission to occur in the presence of the drug and high extracellular 5-HT [55, 56]. Thus, therapeutic effects were felt when extracellular 5-HT levels increased.

The use of SSRIs has been rapidly increasing and they have become a focus for environmental researchers following their detection in the environment [57]. Brooks et al. [58] evaluated the acute effects of fluoxetine on algae (*Pseudokirchneriella subcapitata*), *Ceriodaphnia dubia*, *Daphnia magna*, *Pimephales promelas*, *Hyalella azteca* and *Chironomus tentans*, while Henry and Black [59] looked at the effects on western mosquitofish (*Gambusia affinis*). All organisms were adversely affected by the fluoxetine exposure in the laboratory, however, the concentrations were at least an order of magnitude greater than those reported in municipal effluent. Other effects noted in the laboratory included developmental abnormalities in Japanese medaka (*Oryzias latipes*) with minimal effects on number of eggs produced, fertilized, or hatched when exposed to fluoxetine [60]; and increased spawning in zebra mussels (*Dreissena polymorpha*) [61], increased parturition in fingernail clams [62], and reduced mean number of neonates produced in *Ceriodaphnia dubia* [63] when exposed to various SSRIs.

Exposure duration could play just as much a role in the effects of fluoxetine exposure as the exposure concentration, especially considering the role of autoreceptors in regulating neurotransmission. It is possible that initially, SSRI exposure could lead to decreased 5-HT levels from activation of autoreceptors; but prolonged exposures could cause autoreceptor desensitization allowing for 5-HT levels to increase. Therefore, measuring 5-HT levels along with other endpoints may be important since both increases

and decreases in these levels have been noted depending on the species and duration [54]. For example, it was found that a 7-day fluoxetine exposure reduced *Pimephales promelas* feeding rates in a dose dependent manner, but a 21-day fluoxetine exposure increased *Daphnia magna* grazing rates, though not significantly [64]. These results could reveal an effect of exposure duration and/or species variability, but the authors did not measure serotonin levels, nor did they monitor feeding/grazing over the course of exposure. Therefore, aside from behavioral observations, no real conclusion can be drawn on biochemical changes as a result of the exposure or the duration.

Many behavioral studies evaluating the effects of SSRIs on aquatic organisms have not compared 5-HT levels with observed behavioral changes. It has been usually assumed that serotonin levels increased regardless of what is know about the delayed therapeutic effect of SSRIs. However, this may not always be a correct assumption. For example, a behavioral study with *Betta splendens* showed decreased territorial aggression following acute treatment with 5-HT [65]. This suggested that increased 5-HT levels lead to decreased aggression. Therefore, since the goal of SSRIs is to increase 5-HT levels, it may be assumed that fluoxetine exposure would also decrease aggression. However, when *Betta splendens* were exposed chronically to fluoxetine, they neither exhibited significantly decreased aggression nor increased serotonin levels [65]. Instead, Clotfelter et al. [65] found that the exposure reduced serotonin and 5-HIAA levels, which is actually consistent with long-term exposures for a number of rodent studies. This again supports the importance of exposure duration when interpreting the effects of reuptake-inhibiting drugs.

Behavioral bioassays

Behavioral toxicity occurs when a contaminant or other stressor induces changes that exceed the normal range of variability [2]. Sometimes, this can be observed at levels much lower than the LC50. A single behavioral parameter can be more comprehensive than a physiological or biochemical parameter, but behavioral bioassays have still not reached the stage where they are fully accepted as part of formal testing procedures [23]. The extent to which behavioral studies could be used in risk assessment depends on the validity and understanding of the biochemical effect of the chemical [6]. Still, behavioral tests have been frequently used to identify and characterize chemical-induced alterations in endocrine and nervous system functions, and better predict exposure concentrations that impact ecological fitness - not just survival. There are a number of behavioral assays developed to assess sublethal effects of environmental stressors in fish including reproduction, avoidance, schooling, aggression, swimming, predator avoidance, and feeding. However, it should be mentioned that generalizations regarding any behavioral response to aquatic contaminants are difficult to make due to the variety of species and experimental designs used for each test [2].

The most described predictor of population level effects is to measure reproductive success [5]. Since reproduction is extremely important for population success, it is likely the most relevant for predicting ecological consequences of contamination [2]. Reproduction results from a variety of behaviors including migration to reproductive habitats, establishment of territories, reception and response to courtship, spawning, nest preparation and defense, and parental care [2]. Tests can be performed

for analyzing these behaviors/interactions along with other reproductive measurements like time-to-maturation, population sex ratios, expression of secondary sexual characteristics, clutch size, and percent hatch/survival. Impairment of any of these behaviors could reduce reproductive success and ultimately harm the population.

Many contaminants can also induce avoidance responses. This behavioral response has been observed for over 80 years [23]. Avoidance of unfavorable habitats can be induced by a contaminant, but the opposite could also occur. If a chemical attracts an organism, it could leave it vulnerable to injury or death [2]. This response can be measured by assessing habitat selection of treated and untreated organisms. Treated organisms could be more inclined or less inclined to avoid unfavorable conditions, or less responsive to present danger (i.e. predators, extreme temperatures) [2]. However, pre-exposure to a contaminant could skew responses, leading to an acclimation or desensitization to the chemical(s). This could lessen the behavioral response and underestimate the concentration that would elicit such a response [23].

Social interactions among fish such as schooling and aggression can also be impaired by environmental contaminants. Schooling is highly evolved among fishes for increasing habitat surveillance and providing protection from predators [2]. Many contaminants impair the schooling behavior of fish so some methods for measuring schooling behavior include measuring distance between individuals, orientation within a school, and latency with which the school forms or tighten [2]. Methods for measuring aggression include monitoring changes in posture or coloration, and movement toward or contact between conspecifics [2]. Competition among individuals, species, or age classes

can occur when resources needs overlap, making aggression necessary for survival. Therefore, the more aggressive organism will be further ecologically fit with the capability to win food, shelter, or a mate [2].

Swimming behavior is another fundamental behavior that can be disturbed by environmental contaminants. This behavior is extremely important because it is fundamental to feeding, competition, predator avoidance, and reproduction [2]. Swimming includes frequency and duration of movements, speed and distance traveled, frequency and angle of turns, position in the water column, form and pattern of swimming, orientation to water flow, and the capacity to swim against a current [2]. Since many of these variables are interrelated, they can be measured simultaneously. Swimming behaviors, like other behaviors, vary across species and life stage, so test methods must be tailored accordingly [2].

An altered ability to detect or respond to predators can increase an organism's vulnerability to predation. Therefore, predator avoidance is another useful behavioral measurement of environmental stress and can be measured by subjecting equal numbers of exposed and unexposed prey to a predator under ideal conditions and observing which prey population is more susceptible to predation. While this can be useful for determining effective concentrations, the link between contaminant exposure and predator avoidance is not as straightforward. Sometimes exposed prey may become inactive or have reduced mobility making them less obvious to a predator, thereby making the unexposed prey more obvious [2].

Feeding behaviors are important for development, fitness, and long-term viability of an organism [2]. There are multiple aspects of feeding that can be impaired by environmental stressors and contaminants leading to reduced growth and survival. These include abilities for foraging, detecting, pursuing, capturing, and consuming food. Methods for measuring these behaviors include orientation to food, movement toward and striking activities, prey selectivity, feeding efficiency (number of prey attacked and captured), prey-handling time, strike and capture frequency (including spits and misses), and reaction distance [2]. Although there are numerous methods for measuring changes in feeding in several species, many of these behaviors are interconnected, so measuring multiple aspects of feeding is possible within a single test design [2].

The goal of this dissertation was to better characterize the relationship between brain chemistry and behavior in hybrid striped bass. This goal was achieved through the following objectives:

1. Characterize the changes in predatory behavior as a function of reduced brain acetylcholinesterase caused by diazinon exposure.
2. Characterize the changes in predatory behavior as a function of reduced brain serotonin caused by short-term exposure to fluoxetine.
3. Characterize the changes in predatory behavior and brain serotonin associated with long-term exposure to fluoxetine.

References

1. Chapman PM. 2002. Integrating toxicology and ecology: putting the “eco” into ecotoxicology. *Mar. Pollut. Bull.* 44:7-15.

2. Little EE. 2002. Behavioral measures of environmental stressors in fish. In: Adams SM (Ed.). *Biological Indicators of Aquatic Ecosystem Stress*. American Fisheries Society, Bethesda, MD. pp. 431-472.

3. Little EE, Fairchild JF, DeLonay AJ. 1993. Behavioral methods for assessing impacts of contaminants on early life stage fishes. *Am. Fish. Soc. Symposium* 14:67-76.

4. Bechtel W. 2005. The challenge of characterizing operations in the mechanisms underlying behavior. *J. Exp. Anal. Behav.* 84:313-325.

5. Scott GR, Sloman KA. 2004. The effects of environmental pollutants on complex fish behavior: integrating behavouiral and physiological indicators of toxicity. *Aquat. Toxicol.* 68:369-392.

6. Tilson HA. Neurobehavioral methods used in neurotoxicological research. *Toxicol. Lett.* 68: 231-240.

7. Weis JS, Smith G, Zhou T, Santiago-Bass C, Weiss P. 2001. Effects of contaminants on behavior: biochemical mechanisms and ecological consequences. *BioScience* 51:209-217.

8. Crisp TM, Clegg ED, Cooper RL, Wood WP, Anderson DG, Baetcke KP, Hoffmann JL, Morrow MS, Rodier DJ, Schaeffer JE, Touart LW, Zeeman MG, Patel YM. 1998. Environmental endocrine disruption: an effects assessment and analysis. *Environ. Health Persp.* 106:11-56.

9. Maltby L. 1999. Studying stress: the importance of organism-level responses. *Ecol. Appl.* 9:431-440.

10. Colborn T, vom Saal FS, Soto AM. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. *Environ. Health Persp.* 101:378-384.

11. Crosby DG. 1998. *Environmental Toxicology and Chemistry*. Oxford University Press, New York, NY.

12. Larson SJ, Capel PD, Majewski MS. (Eds.). 1997. *Pesticides in Surface Waters*. Ann Arbor Press, Chelsea, MI.

13. Hoffman DJ, Rattner BA, Burton GA, Cairns J. (Eds.). 2003. *Handbook of Ecotoxicology*. 2nd Edition. Lewis Publishers; CRC Press, Boca Raton, FL.

14. Kimbrough RA, Litke DW. 1996. Pesticides in streams draining agricultural and urban areas in Colorado. *Environ. Sci. Technol*. 30:908-916.

15. Gilliom RJ, Hamilton PA. 2006. Pesticides in the nation's streams and ground water, 1992-2001- a summary. USGS Fact Sheet 2006-3028.

16. Halling-Sorensen B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lutzhoft HC, Jorgensen SE. 1998. Occurrence, fate, and effects of pharmaceutical substances in the environment – a review. *Chemosphere* 36:357-393.

17. Koplan DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic watewater contaminants in US streams, 1999-2000: a national reconnaissance. *Environ. Sci. and Technol.* 36:1202-1211.

18. Christensen FM. 1998. Pharmaceuticals in the environment – a human risk? *Regul. Toxicol. Pharm.* 28:212-221.

19. Daughton CG. 2001. Pharmaceuticals and personal care products in the environment: overreaching issues and overview. In: Daughton CG, Jones-Lepp TL. (Eds.). *Pharmaceuticals and Personal Care Products in the Environment.* American Chemical Society, Washington, DC. pp. 2-38.

20. Furlong ET. 2006. A Happy Medium? Antidepressants in Aquatic Systems. Annual Meeting of Society of Environmental Toxicology and Chemistry, Montreal, QB.

21. Brooks BW, Chambliss K, Stanley JK, Ramirez A. 2005. Determination of selected antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* 24:464-469.

22. Dove A. 2006. Drugs down the drain. *Nat. Med.* 12:376-377.

23. Walker CH, Hopkin SP, Sibly RM, Peakall DB. (Eds.). 1996. *Principles of Ecotoxicology.* 2nd Edition. Taylor and Francis Group, London, UK.

24. Brodal P. 2004. Functional properties of neurons. In: *The Central Nervous System.* 3rd Edition. Oxford University Press, Oxford, UK.

25. Little EE. 1987. Behavior and nervous system function. In: Heath AG (Ed.). *Water Pollution and Fish Physiology.* CRC Press, Boca Raton, FL. pp 181-200.

26. Voet D, Voet JG. (Eds.). 2004. *Biochemistry.* 3rd Edition. John Wiley & Sons, Hoboken, NJ.

27. De la Torre JC. 1972. Metabolism of monoamines. In: *Dynamics of Brain Monoamines*. Plenum Press, New York, NY.

28. Rothman RB, Baumann MH. 2003. Monoamine transporters and psychostimulant drugs. *Eur. J. Pharmacol.* 479: 23-40.

29. Øverli Ø, Winberg S, Damsgård B, Jobling M. 1998. Food intake and spontaneous swimming activity in Arctic char (*Salvenlinus alpinus*): role of brain serotonergic activity and social interactions. *Can. J. Zool.* 76:1366-1370.

30. Lin X, Volkoff H, Narnaware Y, Bernier NJ, Peyon P, Peter RE. 2000. Brain regulation of feeding behavior and food intake in fish. *Comp. Biochem. Phys. A* 126:415-434.

31. Sari, Y. 2004. Serotonin$_{1B}$ receptors: from protein to physiological function and behavior. *Neurosci. Biobehav. R.* 28:565-582.

32. Sloman KA, Lepage O, Rogers JT, Wood CM, Winberg S. 2005. Socially-mediated differences in brain monoamines in rainbow trout: effects of trace metal contaminants. *Aquat. Toxicol.* 71:237-247.

33. Morgan MJ, Kiceniuk JW. 1990. Effect of fenitrothion on the foraging behavior of juvenile Atlantic salmon. *Environ. Toxicol. Chem.* 9:489-495.

34. Ferrari A, Venturino A, Pechne de D'Angelo AM. 2004. Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphosmethyl, parathion, and carbaryl exposure in the goldfish (*Carassius auratus*). *Ecotox. Environ. Safe.* 57:420-425.

35. Weiss CM. 1972. Physiological effect of organic phosphorus insecticides on several species of fish. *Trans. Am. Fish. Soc.* 90:143-152.

36. Pan G, Dutta HM. 1998. The inhibition of brain acetylcholinesterase activity of juvenile largemouth bass *Micropterus salmoides* by sublethal concentrations of diazinon. *Environ. Res. A.* 79:133-137.

37. Sancho E, Ferrando MD, Anereu E. 1997. Response and recovery of brain acetylcholinesterase activity in the European eel, *Anguilla anguilla*, exposed to fenitrothion. *Ecotox. Environ. Safe.* 38:295-209.

38. Beauvais SL.,Jones SB, Brewer SK, Little EE. 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. *Environ. Toxicol. Chem.* 19:1875-1880.

39. Fulton MH, Key PB. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. *Environ. Toxicol. Chem.* 20:37-45.

40. Keizer J, D'Agostion G, Nagel R, Volpe T, Gnemi P, Vittozzi L. 1995. Enzymological differences of AChE and diazinon hepatic metabolism: correlation of in vitro data with the selective toxicity of diazinon to fish species. *Sci. Total Environ.* 171:213-220.

41. Coppage DL. 1972. Organophosphate pesticides: specific level of brain AChE inhibition related to death in sheepshead minnows. *Trans. Amer. Fish. Soc.* 3:534-536.

42. Dembélé K, Haubruge E, Gaspar C. 2000. Concentration effects of selected insecticides on brain acetylcholinesterase in the common carp (*Cyprinus carpio* L.). *Ecotox. Environ. Safe.* 45:49-54.

43. Dutta HM, Munshi SD, Dutta GR, Singh NK, Adhikari S, Richmonds CR. 1995. Age related differences in the inhibition of brain acetylcholinesterase activity of Heteropneustes fossilis (Bloch) by malathion. *Comp. Biochem. Physiol. A* 111A:331-334.

44. Naddy RB, Klaine SJ. 2001. Effect of pulse frequency and interval on the toxicity of chlorpyrifos to *Daphnia magna*. *Chemosphere* 45:497-506.

45. Moser VC. 2000. Dose-response and time-course of neurobehavioral changes following oral chloryprifos in rats of different ages. *Neurotoxicol. Teratol.* 22:713-723.

46. Üner N, Oruç EO, Sevgiler Y, Sahin N, Durmaz H, Usta D. 2006. Effects of diazinon on acetylcholinesterase activity and lipid peroxidation in the brain of *Oreochromis niloticus. Environ. Toxicol. Pharm.* 21:241-245.

47. Maxwell LB, Dutta HM. 2005. Diazinon-induced endocrine disruption in bluegill sunfish, *Lepomis macrochirus*. *Ecotox. Environ. Safe.* 60:21-27.

48. Dutta HM, Meijer HJM. 2003. Sublethal effects of diazinon on the structure of the testis of bluegill, *Lepomis macrochirus*: a microscopic analysis. *Environ. Pollut.* 125:355-360.

49. Cripe GM, Goodman LR, Hansen DJ. 1984. Effect of chronic exposure to EPN and guthion on the critical swimming speed and brain acetylcholinesterase activity of *Cyprinodon variegatus*. *Aquat. Toxicol.* 5:255-266.

50. Kwak IS, Chon TS, Kang HM, Chung NI, Kim JS, Koh SC, Lee SK, Kim YS. 2002. Pattern recognition of the movement tracks of medaka (*Oryzias latipes*) in response to sub-lethal treatments of an insecticide using artificial neural networks. *Environ. Pollut.* 120:671-681.

51. Sandahl JF, Baldwin DH, Jenkins JJ, Scholz NL. 2005. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. *Environ. Toxicol. Chem.* 24:136-145.

52. Pavlov DD, Chuiko GM, Gerassimov YV, Tonkopiy VD. 1992. Feeding behavior and brain acetylcholinesterase activity in bream (Abarmis brama L.) as affected by DDVP, an organophosphorus insecticide. *Comp Biochem Physiol C* 103:563-568.

53. Hiemke C, Härtter S. 2000. Pharmacokinetics of selective serotonin reuptake inhibitors. *Pharmaco. Therapeut.* 85:11-28.

54. Frankfurt M, McKittrick CR, Luine VN. 1994. Short-term fluoxetine treatment alters monoamine levels and turnover in discrete brain nuclei. *Brain Res.* 650:127-132.

55. Le Poul E, Boni C, Hanoun N, Laporte A, Laaris N, Chauveau J, Hamon M, Lanfumey L. 2000. Differential adaptation of brain 5-HT_{1A} and 5-HT_{1B} receptors and 5-HT transporter in rats treated chronically with fluoxetine. *Neuropharmacology* 39:110-122.

56. Dawson LA, Nguyen HQ, Smith DI, Schechter LE. 2000. Effects of chronic fluoxetine treatment in the presence and absence of (±) pindolol: a microdialysis study. *Brit. J. Pharmacol.* 130:797-804.

57. Vasskog T, Berger U, Samuelsen PJ, Kallenborn R, Jensen E. 2006. Selective serotonin reuptake inhibitors in sewage influents and effluents form Tromsø, Norway. *J. Chromatogr. A* 1115:187-195.

58. Brooks BW, Turner PIK, Stanley JK, Weston JJ, Glidewell EA, Foran CM, Slattery M, La Point TW. Huggett DB. 2003. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52:135-142.

59. Henry TB, Black MC. 2008. Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western mosquitofish. *Arch. Environ. Contam. Toxicol.* 54:325-330.

60. Foran CM, Weston J, Slattery M, Brooks BW, Huggett DB. 2004. Reproductive assessment in Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. *Arch. Environ. Contam. Toxicol*. 46:511-517.

61. Fong PP. 1998. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. *Biol. Bull*. 194:143-149.

62. Fong PP, Huminski PT, D'Urso LM. 1998. Induction and potentiation of parturition in fingernail clams (*Sphaerium striatinum*) by selective serotonin re-uptake inhibitors (SSRIs). *J. Exp. Zool*. 280:260-264.

63. Henry TB, Black MC. 2004. Acute and chronic toxicity of five selective serotonin reptake inhibitors in *Ceriodaphnia dubia. Environ. Toxicol. Chem.* 23:2229-2233.

64. Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere* 69:9-16.

65. Clotfelter ED, O'Hare EP, McNitt MM, Carpenter RE, Summers CH. 2007. Serotonin decreases aggression via 5-HT1A receptors in the fighting fish *Betta splendens. Pharmacol Biochem. Behav.* 87:222-231.

CHAPTER 2: BEHAVIORAL AND BIOCHEMICAL EFFECTS OF DIAZINON IN HYBRID STRIPED BASS

Abstract

The effects of environmental stimuli on biochemical processes may influence behavior. Environmental contaminants that alter behavior can have major impacts on populations as well as community structures by changing species' interactions. One important behavior is the ability to capture prey. We hypothesized that sublethal exposure to diazinon, an organophosphate pesticide, may lead to feeding behavior abnormalities in hybrid striped bass (*Morone saxatilis x M. chrysops*) through inhibition of brain acetylcholinesterase (AChE) activity. This can potentially reduce organism survival by affecting its ability to find and capture food. To test this hypothesis, bass were exposed to diazinon for six d, followed by a six-d recovery period in clean water. Brain AChE activity and the ability of bass to capture prey fathead minnows were measured every third day. Exposed fish exhibited a concentration- and duration-dependent decrease in ability to capture prey. While bass in all diazinon treatment groups had significantly inhibited brain AChE activity, only the medium and high treatment groups showed a dose- and time-dependent increase in time to capture prey. Acetylcholinesterase activity also decreased in an exposure duration- and concentration-dependent manner. The AChE levels in exposed fish did not recover to control levels during the 6-d recovery period. These results suggest that sublethal exposure to AChE-inhibiting substances may decrease the ecological fitness of hybrid striped bass by reducing their ability to capture prey.

Introduction

Pesticides are commonly found in the aquatic environment at concentrations that may impact aquatic life, especially in areas where watersheds are dominated by agriculture, urban, or mixed land uses ([1]; http://pubs.usgs.gov/fs/2006/3028). Organophosphate pesticides (OPs) can enter aquatic systems through multiple routes including accidental spillage, discharge of untreated effluents, spray drift, and surface runoff [2]. Once OPs make their way into aquatic systems, they can create potentially toxic environments for non-target species. The mode of action for OPs is to inhibit the enzyme acetylcholinesterase (AChE). Acetylcholinesterase is responsible for removing the neurotransmitter acetylcholine from the synaptic cleft, thus inhibiting AChE can create an accumulation of endogenous acetylcholine in nerve tissues and effector organs [3], resulting in a continuous firing of nerve impulses. This disruption of normal nervous system function can lead to convulsions, paralysis, and eventually death [4]. Inhibition of AChE activity has been used as a biomarker of exposure for OP toxicity in terrestrial and aquatic organisms [2].

Diazinon is an OP that has been extensively used to control a wide variety of insects for domestic and agricultural purposes [5]; http://fl.water.usgs.gov/Gafl/Abstracts/ofr93478/ofr93478.html). While there has been a significant decrease in environmental diazinon concentrations due to a phase-out for nonagricultural uses beginning in 2002 [1], diazinon has been frequently detected as high as 1.4 µg/L [6] in aquatic environments. The presence of pesticides like diazinon could impair the ecological fitness of an organism by altering behaviors such as searching for

and capturing food, avoiding predators, and reproducing. Decreased fitness at the individual level could ultimately impact the population. Because such behaviors are underlined by mechanisms at the biochemical level [7], assessing behavioral changes in organisms exposed to contaminants may allow researchers to better interpret biochemical changes as well as understand potential consequences at the population or community level.

The purpose of the present study was to characterize the relationship between brain AChE and feeding behavior in hybrid striped bass (*Morone saxatilis x M. chrysops*) exposed to diazinon for 6 d followed by a 6-d recovery period. Results of this study demonstrate a relationship between changes in brain biochemistry and an ecologically relevant behavior. Understanding such a relationship is important because it allows us to better predict population level effects from a biochemical response.

Methods and Materials

Test chemicals

Diazinon, acetylcholine iodide (ATCI), 5,5'-disthiobis(2-nitrobenzoic acid) (DTNB), and 0.1M phosphate buffered saline (PBS) were purchased through Sigma Aldrich (St. Louis, MO, USA). Reagent grade $NaHCO_3$ was purchased from Fisher Scientific (Fairlawn, NJ, USA).

Fish

Hybrid striped bass were chosen as the predator species because they grow rapidly, are resilient to handling stress, are amenable to laboratory culture, and are often stocked as sport fish and for use in aquaculture ([8], [9];

http://www.lsuagcenter.com/en/communications/publications/newsletters/lagniappe/). Bass were obtained from Southland Fisheries (Hopkins, SC, USA). Fish were housed in 450 L circular flow-through holding tanks at Clemson University's Institute of Environmental Toxicology and fed a pellet food (Zeigler Brothers, Gardners, PA, USA) daily. Larval fathead minnows (*Pimephales promelas*) were obtained from a culture maintained at Clemson University's Institute of Environmental Toxicology and reared until they were the appropriate size (~4 cm) to be used in the tests.

Experimental design

Hybrid striped bass were exposed to diazinon for 6 d under static conditions followed by a 6-d recovery period, for a total of 12 d. Diazinon concentrations were tested at levels greater than those found in the environment in order to more clearly quantify potential impacts of diazinon on our chosen endpoints. Four tests were conducted for periods of 3 d, 6 d, 9 d, or 12 d. The four treatment groups had five replicate bass per test, for a total of 20 bass per test. Bass were fed live prey on days 0, 3, 6, 9, and 12, and feeding behavior was quantified. Fish brains were extracted for acetylcholinesterase activity at the end of each test (see below). Hence, four tests were conducted for 3, 6, 9, or 12 d in which bass were fed every third day and brains were harvested at the end of each test.

Hybrid striped bass (mean ± standard deviation [SD]; weight 166.0 $\pm$ 37.3 g; length 21.4 $\pm$ 2.3 cm) were randomly placed (one bass per tank) into twenty-four 80-L aquaria (30.5 cm x 30.5 cm x 90 cm) operated under flow through conditions (0.23 liters per min). Bass that did not eat all four minnow prior to exposure initiation were

eliminated from the test to reduce any confounding factors. Therefore, we stocked four extra tanks to be sure that there we would have 20 useable bass for a complete test. Bass removed from the test were returned to the holding tank for potential use in subsequent tests.

Bass were allowed to acclimate for 7 d prior to test initiation. A nearby lake, Lake Hartwell (SC, USA), was the source of test waters (mean ± SD; pH 6.36 $\pm$ 0.12, hardness 24 mg/L as $CaCO_3$, alkalinity 10 mg/L as $CaCO_3$). Water temperature was controlled using a mixing valve (M & M Control Services, Grayslake, IL, USA) that regulated a mixture of ambient and chilled water (ambient water circulated through an in-line chiller) or heated water (ambient water circulated through an in-line heater) depending on the ambient conditions (summer or winter months, respectively) to facilitate achieving a desired temperature of approximately 25°C (mean ± SD; 25.1 ± 1.1°C). Water was then pumped through a multi-resin filtration system (Water and Power Technologies Columbia, SC, USA) to remove suspended solids and any other possible contaminants prior to entering experimental tanks. During exposure periods when waters were static, water temperatures were controlled by ambient air temperature. Diazinon exposure lasted a maximum of 6 d under static conditions, followed by a maximum of 6 d for recovery (flow was turned back on at a rate of 0.23 liter per min), for a total of 12 d.

Bass were fed four fathead minnows every third day during the acclimation period. This feeding regime ensured the bass were hungry and were accustomed to eating live minnows. Bass were fed on days 1, 4, and 7 of the acclimation period so that

the last day of acclimating coincided with day 0 of the diazinon exposure. Feeding behavior (time to capture prey) of bass was quantified every third day during the test.

Prior to each feeding, air stones were removed from each tank, and water flow was also turned off during the recovery period. Following this, researchers waited at least 2 min before adding prey for acclimation just in case bass were startled when air stones were removed. Four fathead minnows were dropped into an exposure tank and the time to eat each prey was recorded. Minnows used in the tests were visually estimated to be approximately 4 cm. Minnows of similar size were preselected prior to feeding to ensure that no extremely large or small minnows were used to skew appetite satiation. Minnows were then randomly selected and fed to bass without regard to treatment. Bass were observed until all minnows were consumed or for a maximum of 25 min. Any uneaten minnows after 25 min were removed from the tank, and air stones replaced. During the recovery period, water flows were also turned back on following each feeding. For statistical purposes, uneaten minnows were assigned a value of 1500 s (25 min).

Diazinon exposure

At test initiation, water flow was turned off and an appropriate quantity of diazinon stock dissolved in acetone was added to each aquarium to achieve nominal treatment concentrations of 50, 150, and 200 µg/L. While reported median effective concentration (EC50) and median lethal concentration (LC50) values for fish range from 248 µg/L for bluegill (*Lepomis macrochirus*) to 6,970 µg/L for fathead minnows (*Pimephales promelas*) [10], preliminary studies performed in our laboratory found that

nominal concentrations of 300 µg/L resulted in significant deaths in hybrid striped bass following a 6-d exposure to diazinon. Limited mortality in the highest treatment (200 µg/L) slightly decreased the number of replicates for that treatment.

One concentrated stock of diazinon was prepared for all tanks in order to reduce tank-to-tank variability within treatments. Final acetone volumes in each tank, which increased with increasing treatment level, did not exceed 280 µl in 80 L of water. This is less than 0.0004% of the test water. The Acetone Material Safety Data Sheet (Fisher Diagnostic, Middleton, PA. www.cleanersolutions.org/msds/Acetone%20MSDS.htm) indicated the acetone LC50 for fish was greater than 5,000 mg/L. Concentrations of acetone in our individual tanks of the present study did not exceed 2.8 mg/L. Previous research in our laboratory with a number of aquatic organisms has indicated that this concentration does not cause effects (S.J. Klaine, personal communication). Test waters were allowed to equilibrate for 1 to 2 h prior to taking 50 ml water samples for diazinon analysis. These water samples were filtered through 6 ml C_{18} solid phase extraction columns (HyperSep C18, Thermo Electon, Bellefonte, PA, USA) that were preconditioned with 6 ml acetone, 6 ml methanol, and 6 ml deionized water. Extraction columns were dried for at least 30 min at room temperature on a vacuum manifold (JT Baker, Philipsburg, NJ, USA) before eluting diazinon with ethyl acetate. Control and low diazinon treatment samples were extracted in 10 ml ethyl acetate while the medium and high concentration samples were extracted in 25 ml. Samples were stored at -20°C until analyzed by gas chromatography.

Diazinon half-life was previously determined to be approximately 48 h under test conditions. Therefore, waters were spiked at half the original diazinon concentration on days 2 and 4 of the exposure period. Water samples for diazinon analysis were taken on day 0, day 2 before spiking, day 2 after spiking, day 4 before spiking, day 4 after spiking, and day 6 to monitor fluctuating diazinon concentrations.

Diazinon analysis

Extracts (1µl) were analyzed via Hewlett-Packard 5890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with a flame photometric detector operated in 'P' mode for 10.5 min each on a 5 meter HP-1 (100% dimethylpolysilozane coating) column (Agilent Technologies). Injection temp was 225°C and the detector temperature was 200°C. Oven was initially set at 60°C, held for 0.5 min, then increased 20°C/min to 180°C and held for 4 min.

Brain tissue preparation

Following timed feedings, bass were euthanized in buffered MS-222 (Tricaine methanesulfonate; Western Chemical Ferndale, WA, USA) and brains were quickly removed and put on dry ice prior to storage at -70°C. Brains were thawed, homogenized in 0.5 ml 0.1M PBS buffer, and diluted to 3 ml. Homogenates were centrifuged at 5,000 rpm and 4°C for 20 min to remove cellular debris. Supernatant was stored at -70°C until used for bioassays.

Protein assay

Protein concentrations were determined using a BCA (bicinchoninic acid) Protein Assay Kit (Pierce™, Rockford, IL, USA). Brain homogenates were diluted 1:2 to 1:5 in

0.1M PBS prior to running the assay. Acetylcholinesterase activity was normalized using protein concentrations.

Acetylcholinesterase assay

Acetylcholinesterase analysis was modified from Ellman et al. [11]. Acetylcholine iodide was prepared at 21.68 mg/ml in 0.1M PBS. Ellman's reagent was prepared at 3.96 mg/ml DTNB and 1.50 mg/ml $NaHCO_3$. The working buffer contained 0.65% acetylcholine iodide (ACTI), 3.25% Ellman's reagent, and 96.10% 0.1M PBS. Four µl brain homogenate was added in duplicate in a 96-well plate to which 246 µl working reagent was added to all wells for a total of 250 µl per well. Absorbance at 412 nm was read in 5 min intervals over 30 min on a SpectraMax® 190 plate reader (Molecular Devices, Sunnyvale, CA, USA) using SoftMaxPro software (Molecular Devices).

Data analysis

We used Statistical Analysis Software® 9.1 (SAS, Cary, NC, USA) to analyze time to capture prey using a two-factor analysis of variance (ANOVA) model with the independent variables treatment and day. Multiple pair-wise comparisons among and within treatment, day, and treatment-by-day terms were performed using the LSMEANS (least square means) option within PROC GLIMMIX (General Linear Model for Mixture Distributions), an analyses matrix that uses joint modeling for multivariate data.

Acetylcholinesterase data were evaluated using two-factor ANOVA models with independent variables of treatment and day. We used PROC GLIMMIX with LSMEANS statement to determine statistical differences among and within treatment,

day, and treatment-by-day interactions, rather than using PROC GLM (ANOVA analyses) with multiple MEANS and LSMEANS statements to determine significant differences among and within the independent variables. An $\alpha = 0.05$ was used as the level of significance for both time to capture prey and AChE levels with both PROC GLM and PROC GLIMMIX analyses.

Results

Diazinon concentrations

Measured diazinon concentrations were approximately 40 to 50% of the anticipated nominal concentrations likely due to hydrolysis, photolysis, and some sorption to the aquaria and debris in aquaria. Extraction efficiencies were 90 to 100% (data not shown). Measured concentrations (mean ± standard error [SE]) throughout the exposure period for the low (50 µg/L), medium (150 µg/L), and high (200 µg/L) treatments were 19.1 ± 0.7, 64.0 ± 2.0, and 101.9 ± 1.4 µg/L, respectively. These values represent the mean of all replicates of the four tests following each spiking. Measured diazinon concentrations were used in all data analyses.

Acetylcholinesterase

Results from the present study showed a dose-response relationship between AChE inhibition and diazinon exposure concentration. Brain AChE activities in all treatments were significantly different from each other at all time points after day 0 with the exception of day 12 (Figure 2.1). Acetylcholinesterase activity continued to decrease significantly between days 3 and 6 for the low and medium treatment groups, but not the high treatment. Hybrid striped bass in the present study demonstrated 86.4% AChE

inhibition (6.1 ± 0.6 pmol/mg protein/min) after just 3 d of exposure to the highest diazinon concentration (mean ± SE; 101.9 ± 1.4 µg/L) as compared to controls on that day (44.7 ± 4.1 pmol/mg protein/min), and these levels remained fairly constant through day 6 (5.6 ± 0.5 pmol/mg protein/min). Acetylcholinesterase activity in bass in the low treatment (mean ± SE; 19.1 ± 0.7 µg/L) was decreased by 51.1% (21.9 ± 1.2 pmol/mg protein/min) and 66.3% (13.8 ± 1.6 pmol/mg protein/min) on days 3 and 6, respectively, while AChE activity in bass in the medium treatment (64.0 ± 2.0 µg/L) decreased by 75.5% (11.0 ± 0.9 pmol/mg protein/min) and 82.2% (7.3 ± 0.3 pmol/mg protein/min) on days 3 and 6, respectively.

Acetylcholinesterase activity began to increase during the recovery period in diazinon treated bass. While AChE activity increased significantly by day 12 (day 6 of recovery) as compared to day 6, enzyme activities did not return to pre-exposure levels. By the third day of the recovery period (day 9), only AChE activity in bass in the medium treatment was significantly increased as compared to the end of the exposure on day 6. Acetylcholinesterase levels in the medium treatment increased by 8.3% between days 6 and 9 to 10.1 ± 0.7 pmol/mg protein/min. Brain AChE activities in low and high treatments on day 9 were only increased by 2.5% (14.1 ± 0.7 pmol/mg protein/min) and 3.3% (6.6 ± 0.5 pmol/mg protein/min), respectively. By the sixth day of recovery (day 12) however, all treatment groups showed significantly greater AChE activity as compared to day 6. The low, medium, and high treatment groups increased by 20.3% (22.9 ± 1.2 pmol/mg protein/min), 13.4% (13.3 ± 1.9 pmol/mg protein/min), and 11.1% (10.5 ± 1.3 pmol/mg protein/min) as compared to day 6, respectively.

To determine the 6-d EC50 for AChE inhibition, diazinon concentrations were linearized using a log plot and 50% effect concentrations were calculated using the linear equation. The estimated 6-d EC50 was 15.2 ± 1.1 µg/L (Figure 2.2).

Behavior Data: Exposure/Recovery Effects

On day 0, behavior data was recorded prior to initial diazinon exposure. There were no significant differences among treatment groups. At no time point during the test was feeding behavior in the low treatment significantly different from the controls (Figure 2.3) even though they exhibited significantly reduced AChE levels at all time points after day 0. In the medium treatment, time to capture the first prey took significantly longer than controls on day 6 of the exposure period, and throughout the recovery period (days 9 and 12) (Figure 2.3a). Time to capture the second prey fish was only significantly longer than controls on day 6, (Figure 2.3b), indicating that behavioral effects of diazinon were diminishing as recovery time increased. However all time points for bass in the medium treatment for capturing the third prey fish were still significantly different from controls throughout the recovery period (Figure 2.3c). Bass in the high treatment group took significantly longer to eat all prey fish versus the controls at all time points after day 0.

As recovery time increased, time to capture prey decreased significantly for bass in the medium treatment, but not the high treatment. Time to capture prey 1 and 2 in the medium treatment significantly decreased between the end of the exposure on day 6 and the third day of recovery on day 9. While time to capture prey continued to decrease between days 9 and 12, this was not significant. During the recovery period (days 9 and

12), time to capture prey 3 for bass in the medium treatment was not significantly different from day 6. While times to capture prey 1, 2, and 3 decreased for bass in the high treatment during the recovery period (days 9 and 12), they were not statistically different from day 6. See Appendix Table A-1 for mean ± SE values for time to capture prey for each treatment and day.

Discussion

Organophosphate pesticides have been widely used since the 1930s due in part to their rapid degradation in the environment [2]. As a result of their nonpersistence, it can be difficult to extrapolate an environmental concentration to behavioral or biochemical effects on aquatic organisms outside a laboratory scenario. The specificity of OPs for AChE makes AChE activity a widely used biomarker of exposure to such compounds. It is important, however, that we try to establish links between biomarkers and higher levels of biological organization, notably ecologically relevant endpoints [12].

Changes in brain biochemistry can alter important behaviors for survival and fitness. While biochemical changes resulting from contaminant exposure are often cited as potentially detrimental, the implications of these changes on populations are speculative at best. Organisms can compensate for sublethal stressors by altering energy uptake (feeding activity) and expenditure, which could lead to changes at lower biological levels and ultimately affect populations [12]. Therefore, an organism's feeding behaviors may provide a better indication of ecological fitness and the impacts of OP exposure on population dynamics [12] than biomarkers alone. Reduced feeding behavior can be separated into a number of different feeding processes including motivation,

orientation to prey, strikes, and miscues [13]. Prey capture, our chosen behavioral endpoint, is ecologically relevant because it can be related directly to growth and survival [7].

Many researchers who have examined the effects of neurotransmitter inhibitors on aquatic organisms focus on the biochemical responses and neglect to measure behavioral responses in a quantitative manner. Fulton and Chambers [14] evaluated behavior qualitatively in mosquitofish (*Gambusia affinis*) reporting normal behavior three weeks after exposure to an AChE inhibitor. They also found that tadpoles (*Rana sp.*) showed no clinical signs associated with neurotoxicity. Keizer et al. [15] observed symptoms of swollen gills and coordination problems in zebrafish (*Brachydanio rerio*) and guppies (*Poecilia reticulata*) exposed to diazinon, noting that they drifted on their backs at the water surface just before death. Unlike zebrafish, however, the guppies showed these symptoms temporarily and then recovered. A review by Fulton and Key [4] compiled laboratory studies in fish and invertebrates in which AChE inhibition and in some cases, AChE recovery, was used as a biomarker. However, these experiments only assessed biochemical changes; hence, they could not relate compromised AChE inhibition to compromised ecological fitness. These studies underscore the use of AChE activity as a biomarker of exposure, but provide little insight into its utility to be used for the characterization of effects.

Although their behavior was significantly impacted, hybrid striped bass tolerated over 80% AChE inhibition following a 6-d diazinon exposure. Other researchers have reported similar results of AChE inhibition. Coppage [16] reported brain AChE

inhibition by OPs greater than 80% in all fish that survived median lethal exposures, and Ferrari et al. [17] reported AChE inhibition values between 77 and 95% following a 96-h exposure to multiple OPs. In addition, bluegill (*Lepomis macrocharis)* had 95% AChE inhibition within 6 h of diazinon exposure, while fathead minnow, goldfish (*Carassius auratus*), and golden shiner (*Notemigonus crysoleucas*) showed 70, 43, and 40% AChE inhibition, respectively, within 18 h of exposure [18]. Goodman et al. [19] also reported a 71% AChE inhibition in sheepshead minnow (*Cyprinodon variegates*) following a 24-h exposure to diazinon.

Contaminants like diazinon can impair feeding behavior by affecting motivation to feed, search effectiveness, or ability to capture prey. Hybrid striped bass tolerated more than 66% AChE inhibition before a significant effect on feeding behavior was observed, as suggested by the lack of observable, or statistical, behavioral impairments in the low treatment group on day 6 (Figure 2.4). We quantified an increased time to capture prey and a decreased AChE activity as a result of increased exposure concentrations and durations. Plotting our two endpoints at the end of the exposure against each other, we noted a threshold response between biochemistry and behavior at the concentrations tested (Figure 4).

In the present study, we saw a dose-response relationship between AChE inhibition and diazinon exposure concentration. The estimated 6-d EC50 of diazinon on AChE activity (mean ± SE) was 15.2 ± 1.1 µg/L. This value was lower than the estimated 6-d EC50 value for feeding behavior (mean ± SE) 50.1 ± 4.0 µg/L (data not shown). Other investigators have reported acute 96-h LC50 values for diazinon for

various aquatic species including crayfish (*Gammarus fasciatus*) 0.2 µg/L [20], *Hyallela azteca* 4.0 µg/L [21], rainbow trout (*Oncorhynchus mykiss)* 90 µg/L, and bluegill (*Lepomis macrocharis)* 170 µg/L [20]. The differences noted in EC50 values between AChE inhibition and behavioral impairments in the present study has also been noted when considering AChE activity and swimming stamina in sheepshead minnows [22], red drum (*Sciaenops ocellatus*) [23], mummichog (*Fundulus heteroclitus*) [23] and salmonids [24]. On the other hand, linear correlations between changes in swimming speed and AChE activity were noted in rainbow trout [25, 26], and Coho salmon (*Oncorhynchus kisutch*) [27, 28].

As with the present study, other researchers have found that several factors are responsible for the effects of OPs on AChE inhibition and recovery in aquatic organisms including the type of OP, exposure duration and concentration, the degree of AChE depression, and species exposed [17, 25, 29, 30]. During the recovery period when bass were in clean water, there were decreases in time to capture prey as compared to day 6 for both the medium and high treatments, however only increased times to capture the first and second prey for bass in the medium treatment were significant (Figure 2.3). When comparing time to capture prey during the recovery period to controls on their respective day, only times to capture prey 2 on days 9 and 12 for the medium treatment were comparable to controls. Although bass improved their prey-capturing ability, AChE levels were still significantly inhibited (Figure 2.1). Similarly, Morgan et al. [29] looked at recovery times for Atlantic salmon (*Salmo salar*) exposed to sublethal concentrations of fenitrothion and found AChE was still depressed by 34% one week following a 7-d

exposure to 213 µg/L, noting that recovery of enzyme activity was related to the degree of initial inhibition. Exposure of goldfish (*Carassius auratus*) to OPs (parathion and carbaryl) also showed little increases in cholinesterase activity during a 96-h recovery period in clean water. The tests further revealed that goldfish required at least 35 d of depuration to substantially recover cholinesterase activity following the exposure [17]. Also, AChE activity in eels (*Anguilla anguilla*) exposed to fenitrothion for 96-h was still significantly inhibited 12 d post-exposure as compared to controls [30].

Fenitrothion is an OP that has been shown to decrease another aspect of feeding: foraging behavior [31]. Researchers found that attack sequences, frequency of ingestion, and reaction distance of juvenile Atlantic salmon were all decreased as compared to controls. The pesticide-exposed salmon waited until their prey moved closer before striking and were more likely to end the sequence before capturing the prey [31]. Latency to first strike and total food strikes during a fixed time interval were also noted in Coho salmon [28]. Although not quantified, similar behavioral abnormalities were observed in the present study as well. Many bass in the medium and high treatments would strike at their prey unsuccessfully due either to not striking quick enough or not being close enough to the prey. After a few failed attempts, bass would no longer strike. Other times, bass would follow a minnow around the tank without attempting to strike, or swim toward the minnow only to turn around, as if to give up before striking in order to conserve energy. Bass in the highest treatment were virtually uninterested in the minnows and were inactive for the most part, even if minnows were within reach with minimal energy expenditure required.

Prey handling time also increased with increasing diazinon concentration causing bass to spend more time and energy consuming the food, thus eating less in a given period of time. Since prey handling depends on coordination of different muscle activities that are under the control of the central nervous system, impairment of nerve transmission may result in a loss of this coordination [32]. While bass in the medium treatment were not as active as the control or low treatments, they were still more active than the high treatments. However, they were noted as having an increased handling time as a result of spitting out some captured prey. Bass in the low treatment exhibited no signs of behavioral impairment despite having significantly reduced AChE activities at all time points measured and would wait anxiously to be fed throughout the duration of the experiments.

Seemingly small impacts on various behaviors can impact both populations and community structures. A decreased efficiency in foraging and prey capture may lead to decreased growth and survival, as well as decreased energy available for reproduction. In order to be ecologically fit, fish must maintain essential behaviors. Dose-response relationships may exist for an endpoint at a given level of organization, but the ecological relevance at higher levels of organization is often unclear or unknown. This uncertainty is perhaps most obvious, and most difficult to address when attempting to establish links between levels of biological organization [33].

Impaired feeding behaviors as a result of OP exposure could significantly decrease the probability of responding to, or capturing prey items. This could result in a decreased growth if the exposure and/or effects were long lasting [31]. However, if

behavioral changes were quickly reversed, there may not be significant impacts on higher levels of organization. As with other sublethal toxic effects, it can be difficult to attribute ecological relevance to sublethal AChE inhibition [27]. The relationship between AChE inhibition and outward signs of intoxiciation can be complex, especially when accounting for species and individual variability [17]. Results of the present study demonstrate that significant AChE inhibition can occur before behavioral changes are observed, and this inhibition can last much longer than the exposure duration. We also noted that the effect of diazinon on feeding behavior was recovering to control levels quicker than AChE activity.

Biomarker assays like AChE activity provide clear indications of contaminant exposure, yet by itself, does not give a clear indication of how these contaminants affect the ecological fitness of an individual. By evaluating an ecologically relevant behavioral endpoint along with AChE activity, it may be possible to better relate this biomarker of organophosphate exposure to ecological fitness. Establishing this relationship would help relate sublethal biochemical changes to population-level effects [26], making AChE activity a more useful endpoint for use in risk assessments.

References

1. Gilliom, RJ, Hamilton PA. 2006. Pesticides in the nations streams and groundwater, 1992-2001- a summary. USGS Fact Sheet 2006-3028. U.S. Geological Survey, Tallahassee, FL.

2. Guilhermino L, Lopes MC, Carvalho AP, Soares AMVM. 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. *Chemosphere* 32:727-738.

3. Pan G, Dutta HM. 1998. The inhibition of brain acetylcholinesterase of juvenile largemouth bass *Micropterus salmoides* by sublethal concentrations of diazinon. *Environ Res Sect A* 79:133-137.

4. Fulton MH, Key PB. 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorus insecticide exposure and effects. Annual Review. *Environ Toxicol Chem* 20:37-45.

5. Berndt MP, Hatzell HH. 2001. Does diazinon pose a threat to a neighborhood stream in Tallahassee, Florida? USGS Fact Sheet FS-143-00. U.S. Geological Survey, Sacramento, CA.

6. Hoffman RS, Capel PD, Larson SJ. 2000. Comparison of pesticides in eight U.S. urban streams. *Environ Toxicol Chem* 19:2249-2258.

7. Weis JS, Smith G, Zhou T, Santiago-Bass C, Weis P. 2001. Effects of contaminants on behavior: Biochemical mechanisms and ecological consequences. *BioScience* 51:209-217.

8. Weirich, CR, Tomasso, JR, Smith TIJ. 1992. Confinement and transport-induced stress in white bass (*Morone chrysops)* x striped bass (*M. saxatilis)* hybrids: Effect of calcium and salinity. *J World Aquacult Soc* 23:49-57.

9. Gothreaux C. 2007. Family profile: Moronidae – The temperate basses. *Lagniappe Fisheries Newsletter* 31:3-5.

10. Giddings JM, Biever RC, Annunziato MF, Hosmer AJ. 1996. Effects of diazinon on large outdoor pond microcosms. *Environ Toxicol Chem* 15:618-629.

11. Ellman GL, Courtney KD, Andres V Jr, Featherstone RM. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol* 7:88-95.

12. Duquesne S. 2006. Effects of an organophosphate on *Daphnia magna* at suborganismal and organismal levels: Implications for population dynamics. *Ecotoxicol Environ Saf* 65:145-150.

13. Little EE, Archeski RD, Flervo B, Kozlovskaya V. 1990. Behavioral indicators of sublethal toxicity in rainbow trout. *Arch Environ Contam Toxicol* 19:380-385.

14. Fulton MH, Chambers JE. 1985. Inhibition of neurotoxic esterase and acetylcholinesterase by organophosphorus compounds in selected ectothermic vertebrates. *Pestic Biochem Physiol* 23:282-288.

15. Keizer J, D'Agostion G, Vittozzi L. 1991. The importance of biotransformation in the toxicity of xenobiotics to fish. I. Toxicity and bioaccumulation of diazinon in guppy (*Poecilia reticulata*) and zebra fish (*Brachydanio rerio*). *Aquat Toxicol* 21:239-254.

16. Coppage DL. 1972. Organophosphate pesticides: Specific level of brain AChE inhibition related to death in sheepshead minnows. *Trans Am Fish Soc* 101:534-536.

17. Ferrari A, Venturion A, Pechen de D'Angelo AM. 2004. Time course of brain cholinesterase inhibition and recovery following acute and subacute azinphosmethyl, parathion and carbaryl exposure in the goldfish (*Carassius auratus*). *Eccotoxicol Environ Saf* 57:420-425.

18. Weiss CM. 1961. Physiological effect of organic phosphorus insecticides on several species of fish. *Trans Am Fish Soc* 90:143-152.

19. Goodman LR, Hansen DJ, Coppage DL, Moore JC, Matthews E. 1979. Diazinon chronic toxicity to, and brain acetylcholinesterase inhibition in, the sheepshead minnow, *Cyprinodon variegatus*. *Trans Am Fish Soc* 108:479-488.

20. Mayer FL, Ellersieck MR. 1986. Manual of acute toxicity: Interpretation and database for 410 chemicals and 66 species of freshwater animals. Resource Publication 160. U.S. Department of the Interior, Fish and Wildlife Service, Washington, DC.

21. Collyard SA, Ankley GT, Hoke RA, Goldenstein T. 1994. Influence of age on the relative sensitivity of *Hyalella azteca* to diazinon, alkylphenol ethoxylates, copper, cadmium, and zinc. *Arch Environ Contam Toxicol* 26:110-113.

22. Cripe GM, Goodman, Hansen DJ. 1984. Effect of chronic exposure to EPN and to guthion on the critical swimming speed and brain acetylcholinesterase inhibition of *Cyprinodon variegates*. *Aquat Toxicol* 5:255-266.

23. Van Dolah RF, Maier PP, Fulton MH, Scott GI. 1997. Comparison of azinphosmethyl toxicity to juvenile red drum (*Sciaenops ocellatus*) and the mummichog (*Fundulus heteroclitus*). *Environ Toxicol Chem* 16:1488-1493.

24. Post G, Leasure R. 1974. Sublethal effect of malathion to three salmonid species. *Bull Environ Contam Toxicol* 12:312-319.

25. Beauvais SL, Jones SB, Parris JT, Brewer SK, Little EE. 2001. Cholinergic and behavioral neurotoxicity of carbaryl and cadmium to larval rainbow trout (*Oncorhynchus mykiss*). *Ecotoxicol Environ Saf* 49:84-90.

26. Beauvais SL, Jones SB, Brewer SK, Little EE. 2000. Physiological measures of neurotoxicity of diazinon and malathion to larval rainbow trout (*Oncorhynchus mykiss*) and their correlation with behavioral measures. *Environ Toxicol Chem* 19:1875-1880.

27. Tierney K, Casselman M, Takeda S, Farrell T, Kennedy C. 2007. The relationship between cholinesterase inhibition and two types of swimming performance in chlorpyrifos-exposed coho salmon (*Oncorhynchus kisutch*). *Environ Toxicol Chem* 26:998-1004.

28. Sandahl JF, Baldwin DH, Jenkins JJ, Scholtz NL. 2005. Comparative thresholds for acetylcholinesterase inhibition and behavioral impairment in coho salmon exposed to chlorpyrifos. *Environ Toxicol Chem* 24:136-145.

29. Morgan MJ, Fancey LL, Kiceniuk JW. 1990. Response and recovery of brain AChE activity in atlantic salmon exposed to fenitrothion. *Can J Fish Aquat Sci* 47:1652-1654.

30. Sancho E, Ferrando MD, Andreu E. 1997. Response and recovery of brain acetylcholinesterase activity in the European eel, *Anguilla anguilla*, exposed to fenitrothion. *Ecotoxicol Environ Saf* 38:205-209.

31. Morgan MJ, Kiceniuk JW. 1990. Effect of fenitrothion on the foraging behavior of juvenile Atlantic salmon. *Environ Toxicol Chem* 9:489-495.

32. Pavlov DD, Chuiko GM, Gerassimov YV, Tonkopiy VD. 1992. Feeding behavior and brain acetylcholinesterase activity in bream (Abarmis brama L.) as affected by DDVP, an organophosphorus insecticide. *Comp Biochem Physiol C* 103:563-568.

33. Sibley PK, Chappel MJ, George TK, Solomon KR, Liber K. 2000. Integrating effects of stressors across levels of biological organization: Examples using organophosphorous insecticide mixtures in field-level exposures. *J Aquat Ecosyst Stress Recovery* 7:117-130.

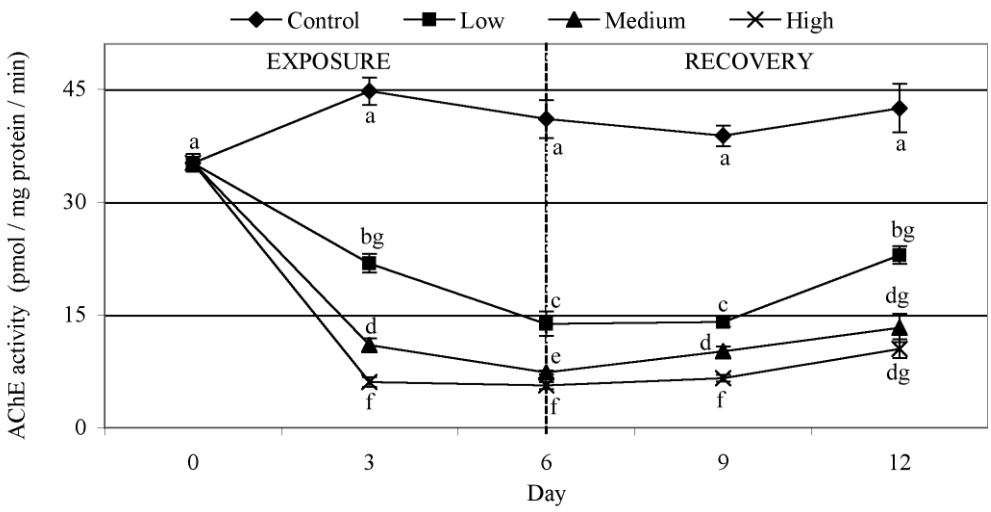

Figure 2.1. Brain acetylcholinesterase activity (AChE) (mean ± SE) in hybrid striped bass during a 6-day waterborne exposure to diazinon, followed by a 6-day recovery period. Means with the same letter are not statistically different from each other. Vertical line at day 6 separates exposure and recovery time points.

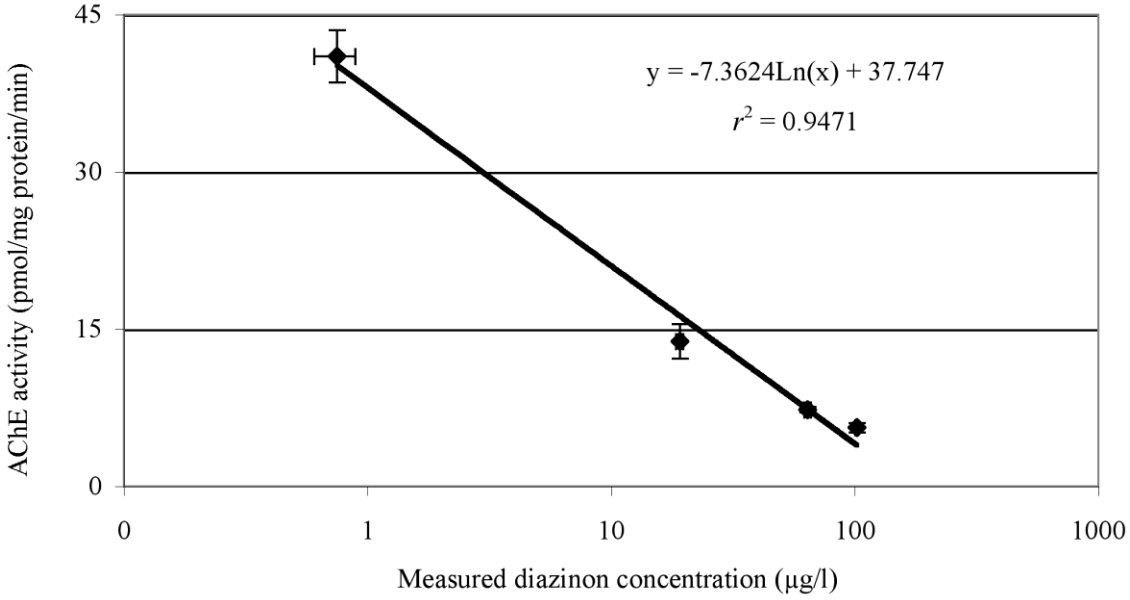

Figure 2.2. Day 6 acetylcholinestrease (AChE) activity (mean ± SE) as a function of measured diazinon concentration (mean ± SE). The estimated 6-day EC50 was 15.2 ± 1.1 µg/L. Error bars indicate 95% confidence intervals.

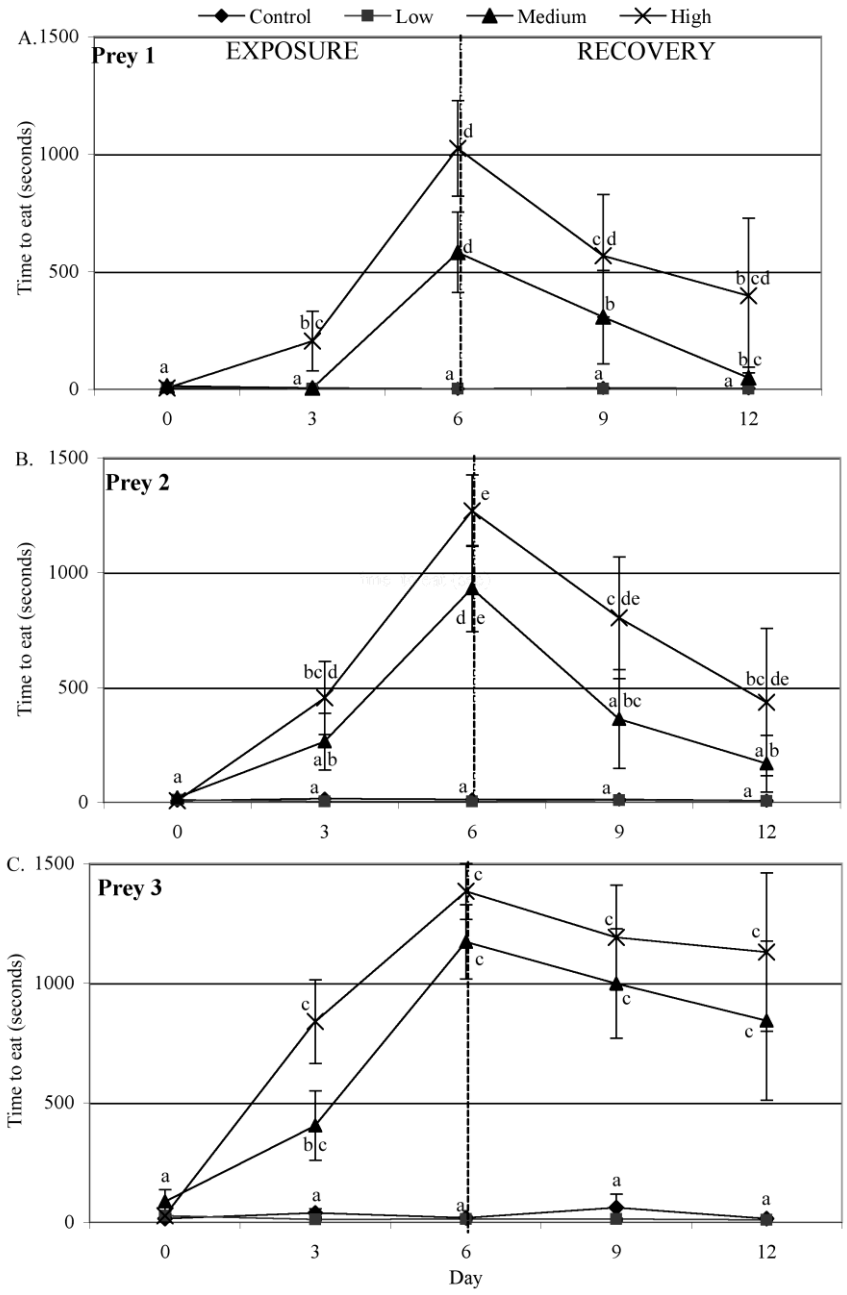

Figure 2.3. Time (mean ± SE) it took hybrid striped bass to capture the first (**A**), second (**B**) and third (**C**) prey fish during a 6-day diazinon exposure followed by a 6-day recovery period. Means with the same letter are not statistically different from each other. Vertical line at day 6 separates exposure and recovery time points.

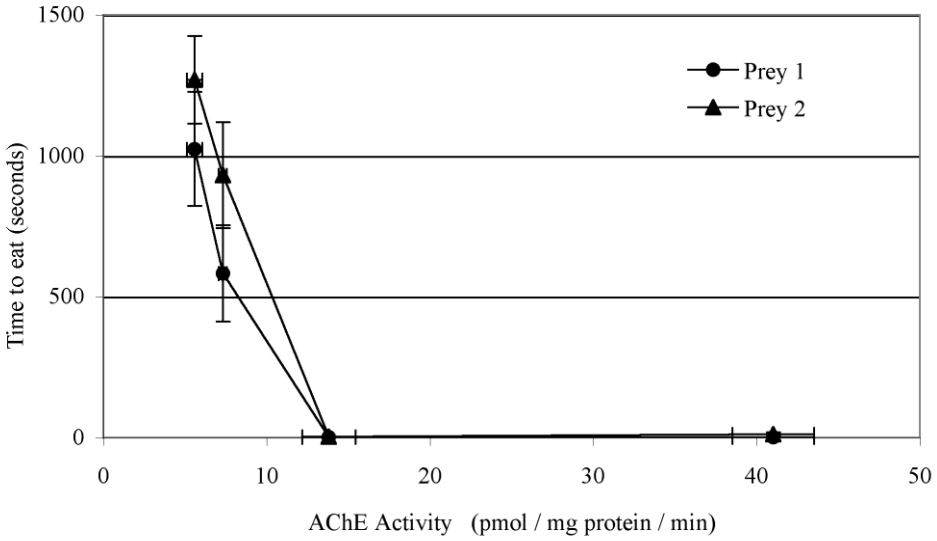

Figure 2.4. Day 6 time to eat prey 1 and prey 2 (mean ± SE) as a function of brain acetylcholinestrease activity (AChE) (mean ± SE) in hybrid striped bass.

CHAPTER 3: BEHAVIORAL AND BIOCHEMICAL RESPONSES OF HYBRID STRIPED BASS DURING AND AFTER FLUOXETINE EXPOSURE

Abstract

Environmental contaminants, including pharmaceuticals, can alter behavior and possibly impact population and community structures. One important behavior that could be impacted is the ability to capture prey. We hypothesized that sublethal fluoxetine exposure may lead to feeding behavior abnormalities in hybrid striped bass (*Morone saxatilis x M. chrysops*). Fluoxetine is an antidepressant that acts as a selective serotonin reuptake inhibitor (SSRI). A change in serotonin levels affects multiple behaviors including feeding, which is an important aspect in ecological fitness. This research characterized the impact of sublethal fluoxetine exposures on the ability of hybrid striped bass to capture fathead minnows (*Pimephales promelas*). Bass were exposed to fluoxetine (0.0 µg/l, 23.2 $\pm$ 6.6 µg/l, 51.4 $\pm$ 10.9 µg/l and 100.9 $\pm$ 18.6 µg/l,) for six days, followed by a six-day recovery period in clean water. Brain serotonin activity and the ability of bass to capture prey were measured every third day. Exposed fish exhibited a concentration- and duration- dependent decrease in ability to capture prey. Increased time to capture prey also correlated with decreases in brain serotonin activity. Serotonin activity also decreased in an exposure time- and concentration-dependent manner, maximally inhibited 23.7%, 28.0%, and 49.1% of control in the low, medium, and high treatments, respectively. Serotonin levels in exposed fish did not recover to control levels during the six-day recovery period. These results suggest that sublethal exposure

to fluoxetine decreases the ability of hybrid striped bass to capture prey and that serotonin can be used as a biomarker of exposure and effect.

Introduction

Many pollutants adversely affect normal endocrine functions, and unfortunately, these chemicals are ubiquitous in the environment [1]. The central nervous system is a target of endocrine-disruptors, so social behaviors under hormonal control such as aggression, dominance, motivation, and activity are often directly impacted by exposure to these contaminants [1]. Behavior is a major link between the organism and its environment [2]. It is both a result and determinant of molecular, physiological, and ecological aspects of toxicology; therefore, it provides insight into various levels of biological organization [3]. Behavioral responses also reflect an organism's ecological fitness and its abilities to avoid predators, select prey items, and reproduce [2]. Alterations in any of these behaviors, coupled with changes in physiology may alter population stability [3].

Fluoxetine (Prozac™) is an antidepressant that acts as a selective serotonin reuptake inhibitor (SSRI). Serotonin functions as a neurotransmitter to regulate a wide range of behaviors including feeding activity, aggressive interactions, sexual behavior, and establishment of social hierarchies [4, 5]. SSRIs, like fluoxetine, act by inhibiting the reuptake of serotonin from the synaptic cleft, and increasing extracellular serotonin levels. They have been effective in improving mood levels and decreasing appetite and aggression. Therefore, SSRIs are commonly prescribed for depression, compulsive behaviors, and personality disorders [6].

Fluoxetine, like most drugs, was developed with the intent of altering biochemistry and having specific pharmacological and physiological functions. Many drugs are polar, nonvolatile, and nonbiodegradable [7, 8] so they tend to escape sedimentation and biological treatment in wastewater treatment plants [5, 7]. They are persistent in order to reach the target site before becoming inactive, but this also increases the possibility of bioaccumulation in aquatic or terrestrial organisms [8]. The high frequency of use and continual output of drugs from wastewater treatment plants and other sources simulates episodic or continuous exposures, rather than acute exposures. Consequently, low levels of fluoxetine (and other pharmaceuticals and personal-care products) have been found in treated sewage effluent, sediment, surface waters, and city water supplies [9]. Although present in the environment at concentration in the ng/l range[10], numerous aquatic species including fish have been found to contain detectable amounts of fluoxetine [11].

Fluoxetine exposure has also been shown to affect various aspects of behavior in aquatic organisms. Studies have shown that exposure to antidepressants can trigger premature spawning in fingernail clams (*Sphaerium striatinum*) [12], and zebra mussels (*Dreissena polymorpha*) [13], decreased fecundity in *Cerodaphnia dubia* [14], decreased growth in algae (*Pseudokirchneriella subcapitata*) [14] and decreased growth and feeding rates in fathead minnows (*Pimephales promelas*) [15].

Assessing the human risk of pharmaceuticals in waters is a high priority; however, the impacts of these drugs on aquatic organisms and communities are also important [7, 9]. A better understanding of toxicological effects of contaminants can be

achieved by integrating behavioral indicators of toxicology with those of other levels [3]. The gol of this study was to (1) determine the effects of waterborne fluoxetine on the feeding behavior of hybrid striped bass (*Morone saxatilis x M. chrysops*); and (2) correlate these changes with changes in brain serotonin levels at various time points of exposure and recovery. Fish are an excellent model for studying effects of environmental pollutants because many ecologically relevant fish behaviors are easily observed and quantified in controlled settings [3]. Feeding behavior was chosen because our previous research demonstrated that changes in this behavior were quantitatively related to neurotoxin exposure and neurotransmitter concentrations [16]. Bass were monitored during a six-day exposure period followed by an additional six days in clean water to observe any latent effects of exposure that may not otherwise be identified at the end of the exposure period. This also allowed us to look at the sensitivity of both biochemical and behavioral endpoints and how these endpoints changed during recovery. Fluoxetine concentrations were tested at levels greater that those found in the environment in order to more clearly quantify potential impacts of fluoxetine on fish brain monoamines and feeding behavior.

Materials and Methods

Test chemicals

Fluoxetine hydrochloride, was generously donated by Fermion (Finland). Perchloric acid, aqueous (0.1 N), was purchased from VWR (USA). Methanol, acetone, triethylamine, acetolnitrile, glacial acetic acid, monochloro-acetic acid, and tetrahydrofuran were purchased from Fisher Scientific (USA). Sodium hydroxide,

sodium octyl sulfate, ethylenediaminetetraacetic acid （EDTA) disodium salt, 5-hydroxyindole-3-acetic acid (5-HIAA), and serotonin creatinine sulfate monohydrate (5-HT) were purchased from Sigma-Aldrich (USA).

Fish

Hybrid striped bass (*Morone saxatilis x M. chrysops*) were obtained from Southland Fisheries near Columbia, SC. Fish were housed in 450 L circular flow-through holding tanks at Clemson University's Institute of Environmental Toxicology and fed a pellet food (Zeigler Brothers, Gardners, PA) daily. Larval fathead minnows (*Pimephales promelas*) were obtained from a culture maintained at Clemson University Institute of Environmental Toxicology and reared until they were the appropriate size (~4 cm) to be used in the tests.

Experimental design

Hybrid striped bass were exposed to fluoxetine for 6 days under static conditions, followed by a 6-day recovery period (water flow was turned back on at a rate of 0.23 liters per minute), for a total of 12 days. Fish were fed on days 0, 3, 6, 9, and 12 and feeding behavior was quantified. Fish brains were extracted for monoamine analysis at the end of each test (see below). Hence, four tests conducted for 3, 6, 9, or 12 days in which bass were fed every third day and brains were harvested at the end of each test.

Hybrid striped bass (average weight: 163.4 $\pm$ 27.4 g; average length: 21.5 $\pm$ 1.2 cm) were randomly placed individually into twenty-four 80-L aquaria operated under flow-through conditions (0.23 liters per minute). Bass were allowed to acclimate for seven days prior to test initiation. A nearby lake, Lake Hartwell (SC, USA), was the

source of test waters (pH = 6.28 $\pm$ 0.17, Hardness 24 mg/L as $CaCO_3$, Alkalinity 10 mg/L as $CaCO_3$). Water was pumped through a multi-resin filtration system (Water and Power Technologies, Columbia, SC) to remove suspended solids prior to entering experimental tanks. Water temperature was controlled using a mixing valve (M & M Control Services, Grayslake, IL) that regulated a mixture of ambient and chilled water (ambient water circulated through an in-line chiller) or heated water (ambient water circulated through an in-line heater) depending on the ambient conditions (summer or winter months, respectively) to facilitate achieving a desired temperature of about 25°C (23.8 $\pm$1.07° C).

Bass were fed four fathead minnows every third day during the acclimation period. This feeding regime ensured the bass were hungry and were accustomed to eating live minnows. Bass were fed on days 1, 4, and 7 of the acclimation period so that the last day of acclimating coincided with day 0 of the fluoxetine exposure. Feeding behavior (time to capture prey) of hybrid striped bass was quantified every third day during the test as well.

Prior to feeding, air stones were removed from each tank at each time point, and water flow was turned off during the recovery period. Four fathead minnows were dropped into an exposure tank and the time to eat each prey was recorded. Minnows used in the tests were visually estimated to be 4 cm. Minnows of similar size were pre-selected prior to feeding to ensure that no extremely large or small minnows were used to skew appetite satiation. Minnows were then randomly selected and fed to bass without regard to treatment. Bass that did not eat all four minnows prior to exposure initiation were eliminated from the test to reduce any confounding factors. Bass were observed

until all minnows were consumed or for a maximum of 25 minutes. Any uneaten minnows after 25 minutes were removed from the tank. For statistical purposes, uneaten minnow was assigned a value of 1500 seconds (25 minutes).

Fluoxetine exposure

Treatments of four nominal fluoxetine concentrations (0, 35, 75, 150 µg/l) were conducted with five replicate tanks per treatment. At test initiation, water flow was turned off and an appropriate quantity of fluoxetine stock dissolved in methanol was added to each aquarium. One concentrated stock solution of fluoxetine was prepared for all tanks in order to reduce tank-to-tank variability within treatments. Final methanol volume did not exceed 1,000 µl in 80 L of water. The toxicity of methanol to fish has been shown to be greater than 15, 000 mg/l [17], and the ASTM standard guide for conducting early life-stage toxicity tests with fishes (ASTM E1241-92) allows methanol as a carrier solvent at concentrations not to exceed 0.1 mg/l. In our experimental setup, methanol concentrations did not exceed 0.01 mg/l.

Test waters were allowed to equilibrate for 1-2 hours prior to taking 250 ml water samples for fluoxetine analysis. These water samples were acidified to pH ~2.5 before being filtered through 6 ml C_{18} solid phase extraction columns (PrepSep™, Fisher Scientific, USA) that were preconditioned with 6 ml acetone, 6 ml methanol, and 6 ml deionized water. Extraction columns were dried for at least 30 minutes at room temperature on a vacuum manifold (JT Baker, Philipsburg, NJ) before eluting fluoxetine with methanol/1% acetic acid. Control, low, and medium fluoxetine treatment samples were diluted to 5 ml in methanol/1% acetic acid, while the high concentration samples

were diluted to 10 ml. Samples were stored at -20°C until analyzed by HPLC-fluorescence.

Fluoxetine half-life was previously determined to be approximately 72 hours under test conditions. Therefore, waters were spiked with half original fluoxetine concentration on day 3 of exposure period. Water samples for fluoxetine analysis were taken on day 0, day 3 after spiking waters, and day 6 at the end of the exposure.

Fluoxetine analysis

Aqueous fluoxetine concentrations were determined on a Waters 1525 Breeze HPLC pump with a Waters 717 Plus autosampler and Waters 2475 multi-wavelength fluorescence detector (Waters, Milford, MA). Mobile phase was composed of 350 ml acetonitrile, 650 ml water, and 4 ml triethylamine. Mobile phase was adjusted to pH 4 with glacial acetic acid. Flow rate was set at 1 ml/min for a 40 µl injection, and the Alltech Prevail C_{18} column (150 mm, 4.66 I.D.) was set at 30°C. Fluorescence detector was set at 230 nm Ex/ 310 nm Em. Run time per sample was approximately 18 minutes.

Brain tissue preparation

Bass were euthanized in buffered MS-222 and brains were quickly removed and put on dry ice prior for storage at -80°C until processed. Brains were thawed, homogenized for 20 seconds by ultrasonic disruption in 1.0 ml 0.1N perchloric acid containing 0.2 pg/µl DHBA as the internal standard. Homogenates were then centrifuged at 21,000 rpm and 4°C for 20 minutes to remove cellular debris. Supernatant was stored at -80°C until used for bioassays.

Protein assay

Protein concentrations were determined using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Brain homogenates were diluted 1:4 in 0.1N perchloric acid prior to running the assay. Brain monoamine levels were normalized using protein concentrations.

Monoamine analysis

Brain samples were analyzed via HPLC with an electrochemical detector with methods modified from Lin and Pivorun [22]. The chromatographic system consisted of a Bioanalytical System LC-4C amperometric detector, PM-80 pump, and a C18 reverse-phase column (ODS-2 Hypersil 250 mm x 4.6 mm). Aliquots of 30 µl were injected into the sample loop (20 µl) of a rotary injection valve. Flow rate was 1.0 ml/min, with the electrode potential maintained at +0.8 volts versus Ag/AgCl. Mixed monoamine standards was prepared with NE, DA, 5-HIAA, and 5-HT ranging from 150 pg/µg to 10 pg/µg in 0.1N perchloric acid. Each standard also contained 50 pg/µl DHBA as the internal standard. Each sample run took 35 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 Milli-Q water with 15 ml of methanol and 18 ml of tetrahydrofuran. The mobile phase was filtered through a 0.45 µm white nylon Millipore filter before adding methanol and tetrahydrofuran. The mobile phase was degassed via sonication prior to use.

Data analysis

Data for time to capture prey was analyzed using Statistical Analysis Software 9.1 (SAS; Cary, NC, USA) with two-factor ANOVA (analysis of variance) models utilizing treatment and day as the independent variables. Since the time to capture prey data were non-normally distributed with non-homogeneous variances, PROC GLIMMIX (General Linear Model for Mixture Distributions), an analyses matrix that accounts for non-normality and non-homogeneous variances, was used to perform multiple pair-wise comparisons. The LSMEANS statement in PROC GLIMMIX was used to differentiate statistical differences across and within treatment, day, and treatment-by-day interactions.

Monoamine analyses were also performed using two-factor ANOVA models utilizing treatment and day as the independent variables. Although monoamine data were normally distributed with homogeneous variance, PROC GLIMMIX with LSMEANS statement was executed to differentiate statistical differences among and within the independent variables (treatment, day, and the treatment-by-day interaction terms) rather than separate LSMEANS statements within PROC GLM (ANOVA analyses) by day or treatment. An $\alpha = 0.05$ was used with both time to capture prey and monoamine analyses when examining statistical significance of factors using PROC ANOVA and PROC GLIMMIX.

Results

Fluoxetine concentrations

Measured fluoxetine concentrations were approximately 67% of the nominal concentrations most likely due to photolysis, and some sorption to debris in aquaria.

Extraction efficiencies were 90-100% (data not shown). Measured concentrations (mean ± standard deviation) throughout the exposure period for the low (35 µg/l), medium (75 µg/l) and high (150 µg/l) treatments were 23.2 ± 6.6, 51.4 ± 10.9, and 100.9 ± 18.6 µg/l, respectively.

5-HT levels

Brain serotonin levels in low, medium, and high fluoxetine treatments decreased as exposure time increased (Table 1), with maximum depression occurring on day 9 (3 days post exposure). Serotonin levels were depressed 23.7, 28.0, and 49.1% by day 9 in the low, medium, and high treatments, respectively. The low fluoxetine treatment was not significantly different from controls until 3 and 6 days post exposure on days 9 and 12, respectively. Bass in the medium and high treatment groups exhibited significantly depressed serotonin levels at all time points past day 0. Serotonin levels in all treatments began to increase during the recovery period between days 9 and 12. Serotonin in the high treatment decreased significantly between days 6 and 9 with a corresponding increase during the recovery period between days 9 and 12. None of the fluoxetine treated bass recovered completely to control serotonin levels.

To determine the 6-d EC50 for serotonin depression, serotonin levels and fluoxetine concentrations were plotted and 50% effect concentrations were calculated using the polynomial equation. The estimated 6-d EC50 was 24.0 µg/L (Figure 3.1).

5-HIAA Levels

5-HIAA levels were significantly depressed as a result of fluoxetine exposure in all treatment groups (Table 1). On days 3 and 9 (3 days post exposure), all fluoxetine

treatments were significantly different from controls, but not from each other. On day 6, all treatments were significantly different from controls, and the high treatment group was significantly lower than low and medium treatments. On day 12 however (6 days post exposure), while all treatments were significantly different from controls, 5-HIAA levels in the high treatment increased dramatically so that they were significantly greater than low and medium treatment groups.

5-HIAA:5-HT ratio

Both serotonin and 5-hydroxyindoleacetic acid were depressed as a result of fluoxetine exposure. Further, the ratio of 5-HIAA:5-HT was significantly lower in all treatment groups as compared to controls on day 3 (Table 1). On day 6, low and high treatment groups were significantly different from controls, yet they were not significantly different from the medium treatment. On day 9, only the high treatment group was significantly different than controls, due to both continual declines in 5-HT and spikes in 5-HIAA levels. The low fluoxetine group was the only treatment significantly lower than controls at the end of the test on day 12.

Behavior Data: Exposure/Recovery Effects

On day 0, behavior data was recorded prior to initial fluoxetine exposure. There were no significant differences among treatment groups. In the low treatment group, there was a concentration- and duration-dependent effect of fluoxetine on time to capture prey (Figure 3.2). Time to eat increased significantly between capturing the first and second prey fish in the low treatment. By day 12, the sixth day of recovery, time to capture prey decreased to levels comparable to control levels. In the medium treatment,

time to capture prey was significantly different from controls at all time points, with the exception of time to capture prey one on day 12 (Figure 3.2a). Time to capture prey decreased as recovery time increased. In the high treatment, time to capture prey increased through day 12 for the first prey fish, and through day 9 for the second and third prey fish (Figures 3.2b and 3.2c). See Appendix Table A-2 for mean ± SE values for time to capture prey for each treatment and day.

Discussion

SSRIs inhibit 5-HT transporters within minutes, yet it is only after a few weeks of treatment that they exert their full antidepressant effect [19-21]. Therefore, it is the adaptive changes of the 5-HT-containing neurons and receptors that underlie their therapeutic effect, not just the inhibition of 5-HT transporters [19]. Serotonin-containing neurons are endowed with somatodendritic 5-HT_{1A} autoreceptors that exert a negative feedback influence on their firing activity [19]. During prolonged SSRI treatment the 5-HT_{1A} autoreceptors may become desensitized, thus explaining the recovery of normal firing activity of 5-HT-containing neurons [19, 22]. Chronic treatment with SSRIs cause antidepressant effects (a rise in 5-HT concentrations in the terminal region), but acute treatment may not [23]. For example, a two-day treatment with SSRIs decreased the firing activity of 5-HT-containing neurons, but a two-week treatment resulted in neurons progressively regaining their normal firing activity [19].

The results from our study may suggest that 5-HT_{1A} autoreceptors were activated, resulting in a decreased release of serotonin in hybrid striped bass following a 6-day fluoxetine exposure. Serotonin levels in fluoxetine treated bass were decreasing with

increasing exposure duration to fluoxetine (Table 1). It could be hypothesized that if bass in our study were exposed for longer durations, serotonin levels as well as time to capture prey would recover to control levels as a result of the delayed enhancement of 5-HT-mediated transmission following prolonged fluoxetine treatments. This underscores the need for chronic studies since freshwater organisms are likely to be exposed to low levels of fluoxetine over long periods of time.

Following the release of serotonin into the synapse, it continues to stimulate pre- and post-synaptic receptors until it is either taken back up by the presynaptic neuron for reuse, or it is metabolized to 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase [24] in the presynaptic neuron or synaptic cleft. Serotonin turnover is often reported as the ratio of 5-HIAA:5-HT. While it is possible that treatment with SSRIs can increase the ratio due to the decrease in the reuptake of serotonin, and thus metabolism by monoamine oxidases, it has also been shown that stress could increase the 5-HIAA:5-HT ratio, due to increased 5-HIAA levels [25]. However, in the present study, both 5-HIAA and 5-HT levels were reduced in the whole brains of hybrid striped bass exposed to fluoxetine. *Betta splendens* also exhibited declines in 5-HT in the forebrain and 5-HIAA in the forebrain and hindbrain following 14 daily i.p. injections of fluoxetine (4.3 mmol), resulting in a significant reduction in serotonergic activity (5-HIAA:5-HT) in the hindbrain [26]. Yet there were no changes in their aggression. In the present study, 5-HT levels decreased in a dose-dependent manner (Figure 3.1) and 5-HIAA levels decreased with no clear dose-dependent relationship in hybrid striped bass. As a result, we saw a decrease in the 5-HIAA:5-HT ratio during fluoxetine exposures.

Although serotonin levels continued to decrease three days into the recovery period (day 9), 5-HIAA levels in all treatments increased between days 6 and 9 (Table 1), with 5-HIAA levels increasing significantly in the high treatment. This resulted in a spike in the 5-HIAA : 5-HT ratio in bass in the high treatment, rather than a gradual increase in the ratio (Table 1). By day 12, the sixth day of recovery, 5-HIAA levels in the high treatment increased to levels that were significantly greater than the low and medium treatment groups. However recovery of 5-HIAA to control levels had not occurred by day 12 in any treatment.

It has also been shown that food deprivation may increase brain 5-HIAA levels in mammals due to an increased synthesis and metabolism of serotonin [27]. It may be possible that high exposures to fluoxetine, which is effective in decreasing appetite, may have contributed to the sharp increase in 5-HIAA levels seen in bass in the high treatment group between days 6 and 9. In the present study, bass in the high fluoxetine treatment took significantly longer to eat the first and second prey fish as compared to the other treatment groups. They did not eat as many, if any, minnows as compared to the other treatment groups during the exposure and recovery periods. The decreased time to eat exhibited by fish in the low and medium treatments was not seen in the high treatment during the recovery period. (Figures 3.2a and 3.2b). This reduction in food consumption is consistent with reports of suppressed appetite with SSRI treatment [28, 29]. However, fluoxetine elicits this anorexic effect as a result of increased levels of extracellular 5-HT [24, 29, 30], not a decrease in 5-HT levels as seen in our study. We saw a strong negative relationship between serotonin levels and time to capture prey (Figure 3.3).

One explanation for the reduced serotonin levels into the recovery period could be that a reduced energy intake (i.e. reduced feeding) can also significantly reduce 5-HT concentrations in the brain [24]. Bass in all treatments had lower serotonin levels into the recovery period, but bass in the high treatment showed significantly lower serotonin on day 9 (day 3 of recovery) than the other treatments. This could be the result of the bass not eating nearly as many minnow, thus further reducing energy intake.

Another suggestion for the continued decrease in serotonin levels three days into the recovery period (day 9) could be latent effects of fluoxetine. The half-live of fluoxetine has been reported as one to four days in mammals and we should be able to apply this concept to fish [31] since fish have been shown to have high levels of sequence identity for serotonin transporter protein and 5-HT_{1A} receptors genes when compared to humans, rodent, and bovine [32, 33].

Supporting the results of the present study, juvenile *Pimephales promelas* (1-250 µg/l) feeding rates were reduced in a dose dependent manner following a 7-day waterborne fluoxetine exposure. On the other hand, grazing rates of *Daphnia magna* increased with fluoxetine concentrations following a 21-day chronic toxicity test (10-1000 µg/l), though not significantly [15]. This may be the result of desensitized 5-HT_{1A} autoreceptors following an extended exposure duration as discussed earlier. Unfortunately, they did not measure brain serotonin levels to correlate these changes in behavior with biochemical changes. Due to the critical role of serotonin, and the potential for SSRIs to alter an organism's ecological fitness, linking measured

biochemical levels with behavioral changes is important when assessing the impacts of pharmaceuticals on aquatic organisms.

In vertebrates, chronically increased serotonin levels can decrease aggression, resulting in subordinate males having higher serotonergic activity [26, 34]. Clements and Schreck [35] noted a decrease in locomotor activity in Chinook salmon (*Oncorhynchus tshawytscha*) following daily 2.5 mg/kg i.p. fluoxetine injections for 10 days as compared to the saline control. Male bluehead wrasse (*Thalassoma bifasciatum*) injected with fluoxetine daily for 14 days (6 µg/g/day) also exhibited significantly decreased aggression [36]. Even though changes in serotonin levels were not measured, Semsar et al. [36] implied that serotonin levels increased in a dose-dependent manner due to the mode of action of fluoxetine and the behaviors observed. However, results from our study contradict this belief since we saw decreased feeding rates accompanied by decreased whole-brain serotonin levels.

In the present study, bass exposed to high fluoxetine concentrations were observed maintaining their position at the top of the water surface rather than the bottom of the tank like control bass, sometimes with their dorsal fin out of the water. They were also noted to maintain a vertical position in the aquaria; these behaviors persisted throughout both the exposure and recovery periods. Mosquitofish (*Gambusia affinis*) exposed to fluoxetine have also shown abnormal behavior compared to unexposed fish including changing position in the water column so that they were closely associated with the water surface and tended to lay on their sides with little or no swimming movements [37]. Although concentrations used in the present study are above those found in the

environment (typically less than 0.1 µg/l [10]), effects of this nature could have detrimental effects on an organism in the presence of predators.

Behavioral data are useful as predictive indices of population and community-level effects because disruption of essential functions such as predator-prey relationships can become ecologically apparent through population changes when enough individuals are affected [2]. Feeding behavior is crucial for the development, fitness, and long-term viabiliy of an organism and is clearly relevant to the assessment of environmental stressors such as sublethal contaminant exposure [2]. Serotonin has been shown to have a crucial role in many aspects of behavior and biochemical processes. Laboratory studies have shown contradictory results in different species [38]. However, although species sensitivity may be a major contributing factor, concentration, duration, and route of exposure may be just as important. In the present study, there was a dose-dependent response between serotonin levels and fluoxetine concentrations (Figure 3.1) as well as a strong linear relationship between serotonin levels and feeding behavior in bass (Figure 3.3). This linear response was contrary to the non-linear relationship between feeding behavior and brain acetylcholinestrease (AChE) activity in hybrid striped bass exposed to diazinon [16]. AChE activity could be inhibited at concentrations significantly lower than those needed to change behavior. This suggests that there may be excess AChE activity and that bass feeding behavior is not affected until enzyme activity is inhibited by greater than 60%. Results of the present research demonstrate that behavior changes as soon as serotonin levels decrease. These results underscore the use of serotonin as a

biomarker of fluoxetine exposure as well as a biomarker of effect that signals compromised ecological fitness.

References

1. Zala SM, Penn DJ. 2004. Abnormal behaviours induced by chemical pollution: a review of the evidence and new challenges. *Anim. Behav*. 68, 649-664.

2. Little EE. 2002. Behavioral measures of environmental stressors in fish. In: Adams, S.M. (Ed.). *Biological Indicators of Aquatic Ecosystem Stress*. American Fisheries Society. Bethesda, Md., pp. 431-472.

3. Scott GR, Sloman KA. 2004. The effects of environmental pollutants on complex fish behaviour: integrating behavioural and physiological indicators of toxicity. *Aquat. Toxicol.* 68:369-392.

4. Barton BA, Morgan JD, Vijayan MM. 2002. Physiological and condition-related indicators of environmental stress in fish. In: Adams, S.M. (Ed.). *Biological Indicators of Aquatic Ecosystem Stress.* American Fisheries Society. Bethesda, Md., pp. 111-148.

5. Fent K, Weston AA, Caminada D. 2006. Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.* 76:122-159.

6. Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, LaPoint TW. 2003. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* 142:169-183.

7. Bendz D, Paxéus NA, Ginn TR, Loge FJ. 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweeden. *J. Hazard. Mater.* 122(3):1995-204.

8. Halling-Sørensen, B, Nors Nielsen S, Lanzky PF, Ingerslev F, Holten Lützhøft HC, Jørgensen SE. 1998. Occurrence, fate, and effects of pharmaceutical substances in the environment – a review. *Chemosphere* 36(2):357-393.

9. Dove A. 2006. Drugs down the drain. *Nat. Med.* 12:376-377.

10. Koplin DQ, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 199-2000: a national reconnaissance. *Environ. Sci. Technol.* 36:1202-1211.

11. Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ. 2005. Determination of select antidepressants in fish from an effluent-dominated stream. *Environ. Toxicol. Chem.* 24(2):464-469.

12. Fong PP, Huminski PT, D'Urso LM. 1998. Induction and potentiation of parturition in fingernail clams (*Sphaerium striatinum*) by selective serotonin re-uptake inhibitors (SSRIs). *J. Exp. Zool.* 280:260-264.

13. Fong PP. 1998. Zebra mussel spawning is induced in low concentrations of putative serotonin reuptake inhibitors. *Biol. Bull.* 194:143-149.

14. Brooks BW, Turner PK, Stanley JK, Weston JJ, Glidewell EA, Foran CM, Slattery M, LaPoint TW, Huggett DB. 2003. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52:135-142.

15. Stanley JK, Ramirex AJ, Chambliss CK, Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere* 69:9-16.

16. Gaworecki KM, Roberts AP, Ellis N, Sowers AD, Klaine SJ. 2008. Biochemical and behavioral effects of diazinon exposure in hybrid striped bass. (*Envrion. Toxicol. Chem.* in press)

17. Kaviraj A, Bhunia, F, Saha NC. 2004. Toxicity of methanol to fish, crustacean, oligochaete worm, and aquatic ecosystem. *Int. J. Toxicol.* 23:55-63.

18. Lin L-H, Pivorun E.B. 1990. Hypothalamic monoamines and their metabolites in the deermouse, *Peromyscus maniculatus*, during daily torpor. *J. Neural. Transm.* 79:11-18.

19. Blier P, de Montigny D. 1994. Current advances and trends in the treatment of depression. *Trends in Pharmacol. Sci.* 15:220-226.

20. Dawson LA, Nguyen HQ, Smith DI, Schechter LE. 2000. Effects of fluoxetine treatment in the presence and absence of (±)pindolol: a microdialysis study. *Br. J. Pharmacol.* 130:797-804.

21. Le Poul E, Boni C, Hanoun N, Laporte A, Laaris N, Chauveau J, Hamon M, Lanfumey L. 2000. Differential adaptation of brain 5-HT_{1A} and 5-HT_{1B} receptors and 5-HT transporter in rats treated chronically with fluoxetine. Neuropharmacology 39:110-122.

22. Le Poul E, Laaris N, Doucet E, Laporte AM, Hamon M, Lanfumey L. 1995. Early desensitization of somato-dendritic 5-HT_{1A} autoreceptors in rats treated with fluoxetine or paroxetine. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 352:141-148.

23. Invernizzi R, Bramante M, Samanin R. 1994. Chronic treatment with citalopram facilitates the effect of a challenge dose on cortical serotonin output: role of presynaptic 5-HT_{1A} receptors. *Eur. J. Pharmacol.* 260:243-246.

24. Halford JCG, Harrold JA, Lawton CL, Blundell JE. 2005. Serotonin (5-HT) drugs: effects on appetite expression and use for the treatment of obesity. *Curr. Drug Targets* 6:201-213.

25. Winberg S, Nilsson GE, Olsen KH. 1992. The effects of stress and starvation on brain serotonin utilization and starvation on brain serotonin utilization in arctic charr (*Salvelinus alpinus*). *J. Exp. Biol.* 165:229-239.

26. Clotfelter ED, O'Hare EP, McNitt MM, Carpenter RE, Summers CH. 2007. Serotonin decreases aggression via 5-HT_{1A} receptors in the fighting fish *Betta splendens. Pharmacol. Biochem. Behav.* 87:222-231.

27. Feunmayor LD, Garcia S. 1984. The effect of fasting on 5-hyddroxytryptamine metabolism in brain regions of the albino rat. *Br. J. Pharmac.* 83:357-362.

28. Blundell JE, Lawton CL, Halford JCG. 1995. Serotonin, eating behavior, and fat intake. *Obesity Res. 3(Suppl.4)*: 471s-476s.

29. Li DL, Simmons RMA, Iyengar S. 1998. 5HT1A receptor antagonists enhance the functional activity of fluoxetine in a mouse model of feeding. *Brain Res.* 781:121-128.

30. Carlini VP, Gaydou RC, Schioth HB, de Barioglio SR. 2007. Selective serotonin reuptake inhibitor (fluoxetine) decreases the effect of ghrelin on memory retention and food intake. *Regul. Peptides* 140:65-73.

31. Nakamura Y, Yamaoto H, Sekizawa J, Kondo T, Hirai N, Tatarazako N. 2008. The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): acute toxicity in fish larvae and bioaccumulation in juvenile fish. *Chemosphere* 70(5) :865-873.

32. Airhart MJ, Lee DH, Wilson TD, Miller BE, Miller MN, Skalko RG. 2007. Movement disorders and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (PROZAC™). *Neurotoxicol. Teratol.* 29:652-664.

33. Yamaguchi F, Brenner S. 1997. Molecular cloning of 5-hydroxytryptamine (5-HT) type 1 receptor genes from the Japanese puffer fish, *Fugu rubripes*. *Gene* 191:219-223.

34. Perreault HAN, Semsar K., Godwin J. 2003. Fluoxetine treatment decreases territorial aggression in a coral reef fish. *Physiol. Behav.* 79:719-724.

35. Clements S, Schreck CB. 2007. Chronic administration of fluoxetine alters locomotor behavior, but does not potentiate the locomotor stimulating effects of CRH in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *Comp. Biochem. Physiol. A* 147:43-49.

36. Semsar K, Perreault HAN, Godwin J. 2004. Fluoxetine-treated male wrasses exhibit low AVT expression. *Brain Res*. 1029:141-147.

37. Henry TB, Black MC. 2008. Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western mosquitofish. *Arch. Environ. Contam. Toxicol.* 54:325-330

38. Foran CM, Weston J, Slattery M, Brooks BW, Huggett DB. 2004. Reproductive assessment of Japanese medaka (Oryzias latipes) following a four-week fluoxetine (SSRI) exposure. *Arch. Environ. Contam. Toxicol.* 46:511-517.

		Brain Monoamine (pg/µg protein)				
Monoamine	Treatment	Day 0	Day 3	Day 6	Day 9	Day 12
5-HT	Control	30.62 ± 1.46 a	30.66 ± 2.31 a	30.04 ± 0.51 abc	30.21 ± 1.34 ab	29.77 ± 1.29 abc
	Low	30.62 ± 1.46 a	26.50 ± 2.42 c	25.98 ± 1.04 cde	23.05 ± 1.17 efg	24.97 ± 2.13 efg
	Medium	30.62 ± 1.46 a	25.67 ± 1.24 d	22.97 ± 1.64 g	21.74 ± 1.66 g	23.29 ± 0.96 efg
	High	30.62 ± 1.46 a	21.96 ± 0.80 f	21.71 ± 1.13 g	15.37 ± 0.86 h	22.84 ± 1.18 efg
5-HIAA	Control	14.06 ± 0.95 cd	16.72 ± 1.67 ab	14.70 ± 1.05 bc	16.09 ± 1.45 ab	18.13 ± 0.48 a
	Low	14.06 ± 0.95 bc	9.91 ± 1.07 efgh	8.42 ± 0.55 fgh	10.15 ± 0.52 efgh	11.11 ± 0.54 defg
	Medium	14.06 ± 0.95 bc	8.97 ± 0.71 gh	9.00 ± 0.55 gh	11.35 ± 1.12 def	11.84 ± 0.97 de
	High	14.06 ± 0.95 bc	8.41 ± 0.39 hi	6.35 ± 0.63 i	12.07 ± 1.14 cde	14.49 ± 0.91 bc
5-HIAA:5-HT	Control	0.46 ± 0.04 cde	0.55 ± 0.05 abc	0.49 ± 0.04 ace	0.53 ± 0.03 abc	0.61 ± 0.02 ab
	Low	0.46 ± 0.04 acde	0.38 ± 0.05 def	0.32 ± 0.02 df	0.45 ± 0.04 cde	0.45 ± 0.02 cde
	Medium	0.46 ± 0.04 acde	0.35 ± 0.03 def	0.42 ± 0.03 cdef	0.53 ± 0.05 abc	0.52 ± 0.05 ac
	High	0.46 ± 0.04 acde	0.39 ± 0.02 def	0.29 ± 0.02 f	0.81 ± 0.12 g	0.65 ± 0.07 b

Table 3.1: Serotonin (5-HT) and 5-hydroxyindoleaceteic acid (5-HIAA) levels (mean ± SE) in hybrid striped bass brains (pg/µg protein), and 5-HIAA:5-HT ratios following a 6-day fluoxetine exposure followed by a 6-day recovery period. a through i denotes significant differences among treatment and day for each. (On previous page)

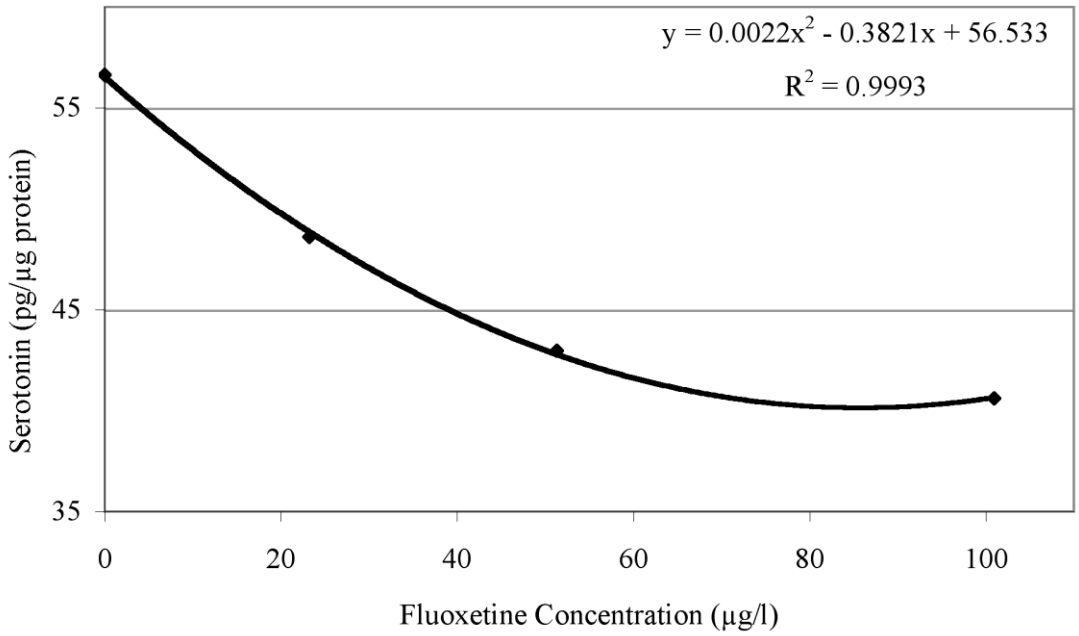

Figure 3.1. Day 6 serotonin (5-HT) levels as a function fluoxetine concentration. The estimated 6-day EC50 was 24.0 µg/l.

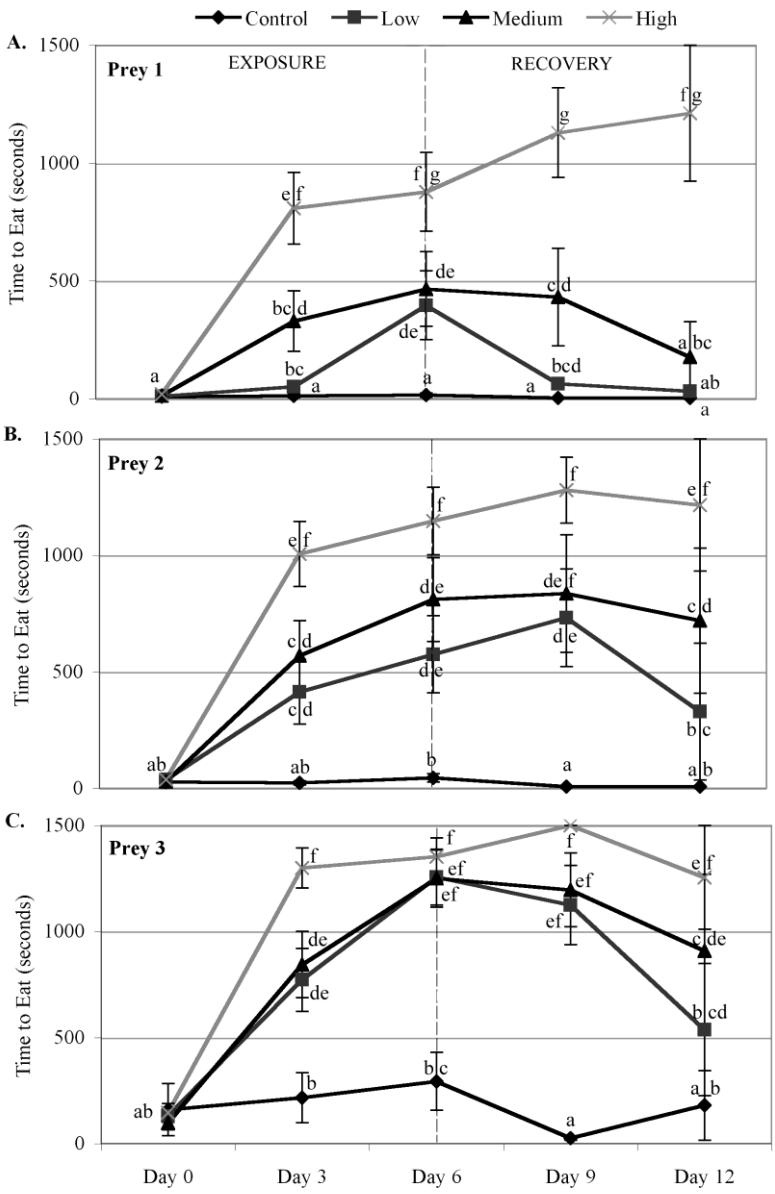

Figure 3.2. Time (mean ± SE) it took hybrid striped bass to capture the first (A) first, (B) second, and (C) third prey fish during as 6-day fluoxetine exposure followed by a 6-day recovery period. The vertical line at day 6 signifies the end of the exposure period and beginning of the recovery period. Means with the same letter are not significantly different from each other.

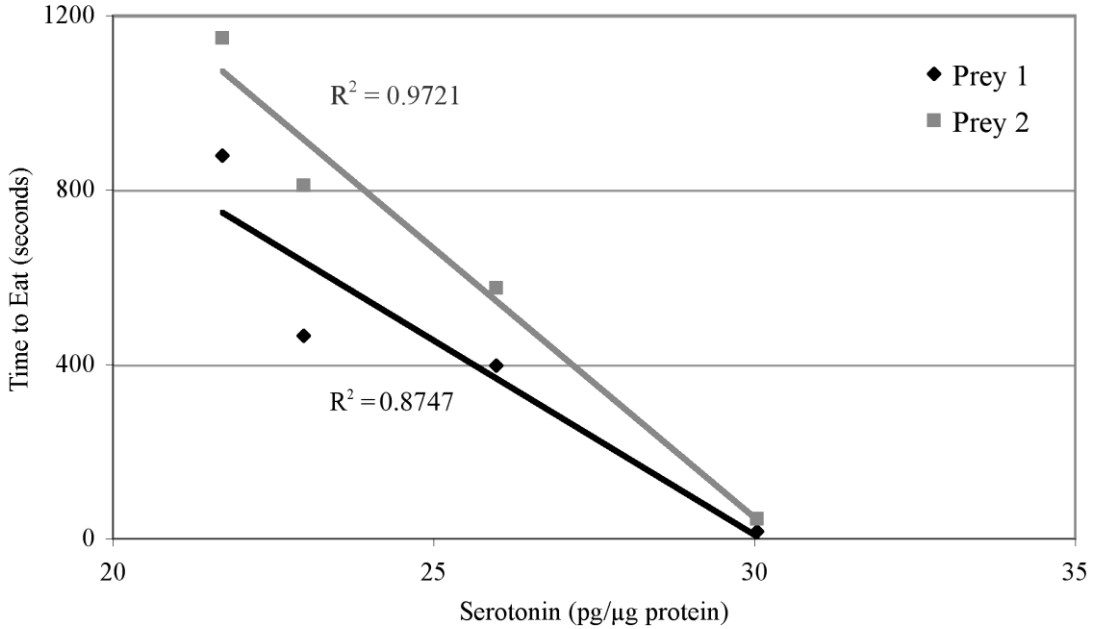

Figure 3.3. Day 6 time to eat prey 1 and prey 2 as a function of brain serotonin levels in hybrid striped bass.

CHAPTER 4: LONG-TERM FLUOXETINE EXPOSURE: BEHAVIORAL AND BIOCHEMICAL EFFECTS

Abstract

Research conducted has shown that pharmaceuticals present in aquatic environments can alter behavior in aquatic organisms. Previous work in our lab has shown that short-term exposure to fluoxetine, a selective serotonin reuptake inhibitor, alters serotonin levels and feeding behavior in hybrid striped bass (*Morone saxatilis x M. chrysops*). The current study characterized effects of long-term fluoxetine exposure on hybrid striped bass. Bass were exposed to fluoxetine (0, 0.1, 1.0, and 10 µg/L) for 27 days. Time to capture prey, *Pimephales promelas*, was measured every third day and brain serotonin levels were measured on days 0, 6, 12, 21, and 27. 5-Hydroxyindoleacetic acid (5-HIAA), norepinehrine, and dopamine levels were also monitored at these times. There were no clear concentration- or exposure duration-dependent changes in feeding behavior, norepinephrine levels, or dopamine levels. While there was no clear dose-response in serotonin levels, there was a significant decrease in 5-HIAA levels in bass in the 10 µg/L fluoxetine treatment as compared to controls. These results suggest that environmentally relevant fluoxetine concentrations do not significantly affect serotonin levels or feeding behavior in hybrid striped bass.

Introduction

A diverse number of pharmaceuticals are produced and used in large quantities around the world both for human and animal consumption (Bound and Voluvoulis, 2004). They are absorbed by an organism after intake and are subject to metabolic

reactions, but metabolism and elimination rates largely depend on the individual, the drug, and the dose. Sometimes, large proportions (up to 90%) of these drugs can leave the body unmetabolized and make their way into wastewater treatment plants (WWTPs) [1, 2]. Many of these drugs are polar, escape sedimentation, and resist biological treatment in WWTPs [3]. Recent studies have found that the incomplete removal of these compounds during the treatment process results in the discharge of these drugs into the aquatic environment [3, 4].

The US Food and Drug Administration (FDA) has been regulating pharmaceuticals in the environment in the USA since 1977 through the environmental review process for New Drug Applications that are submitted. When a new drug is proposed for market, FDA requires risk assessments to estimate concentrations that could be found in the environment as a result of excretion. If this concentration is less than 1 µg/l, the drug is assumed to pose acceptable risk [5, 6]. As a result, the FDA has never turned down a proposed new drug based on estimated environmental concentrations, but no actual testing is conducted after a drug is marketed to see if the estimation was correct [6]. Nevertheless, some small streams are dominated by WWTP effluent and have been found to contain pharmaceutical concentrations exceeding 1 µg/l [7]. Unfortunately, US Environmental Protection Agency water quality criteria do not exist for steroid or non-steroid pharmaceuticals [5].

One class of pharmaceuticals found in the environment is selective serotonin reuptake inhibitors (SSRIs). SSRIs are one of the most prescribed pharmaceuticals in the US (rxlist.com, 2008). The first SSRI, fluoxetine (Prozac™), gained national attention in

1990 when it was referred to, in *Newsweek,* as 'a breakthrough drug in the treatment of depression: a once-a-day medication with acceptable side effects and relatively safe cardiac effects' [8]. Fluoxetine is also prescribed commonly to treat obsessive-compulsive and eating disorders [8]. As an SSRI, fluoxetine blocks serotonin reuptake transporters that return released serotonin back to the presynaptic terminal, in order to elevate serotonin levels and increase serotonin neurotransmission [9, 10].

Serotonin is likely one of the most potent and ubiquitous neuromodulators in plants, invertebrates, and vertebrates [11] playing a role in many essential aspects of life including eating, sleeping, sexual behavior, and mood [8]. Since serotonin regulates so many critical functions, it is possible that exposure to SSRIs could disrupt a number of behavioral functions in non-target species, including appetite [12]. Fluoxetine has been shown to suppress appetite in laboratory animals, and reduce meal size without affecting eating frequency [8].

While only 10% of excreted fluoxetine is as the unmetabolized parent compound [13], the main metabolite, norfluoxetine, has a similar mode of action as the parent compound. While environmental concentrations of norfluoxetine have not been reported, fluoxetine has been reported in surface waters at concentrations as high as 0.012 µg/l [14] and in wastewater effluent from 99 µg/l [14] to 0.540 µg/l [15]. As a result, there are concerns over the potential sublethal effects of fluoxetine on aquatic organisms [12]. While concentrations of pharmaceuticals have typically been found at the ng/l level, their presence is still a concern since they are biologically active [1]. Physiological and behavioral responses are among the issues raised and although the concentrations

detected in effluent of WWTPs are probably too low to affect humans, impacts on aquatic organisms are likely [10]. Pharmaceuticals may be able to biodegrade fairly quickly, but they are continually released into the environment simulating more chronic exposures than acute exposures. Yet, little is known about the long-term low-dose chronic effects of exposure on aquatic organisms.

Fluoxetine has been shown to adversely affect numerous aquatic organisms; however, this occurs at levels at least an order of magnitude higher than reported in the environment [5, 16-18]. Most results reported are that of acute toxicity tests, and many pharmaceuticals are not acutely toxic to aquatic organisms at low concentrations; but they could have significant cumulative effects [3]. Current toxicity-screening data for drugs are usually obtained from standard bioassays that do not target the mode of action of the compound [1], so the danger of some of these drugs could be, and have been, underestimated [19].

Previous research in our lab, demonstrated a linear response between serotonin levels and feeding behavior in hybrid striped bass following a 6-day fluoxetine exposure [20]. However, fluoxetine concentrations used were not environmentally relevant. The purpose of this study was to examine the biochemical and behavioral effects of fluoxetine on the feeding behavior of hybrid striped bass at lower exposure concentrations over a 27-day period.

Materials and Methods

Test chemicals

Fluoxetine hydrochloride, was generously donated by Fermion (Finland). 0.1N perchloric acid (aq), was purchased from VWR (USA). Methanol, acetone, triethylamine, acetolnitrile, glacial acetic acid, monochloro-acetic acid, and tetrahydrofuran were purchased from Fisher Scientific (USA). Sodium hydroxide, sodium octyl sulfate, ethylenediaminetetraacetic acid （EDTA) disodium salt, 3,4-dihydroxybenzylamine hydrobromine (DHBA), 5-hydroxyindole-3-acetic acid (5-HIAA), serotonin creatinine sulfate monohydrate (5-HT), norepinephrine hydrochloride (NE), and dopamine hydrochloride (DA) were purchased from Sigma-Aldrich (USA).

Fish

Hybrid striped bass were obtained from Southland Fisheries near Columbia, SC. Fish were housed in 450 L circular flow-through holding tanks at Clemson University's Institute of Environmental Toxicology and fed a pellet food (Zeigler Brothers, Gardners, PA) daily. Larval fathead minnows (*Pimephales promelas*) were obtained from a culture maintained at Clemson University Institute of Environmental Toxicology and reared until they were the appropriate size (4 to 5 cm) to be used in the tests.

Experimental design

Hybrid striped bass were exposed to fluoxetine for 27 days under static conditions, with a 50% water change every sixth day. Bass were fed every third day on days 0, 3, 6, 9, 12, 15, 18, 21, 24, and 27 and feeding behavior was quantified at these times. Four tests were conducted for 6, 12, 21, or 27 days, where bass were fed every

third day and brains harvested on days 0, 6, 12, 21, or 27, respectively, for quantification of brain monoamine levels.

Hybrid striped bass (16 - 18 months old; average weight 239.8 ± 46.2 g; average length: 24.1 ± 1.4 cm) were randomly placed individually (one bass per tank) into twenty-four 80-L aquaria operated under flow-through conditions (0.23 liters per minute). Bass were allowed to acclimate in aquaria for five days prior to test initiation. A nearby lake, Lake Hartwell (SC, USA), was the source of test waters (pH = 6.5 ± 0.8, Hardness 24 mg/L as $CaCO_3$, Alkalinity 10 mg/L as $CaCO_3$). Water was pumped through a multi-resin filtration system (Water and Power Technologies, Columbia, SC) to remove suspended solids prior to entering experimental tanks. Water temperature was controlled using a mixing valve (M & M Control Services, Grayslake, IL) that regulated a mixture of ambient and chilled water (ambient water circulated through an in-line chiller) or heated water (ambient water circulated through an in-line heater) depending on the ambient conditions (summer or winter months, respectively) to facilitate achieving a desired temperature of about 25°C. During the exposure period while waters were static, water temperatures equilibrated to room temperatures (22.1 ± 1.3° C).

Bass were fed four fathead minnows every third day during the acclimation period. This feeding regime ensured the bass were hungry and were accustomed to eating live minnows. Bass were fed on days 2 and 5 of the acclimation period in the aquaria so that the last day of acclimating coincided with day 0 of the fluoxetine exposure. Feeding behavior (time to capture prey) of hybrid striped bass was then quantified every third day during each test.

Prior to a feeding time point, air stones were removed from each tank. Four fathead minnows were dropped into an experimental tank and the time to eat each prey was recorded. Minnows of similar size (approximately 4 to 5 cm) were pre-selected prior to feeding to ensure that no extremely large or small minnows were used to skew appetite satiation. Minnows were then randomly selected and fed to bass without regard to treatment. Bass that did not eat all four minnows prior to exposure initiation were eliminated from the test to reduce any confounding factors. Bass were observed until all minnow's were consumed or for a maximum of 25 minutes. Any uneaten minnows after 25 minutes were removed from the tank prior to replacing air stones. For statistical purposes, an uneaten minnow was assigned a value of 1500 seconds (25 minutes).

Fluoxetine exposure

Treatments of four nominal fluoxetine concentrations (0, 0.1, 1, 10 µg/l) were conducted with five replicate tanks per treatment. At test initiation, water flows were turned off and an appropriate quantity of fluoxetine stock was added to each aquarium. Every three days, fluoxetine powder was weighed out and dissolved in 5 ml of methanol. This was used to spike three 2-liter stock solutions at various concentrations, one for each treatment level. Methanol volumes ranged from 17.0 to 71.9 µl in the Low stock solutions up to 1, 400 to 3,300 µl in the High stock solutions. Volumes of 100-250 ml from each stock were added to each individual tank to achieve the desired fluoxetine concentration. Final methanol volumes did not exceed 412.5 µl in 80 L of water in test aquaria. The toxicity of methanol to fish has been shown to be greater than 15,000 mg/l [21], and the ASTM standard guide for conducting early life-stage toxicity tests with

fishes (ASTM E1241-92) allows methanol as a carrier solvent at concentrations less than 0.1 mg/l. In our experimental setup, methanol concentrations were much lower than this limit.

Fluoxetine half-life was previously determined to be approximately 72 hours under test conditions. Therefore, waters were spiked every third day with either half original fluoxetine concentration (days 3, 9, 15, and 21) or two-thirds the original fluoxetine concentration (days 6, 12, 18, and 21) when there was also a 50% water change. Since exposure concentrations were so low, it was not feasible to analytically measure individual tank concentrations. Therefore, the stock solutions were used for fluoxetine analyses. For the low, medium, and high stock solutions, 700, 200, and 25 ml, respectively, were acidified to pH ~ 2.5 before being filtered through 6 ml C_{18} solid phase extraction columns (PrepSep™, Fisher Scientific, USA) that were preconditioned with 6 ml acetone, 6 ml methanol, and 6 ml deionized water. Extraction columns were dried for at least 30 minutes at room temperature on a vacuum manifold (JT Baker, Philipsburg, NJ) before eluting fluoxetine with10 ml methanol / 1% acetic acid. Samples were stored at -20°C until analyzed by HPLC-fluorescence.

Fluoxetine Analysis

Aqueous fluoxetine concentrations were determined on Waters 1525 Breeze HPLC pump with a Waters 717 Plus autosampler and Waters 2475 multi-wavelength fluorescence detector (Waters, Milford, MA). Mobile phase was composed of 350 ml acetonitrile, 650 ml water, and 4 ml triethylamine. Mobile phase was adjusted to pH 4 with glacial acetic acid. Flow rate was set at 1 ml/min for a 40 µl injection, and the

Alltech Prevail C_{18} column (150 mm, 4.66 I.D.) was set at 30°C. Fluorescence detector was set at 230 nm Ex/ 310 nm Em. Run time per sample was approximately 18 minutes.

Brain tissue preparation

Bass were euthanized in buffered MS-222 and brains were quickly removed and put on dry ice prior for storage at -80°C until processed. Brains were thawed, weighted, and diluted in 1.0 ml 0.1N perchloric acid containing 50 pg/µl DHBA, the internal standard. Samples were then homogenized for 20 seconds by ultrasonic disruption and centrifuged at 21,000 rpm and 4°C for 20 minutes to remove cellular debris. Supernatant was aliquoted and stored at -80°C until used for analyses.

Protein Assay

Protein concentrations were determined using a BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA). Brain homogenates were diluted 1:4 in 0.1N perchloric acid prior to running the assay. Brain monoamine levels were normalized using protein concentrations.

Monoamine Analysis

Brain samples were analyzed via HPLC with an electrochemical detector with methods modified from Lin and Pivorun [22]. The chromatographic system consisted of a Bioanalytical System LC-4C amperometric detector, PM-80 pump, and a C18 reverse-phase column (ODS-2 Hypersil 250 mm x 4.6 mm). Aliquots of 30 µl were injected into the sample loop (20 µl) of a rotary injection valve. Flow rate was 1.0 ml/min, with the electrode potential maintained at +0.8 volts versus Ag/AgCl. Mixed monoamine standards was prepared with NE, DA, 5-HIAA, and 5-HT ranging from 150 pg/µg to 10

pg/µg in 0.1N perchloric acid. Each standard also contained 50 pg/µl DHBA as the internal standard. Each sample run took 35 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 Milli-Q water with 15 ml of methanol and 18 ml of tetrahydrofuran. The mobile phase was filtered through a 0.45 µm white nylon Millipore filter before adding methanol and tetrahydrofuran. The mobile phase was degassed via sonication prior to use.

Data analysis

Data for time to capture prey was analyzed using Statistical Analysis Software 9.1 (SAS; Cary, NC, USA) with two-factor ANOVA (analysis of variance) models utilizing treatment and day as the independent variables. Since the time to capture prey data were non-normally distributed with non-homogeneous variances, PROC GLIMMIX (General Linear Model for Mixture Distributions), an analyses matrix that accounts for non-normality and non-homogeneous variances, was used to perform multiple pair-wise comparisons. The LSMEANS statement in PROC GLIMMIX was used to differentiate statistical differences across and within treatment, day, and treatment-by-day interactions.

Monoamine concentrations were analyzed using two-factor ANOVA models utilizing treatment and day as the independent variables. Although monoamine data were normally distributed with homogeneous variance, PROC GLIMMIX with LSMEANS statement was executed to differentiate statistical differences among and within the independent variables (treatment, day, and the treatment-by-day interaction terms) rather than separate LSMEANS statements within PROC GLM (ANOVA analyses) by day or

treatment. An $\alpha = 0.05$ was used with both time to capture prey and monoamine analyses when examining statistical significance of factors using PROC ANOVA and PROC GLIMMIX.

Results

Fluoxetine concentrations

Measured fluoxetine stock concentrations (mean ± standard deviation) throughout the exposure period for the low (0.1 µg/l), medium (1 µg/l) and high (10 µg/l) treatments were 0.08 ± 0.02, 0.87 ± 0.12, and 9.44 ± 0.82 µg/l, respectively.

5-HT levels

There was no clear dose-response relationship for serotonin levels on any day. As compared to controls on day 27, serotonin levels were significantly greater in bass in the high treatment (Table 1, Figure 1a). At no other time point did fluoxetine-treated bass have significantly different serotonin levels as compared to the controls. When analyzing serotonin levels within each treatment across days, there were no significant increases or decreases in serotonin levels, with the exception of the medium treatment group between days 6 and 27 where serotonin levels were significantly greater on day 6 than day 27 (Table 1). In addition, serotonin levels were significantly lower in the control and low treatments on day 27 as compared to all other time points for these treatments (Table 1). In trying to explain this decrease in serotonin levels, brain monoamines in the medium treatment were also lower on day 27, although not significant, suggesting a possible issue with sample storage from that experiment, rather than a consequence of fluoxetine exposure. Therefore, it is not possible to conclude that the significant difference in

serotonin levels between the controls and high treatment on day 27 is a result of fluoxetine exposure.

5-HIAA levels

There were significant decreases in 5-HIAA levels between days 0 and 6 for bass in the control, low, and high treatments, but not the medium treatment (Table 1). There were also significant decreases in 5-HIAA levels on day 27 as compared to day 0 for all treatment groups, including the controls (Table 1). This may be the result of an error in sample storage rather than an observed effect of fluoxetine exposure since there were significant changes in the controls as well. On days 6, 12, and 21, 5-HIAA levels were significantly reduced in the high treatment as compared to the controls on the same day (Table 1, Figure 1b). There was a decreasing trend in 5-HIAA levels between days 6 and 12 in the low and medium treatments, but they were not significant (Table 1). By day 27, there was no significant difference in 5-HIAA activities between any treatment groups.

5-HIAA:5-HT ratio

On day 0, the 5-HIAA:5-HT ratio was significantly greater than all other time points, except for controls on day 27 (Table 1, Figure 1c). This is most likely due to the significantly greater 5-HIAA levels on day 0. Otherwise, the only significant difference as compared to control was seen in the high treatment on days 6 and 12 (Table 1, Figure 1c). However, there were no significant changes in 5-HIAA:5-HT ratio in the high treatment as the exposure duration continued between days 6 and 27.

NE levels

There was no significant difference in NE levels as compared to controls on any day, with the exception of day 27 in which NE levels in the high treatment were significantly greater than control and low treatments (Table 1, Figure 2a). This was most likely due to the significantly lower NE levels in the control and low treatments as compared to any other day, and not the result of fluoxetine exposure. NE levels in bass in the medium treatment were significantly lower on day 27 as compared to days 0 and 6; however, NE levels were not as low as the control or low treatments, nor were they significantly different (Table 1).

DA levels

There were no significant differences in DA levels as compared to controls on any day (Table 1, Figure 2b). However, as mentioned for the other monoamines investigated, there were significant declines in DA levels on day 27 in the control and low treatment groups as compared to any other day (Table 1).

Behavior data

In general, time to capture prey was not significantly impaired at the fluoxetine concentrations chosen for this test (Figure 3). There were, however, a few significant increases in time to capture prey in the high treatment as compared to controls. Time to eat prey 1 was significantly longer on days 6, 15, and 24; time to eat prey 2 was significantly longer on days 6 and 15; time to eat prey 3 was significantly longer on days 6, 12, and 15. These significant differences are most likely the result of a few outlier bass, by which their increased time to eat greatly skewed the means. When looking at the

effect of exposure-duration on each treatment, there were no significant differences across days, including in the high treatment. Overall, the feeding behavior of bass was not affected at the concentrations tested. See Appendix Table A-3 for mean ± SE values for time to capture prey for each treatment and day.

Discussion

Selective serotonin reuptake inhibitors have become very popular due to their effectiveness in treating depression, obsessive-compulsive disorders, and eating disorders. In 2008, there were seven identified antidepressants among the top 200 most prescribed drugs in the US that regulate the reuptake of serotonin on the presynaptic terminal (rxlist.com, 2008). This includes both SSRIs and selective serotonin and norepinephrine reuptake inhibitors (SSNRIs). Although detected environmental concentrations of individual SSRIs are low, the aquatic exposure might be a function of their combined concentrations, taking into consideration that SSRIs likely have the same mode of action [17]. Thus, total environmental concentrations may be approaching values that are shown to impact non-target species [23]. It has already been shown that some SSRIs can accumulate in fish in effluent-dominate streams, including fluoxetine [24].

In the laboratory, fluoxetine been shown to cause a number of behavioral effects in aquatic organisms including induced spawning in mussels [16], reduced reproduction in *C. dubia* [9], and reduced feeding behavior in hybrid striped bass [20]. However, all these results were determined using fluoxetine concentrations that were greater than what is found in the environment. When looking at the effects at lower, more environmentally

relevant concentrations, the effects were not as profound. In the present study, we saw no significant effects of fluoxetine on feeding behavior in bass exposed to 0.1 1.0, or 10.0 µg/l over the 27-day exposure. There were a few time points in which feeding behavior in the highest treatment was significantly different from controls, but it cannot be concluded if this was a result of fluoxetine exposure, or just individual variability. Overall, there was no clear dose-dependent response in feeding behavior across treatment for any time point.

It has been generally concluded that environmentally relevant concentrations of fluoxetine is not likely to cause acute toxicity to aquatic organisms. However, as a result of the continuous human use of fluoxetine and the incomplete removal of SSRIs and other pharmaceuticals from WWTPs, there will likely be a continuous release of low concentrations into the environment [17]. Therefore, chronic toxicity tests to determine long-term effects should be routine when evaluating these chemicals. Henry and Black [18] found that chronic exposure of western mosquitofish (*Gambusia affinis*) to low concentrations of fluoxetine did not affect survival, sex ratio, or development. While there was evidence of decreased expression of sexual characteristics indicating developmental delays from chronic fluoxetine exposure of 71 µg/l, this effect was not seen at lower tested concentrations [18]. Other long-term tests conducted using low fluoxetine concentrations (0.1 to 5.0 µg/l) showed no effect on growth or condition factors, or a number of measured adult reproductive parameters in Japanese medaka (*Oryzias latipes*) [25]. In addition, long-term fluoxetine treatment (14 daily i.p. injections

at 4.3 mmol) of male *Betta spendens* did not significantly decrease their territorial aggression [26].

The results of the present study further support the notion that there are minimal effects of long-term fluoxetine exposure on behavior. There were no clear behavioral effects on feeding as a result of exposure duration in bass exposed to fluoxetine (0.1 to 10.0 µg/l) for 27 days. In the high treatment there were some observed behavioral effects of fluoxetine in which some bass were slightly diagonal in the water column, but this did not impair their ability to capture prey once minnows were dropped into the aquarium. Bass in all treatments, including the high treatment, were observed to be anxiously waiting to be fed. Contrary to our observations, Henry and Black[18] noted that there was a slight increase in lethargy when mosquitofish were chronically exposed to fluoxetine (0.5 to 5.0 µg/l), indicating slight behavioral changes [18]. However, this could be attributed to differences in species sensitivity.

Serotonin is involved in a number of physiological processes, including control of meal size or satiation, and fluoxetine has been shown to have properties of satiety enhancing agents, advancing the behavioral satiety sequence in rats [8]. Thus fluoxetine exposure has the potential to alter serotonin levels and hunger in aquatic species as well, ultimately resulting in consequences on organism and population [17]. Unfortunately, attempts to provide a unified function for serotonin are elusive since the literature on this one chemical system is filled with apparent contradictions [11] with species to species variability including teleosts [27].

While fluoxetine was developed with the intent to increase serotonin levels at the synapse, there is a delayed onset of this therapeutic effect for 2-4 weeks [28, 29]. As a compensatory mechanism following the blockage of reuptake pumps, inhibitory 5-HT autoreceptors are activated leading to decreased serotonin neuronal activity and neurotransmitter release, thus preventing an increase in serotonin concentrations [30]. It has been shown that desensitization of 5-HT_{1A} and 5-HT_{1B} autoreceptors that control serotonin release occurs after long-term treatment with SSRIs [28-30], thus eventually allowing the release of serotonin while in the presence of SSRIs. This process leads to the delayed therapeutic effect.

Many aquatic toxicity tests involving fluoxetine are preformed without verifying changes in serotonin levels in the organism, assuming that serotonin levels would increase in the presence of fluoxetine. One rat study involving fluoxetine noted elevated serotonin levels within 30 minutes of exposure and persisted for four hours [31], but they did not continue monitoring serotonin levels past this point. Since fluoxetine binds immediately to the transporters [32], it may be possible that this initial increase in serotonin levels occurred before autoreceptors were desensitized. When we looked at serotonin levels three and six days following the initial fluoxetine exposure in bass, we found that bass exhibited a decrease in serotonin levels [20]. We attributed this to activated 5-HT autoreceptors that decreased the release of serotonin into the synapse. We hypothesized that if we carried out the exposure over a longer period of time, we would possibly see serotonin levels return to control levels and maybe surpass control serotonin

levels. However, at the fluoxetine concentrations used in the present study, we did not observe this effect.

While there was a decrease in serotonin levels on day 12 of the fluoxetine exposure, it was neither dose-dependent nor significant. In addition, we did see significantly elevated serotonin levels in the high treatment group as compared to controls on day 27 and it may be an effect of the long-term fluoxetine exposure, but it is also possible that this is the result of unusually low monoamine levels in the control and low treatment groups on that day. There were no significant changes in serotonin levels in the high treatment at any time points tested (days 0, 6, 12, 21, and 27), so the latter may be true. Differences in basal monoamine levels observed among experiments were likely a result of the fact that each experiment was conducted at different times and sample preparations were not conducted all at the same time. Additional research would need to be performed to further speculate the effects of fluoxetine exposure on hybrid striped bass.

Since 5-HIAA is the metabolite of serotonin, there would also be a decrease in 5-HIAA levels noted in the presence of fluoxetine since there is less serotonin at the synapse until the autoreceptors are desensitized. In the present study, there was significantly less 5-HIAA in bass in the high treatment as compared to controls on days 6, 12, and 21. This would be consistent with a decreased firing of 5-HT neurons due to the activation of autoreceptors in the presence of fluoxetine, but there was not a decrease in serotonin levels to fully support this conclusion.

Other studies in mice have shown a reduction in the 5-HIAA:5-HT ratio after acute, subacute, and chronic treatments [33]. They noted a persistence of decreased serotonin turnover (5-HIAA:5-HT) during the fluoxetine administration. In the present study, there were significant decreases in serotonin turnover noted as compared to controls, but only differences on days 6 and 21 in the high treatment. It is also important to mention here that there was no significant change in serotonin turnover in the high treatment between days 6 and 27. The serotonin turnover rate (5-HIAA:5-HT ratio) was significantly higher on day 0 as compared to any other day, but this is most likely the result of the high basal levels of 5-HIAA on that day.

Although it is supposed to be selective for serotonin, fluoxetine exposure has been shown to increase extracellular norepinephrine and dopamine in rats [31]. In the present study, there were no significant differences in norepinephrine or dopamine levels in bass treated with fluoxetine as compared to the controls, with the exception on day 27. Bass in the high treatment exhibited significantly greater norepinephrine levels as compared to the control and low treatments and significantly greater dopamine levels than the low treatment, but not controls, on that day. Again, we cannot confirm whether these findings were a result of the fluoxetine exposure, or a result the significant decrease monoamine levels in the control and low treatments on that day as compared to all other time points.

While it is important to determine the effects of fluoxetine on aquatic organisms at environmentally relevant concentrations, it is also important to know what effects could occur at higher exposure concentrations. Individual pharmaceuticals occurring at low levels in the environment may exhibit synergistic and cumulative effects [3]. Using

only standardized tests, the effects of pharmaceuticals in the environment could be underestimated. Since the FDA is not entirely concerned with drugs present in the environment below 1 µg/l, additive effects of drugs with similar modes of action, and interactions of drugs with different modes of actions are not considered [5]. If tests were tailored toward the mode of action of a compound, the effects level could be significantly lowered [19].

While regulations for drugs in the environment are only beginning to be considered, they are no different from other chemicals. They still have the potential to cause adverse effects to aquatic organisms and communities, especially since many are known to make their way through WWTPs. Although research results have shown that there appears to be minimal behavioral and/or biochemical effects to individual SSRI exposure scenarios, this should not be interpreted to suggest there are no effects of exposure. It is likely that continual pharmaceutical exposure to aquatic organisms could result in effects that go unnoticed until they become irreversible. The effects could accumulate slowly, and as a result changes may be attributed to natural, ecological succession [34] rather than the problem at hand. It is important to run chronic exposure tests, recognizing that most pharmaceutical active ingredients are not acutely toxic, but may have long-term effects on organisms. It is also important to continue expanding this research using mixtures of pharmaceuticals, especially those that have the same mode of action. We know that individual pharmaceuticals have shown effects at concentrations greater than found in the environment; next we need to explore the effects of low-level

mixtures of SSRIs with the same speculated mode of action to more accurately rule out or define adverse effects of exposure to aquatic organisms.

References

1. Bound JP, Voulvoulis, N. 2004. Pharmaceuticals in the aquatic environment – a comparison of risk strategies. *Chemosphere* 56:1143-1155.

2. Carballa M, Omil F, Lema JM, Llompart M, García-Jares C, Rodríguez I, Gómez M, Ternes T. 2004. Behavior of pharmaceuticals, cosmetics and hormones in a sewage treatment plant. *Water Res.* 38:2918-2926.

3. Bendz D, Paxéus NA, Ginn TR, Loge FJ. 2005. Occurrence and fate of pharmaceutically active compounds in the environment, a case study: Höje River in Sweden. *J. Hazard. Mater.* 122:195-204.

4. Ternes TA. Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.* 32:3245-3260.

5. Brooks BW, Turner PK, Stanley JK, Weston JJ, Glidewell EA, Foran CM, Slattery M, La Point TW, Huggett DB. 2003. Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere* 52:135-142.

6. Raloff J. 1998. Drugged Waters. *Sci. News*. 153:187-189.

7. Cleuvers M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combined effects. *Toxicol. Lett.* 142:185-194.

8. Wong DT, Bymaster FP, Engleman EA. 1995. Prozac™ (Fluoxetine, Lilly 110140), the first selective serotonin uptake inhibitor and an antidepressant drug: twenty years since its first publication. *Life Sci.* 57:411-441.

9. Henry TB, Kwon JW, Armbrust KL, Black MC. 2004. Acute and chronic toxicity of five selective serotonin reuptake inhibitors in *Ceriodaphnia dubia*. *Environ. Toxicol. Chem.* 23:2229-2233.

10. Fong PP. 2001. Antidepressants in aquatic organisms: a wide range of effects. In: Daughton CG, Jones-Lepp TL. (Eds.). *Pharmaceuticals and Personal Care Products In the Environment.* American Chemical Society, Washington, D.C. pp.264-281.

11. Azmitia, EC. 1999. Serotonin neurons, neuroplasticity, and homeostasis of neural tissue. *Neurophyschopharmacology 21 Supp.* (33S-45S)

12. Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, La Point TW. 2003a. Aquatic ecotoxicology of fluoxetine. *Toxicol. Lett.* 142:169-183.

13. Hiemke C, Härtter S. 2000. Pharmacokinetics of selective serotonin reuptake inhibitors. *Pharmacol. Therapeut.* 85:11-28.

14. Koplin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: a national reconnaissance. *Environ. Sci. Technol.* 36:1202-1211.

15. Weston JJ, Huggett DB, Rimoldi J, Foran CM, Stattery M. 2001. Determination of fluoxetine (Prozac™) and norfluoxetine in the aquatic environment. Annual Meeting of the Society of Environmental Toxicology and Chemistry, Baltimore, MD.

16. Fong, PP, Huminski PT, D'Urso. 1998. Induction and potentiation of parturition in fingernail clams (*Sphaerium striatinum*) by selective serotonin re-uptake inhibitors (SSRIs). *J. Exp. Zool*. 280:260-264.

17. Henry TB, Black MC. 2007. Mixture and single-substance acute toxicity of selective serotonin reuptake inhibitors in *Cerodaphnia dubia. Environ. Toxicol. Chem.* 26:1751-1755.

18. Henry TB, Black MC. 2008. Acute and chronic toxicity of fluoxetine (selective serotonin reuptake inhibitor) in western mosquitofish. *Arch. Environ. Contam. Toxicol.* 54:325-330.

19. Henschel KP, Wenzel A, Diedrich M, Fliedner A. 1997. Environmental hazard assessment of pharmaceuticals. *Regul. Toxicol. Pharm.* 25:220-225.

20. Gaworecki KM, Klaine SJ. 2008. Behavioral and biochemical responses of hybrid striped bass during and after fluoxetine exposure. *Aquat. Toxicol. in press.*

21. Kaviraj, A., Bhunia, F., Saha, N.C., 2004. Toxicity of methanol to fish, crustacean, oligochaete worm, and aquatic ecosystem. *Int. J. Toxicol*. 23, 55-63.

22. Lin L-H, Pivorun EB. 1990. Hypothalamic monoamines and their metabolites in the deermouse, Peromyscus manicultatus, during daily torpor. *J. Neural Transm. [Gen. Sec.]* 79:11-18.

23. Vasskog T, Berger U, Samuelsen, PJ, Kallenborn R, Jensen E. 2006. Selective serotonin reuptake inhibitors in sewage influents and effluents from Tromsø, Norway. *J. Chromatogr. A* 1115:187-195.

24. Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE., Johnson RD, Lewis RJ. 2005. Determination of select antidepressants in fish from and effluent-dominated stream. *Environ. Toxicol. Chem.* 24:464-469

25. Foran CM, Weston J, Slattery M, Brooks BW, Huggett DB. 2004. Reproductive assessment of Japanese medaka (*Oryzias latipes*) following a four-week fluoxetine (SSRI) exposure. *Arch. Environ. Contam. Toxicol.* 46:511-517.

26. Clotfelter ED, O'Hare EP, McNitt MM, Carpenter RE, Summers CH. 2007. Serotonin decreases aggression via 5-HT1A receptors in the fighting fish *Betta splendens. Pharmacol. Biochem. Behav.* 87:222-231.

27. Airhart MJ, Lee DH, Wilson TD, Miller BE, Miller MN, Skalko RG. 2007 Movement disorder and neurochemical changes in zebrafish larvae after bath exposure to fluoxetine (Prozac™). *Neurotoxiol. Teratol.* 29: 652-664.

28. Dawson LA, Nguyen HQ, Smith DI, Schechter LE. 2000. Effects of chronic fluoxetine treatment in the presence and absence of (±)pindolol: a microdialysis study. *Brit. J.Pharmacol.* 130:797-804.

29. Newman, ME, Shalom G, Ran A, Gur E, Van de Kar LD. 2004. Chronic fluoxetine-induced desensitizationof 5-HT_{1A} and 5-HT_{1B} autoreceptors: regional differences and effects of WAY-100635. *Eur. J. Pharmacol.* 486:25-30.

30. Invernizzi R, Bramante M, Samanin R. 1994. Chronic treatment with citalopram facilitiate the effect of a challenge dose on cortical serotonin output: role of presynaptic 5-HT_{1A} receptors. *Eur. J. Pharmacol.* 260:243-246.

31. Bymaster FP, Zhang W, Carter PA, Shaw J, Chernet E, Phebus L, Wong DT, Perry KW. 2002. Fluoxetine, but not other selective serotonin uptake inhibitors, increases norepinephrine and dopamine extracellular levels in prefrontal cortex. *Psychopharmacology* 160:353-361.

32. Le Poul E, Boni C., Hanoun N, Laporte AM, Laaris N, Chauveau J, Hamon M, Lanfumey L. 2000. Differential adaptation of brain 5-HT1A and 5-HT1B receptors and 5-HT transporter in rats treated chronically with fluoxetine. *Neuropharmacology* 39:110-122.

33. Hall JM, Anderson GM, Cohen DJ. 1995. Acute and chronic effects of fluoxetine and haloperidol on mouse brain serotonin and norepinephrine turnover. *Life Sci.* 57:791-801.

34. Daughton CG, Ternes TA. 1999. Pharmaceuticals and personal care products in the environment: agents of subtle change. *Environ. Health Persp.* 107:907-938.

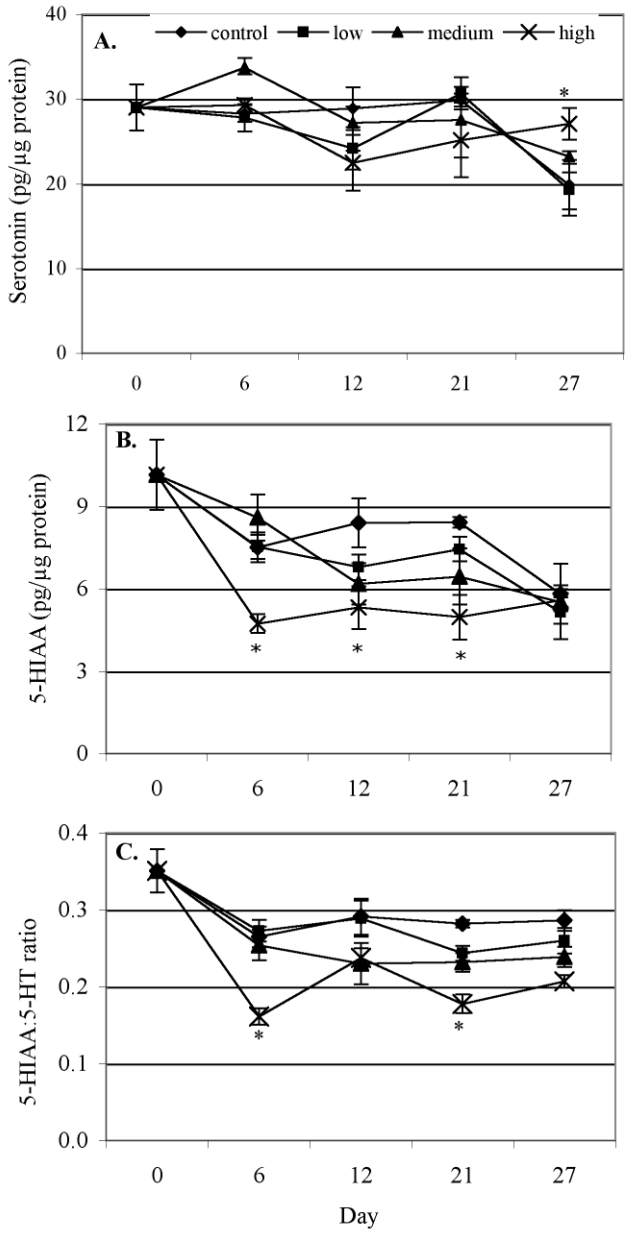

Figure 4.1. Serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) levels (mean ± SE) in hybrid striped bass brains, and 5-HIAA:5-HT ratios during a 27-day fluoxetine exposure. * denotes significant differences (α=0.05) from the control on the respective day.

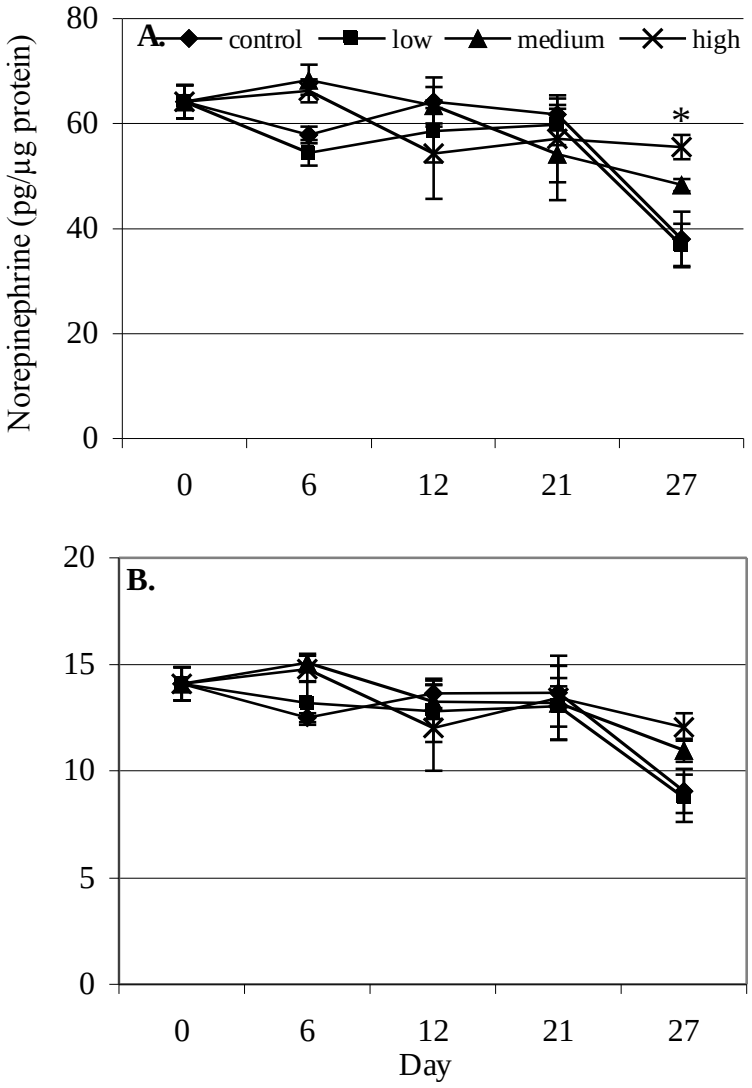

Figure 4.2. Norepinephrine (A.) and dopamine (B.) levels (mean ± SE) in hybrid striped bass brains during a 27-day fluoxetine exposure. * denotes significant differences (α=0.05) from the control on the respective day.

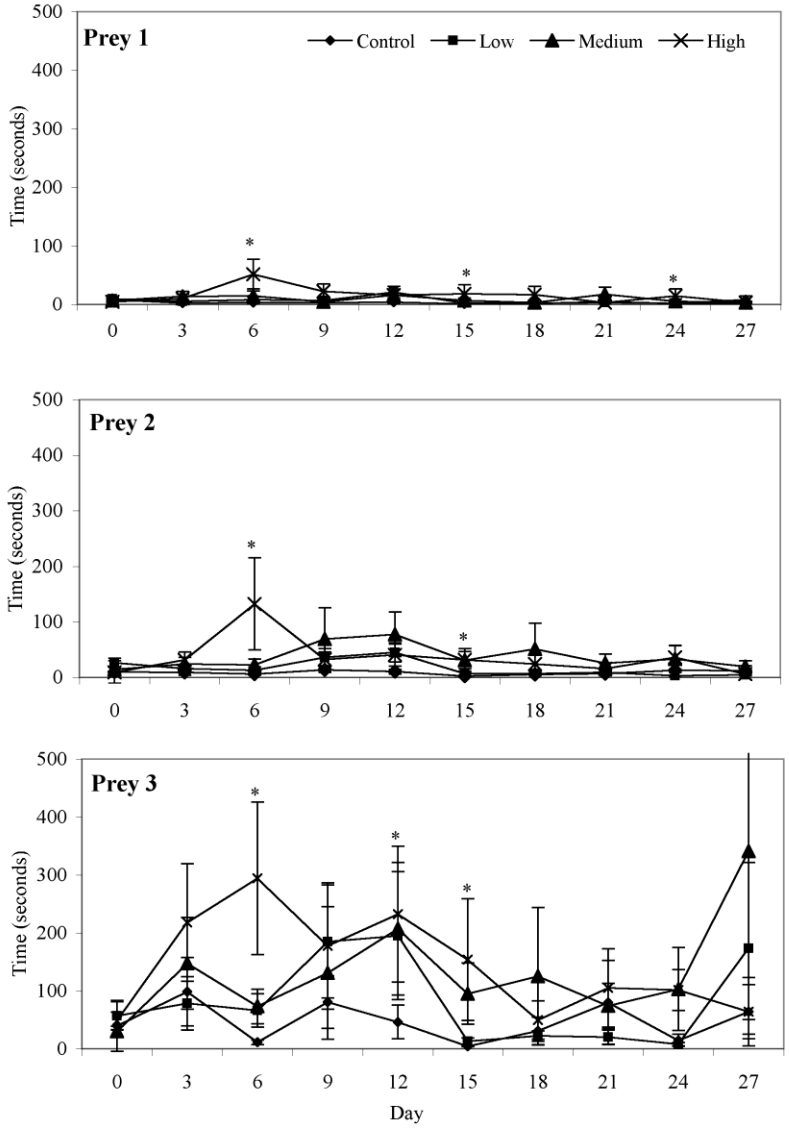

Figure 4.3. Time (mean ± SE) it took hybrid striped bass to capture prey 1, 2, and 3 during a 27-day fluoxetine exposure. * denotes days when time to capture prey in the high and medium treatments, respectively, were significantly different ($\alpha = 0.05$) from controls.

Brain Monoamine (pg/µg protein)						
Monoamine	Treatment	Day 0	Day 6	Day 12	Day 21	Day 27
5-HT	Control	29.0 ± 2.7 abcd	28.3 ± 1.0 abcd	28.9 ± 2.5 abcd	29.8 ± 0.8 abc	19.9 ± 2.9 e
	Low	29.0 ± 2.7 abcd	27.8 ± 1.6 abcd	24.2 ± 2.5 bcde	30.7 ± 1.9 ab	19.3 ± 3.1 e
	Medium	29.0 ± 2.7 abcd	33.7 ± 1.1 a	27.2 ± 1.9 abcd	27.5 ± 3.9 abcd	23.2 ± 0.7 cde
	High	29.0 ± 2.7 abcd	29.3 ± 0.8 abcd	22.4 ± 3.3 de	25.1 ± 4.4 bcde	27.1 ± 1.9 abcd
5-HIAA	Control	10.1 ± 1.3 a	7.5 ± 0.5 bcd	8.4 ± 0.9 abc	8.4 ± 0.2 abc	5.8 ± 1.1 def
	Low	10.1 ± 1.3 a	7.5 ± 0.4 bcd	6.8 ± 0.5 bcdef	7.4 ± 0.4 bcde	5.1 ± 1.0 f
	Medium	10.1 ± 1.3 a	8.6 ± 0.8 ab	6.2 ± 0.8 cdef	6.4 ± 1.0 bcdef	5.5 ± 0.2 def
	High	10.1 ± 1.3 a	4.7 ± 0.3 f	5.3 ± 0.8 ef	5.0 ± 0.8 f	5.6 ± 0.4 def
5-HIAA:5-HT	Control	0.35 ± 0.03 a	0.27 ± 0.01 bc	0.29 ± 0.02 b	0.28 ± 0.01 bc	0.27 ± 0.01 ab
	Low	0.35 ± 0.03 a	0.27 ± 0.01 bc	0.29 ± 0.02 bc	0.24 ± 0.01 bcd	0.26 ± 0.02 bcd
	Medium	0.35 ± 0.03 a	0.25 ± 0.02 bcd	0.23 ± 0.03 cde	0.23 ± 0.01 bcde	0.24 ± 0.01 bcde
	High	0.35 ± 0.03 a	0.16 ± 0.01 e	0.24 ± 0.01 bcde	0.18 ± 0.01 de	0.21 ± 0.01 bcde
NE	Control	64.1 ± 3.2 ab	57.9 ± 1.6 abc	64.1 ± 4.7 ab	61.7 ± 3.1 abc	38.0 ± 5.2 d
	Low	64.1 ± 3.2 ab	54.5 ± 2.4 bc	58.6 ± 5.9 abc	59.8 ± 3.8 abc	36.7 ± 4.2 d
	Medium	64.1 ± 3.2 ab	68.4 ± 2.8 a	63.5 ± 3.5 ab	54.2 ± 8.7 bc	48.3 ± 1.1 cd
	High	64.1 ± 3.2 ab	66.2 ± 2.2 ab	54.3 ± 8.7 bc	57.1 ± 8.3 abc	55.5 ± 2.4 abc
DA	Control	14.1 ± 0.8 a	12.5 ± 0.2 ab	13.6 ± 0.7 ab	13.7 ± 0.7 ab	9.0 ± 1.0 cd
	Low	14.1 ± 0.8 a	13.2 ± 1.0 ab	12.8 ± 1.4 ab	13.0 ± 0.9 ab	8.7 ± 1.1 d
	Medium	14.1 ± 0.8 a	15.1 ± 0.4 a	13.2 ± 0.8 ab	13.2 ± 1.7 ab	11.0 ± 0.6 bcd
	High	14.1 ± 0.8 a	14.8 ± 0.6 a	12.0 ± 2.0 abc	13.4 ± 2.0 ab	12.0 ± 0.6 abc

Table 4.1. Serotonin (5-HT), 5- hydroxyindoleacetic acid (5-HIAA), norepinephrine (NE), dopamine (DA) levels (mean ± SE) in hybrid striped bass (pg/µg protein), and the 5-HIAA:5-HT ratio following a 27-day fluoxetine exposure. a through f denotes significant differences among treatment and day for each monoamine or ratio. (On previous page)

CONCLUSIONS

Behavior

(1) There were duration-dependent increases in time-to-capture prey in hybrid striped bass acutely exposed to diazinon or fluoxetine for 6 days.

(2) There were duration-dependent decreases in time-to-capture prey in hybrid striped bass through 6-day recovery periods following acute diazinon or fluoxetine exposures.

(3) There were no concentration- or duration-dependent responses in time-to-capture prey in hybrid striped bass exposed to fluoxetine for 27 days.

Biochemistry

(4) There were concentration- and duration-dependent decreases in brain acetylcholinesterase (AChE) and serotonin levels in hybrid striped bass acutely exposed to diazinon and fluoxetine, respectively, for 6 days

(5) There were concentration- and duration-dependent increases in brain acetylcholinesterase and serotonin levels in hybrid striped bass through 6-day recovery periods from diazinon or fluoxetine exposures, respectively. However, increases were not always significant nor did they all reach control levels at the end of the recovery period.

(6) There were no concentration- or duration-dependent changes in serotonin, norepinephrine, or dopamine levels in hybrid striped bass exposed to fluoxetine for 27-d. 5-HIAA levels were only significantly lower than controls in the highest fluoxetine treatment (10 µg/L).

Relating feeding behavior and brain biochemistry

(7) Feeding behavior recovered more quickly than brain AChE or serotonin activities; 6-day recovery periods were not sufficient for full recovery of either neurotransmitter.

(8) There was a threshold response between brain AChE activity and time-to-capture prey for hybrid striped bass acutely exposed to diazinon.

(9) There was a linear relationship between brain serotonin levels and time-to-capture prey for hybrid striped bass acutely exposed to fluoxetine.

The research in this dissertation demonstrated that relationships between biochemical and behavioral endpoints could be determined. We found that prey capture is an ecologically relevant behavior that can be related to brain neurotransmitter activity. Recognizing that behavioral changes are likely a product of altered biochemistry and understanding this connection may allow more meaningful interpretations of biochemical biomarkers. While there has been an increase in research characterizing various biomarkers, they are not routinely used in risk assessments because their ecological relevance is not as well defined. So once a relationship has been defined, it may possible to use biomarkers as a tool for validating risk. In addition, knowing such a relationship may also aid in predicting more accurately population level impacts from a biochemical endpoint following contaminant exposure.

This bioassay shows potential for evaluating effects of short- and long-term sublethal contaminant exposures on feeding behavior, as well as the ability to monitor

recovery of an organism following exposure. While we know that toxic effects may last longer than the original exposure, we do not usually monitor the recovery of an organism. But this bioasay can successfully reveal prolonged effects and/or improvements in feeding behavior and brain biochemistry, and the sensitivity of each. Overall, the behavioral assay designed for this dissertation can increase our understanding of how changes in brain biochemistry may impact the feeding behavior of hybrid striped bass, and can be further used to understand effects of sublethal exposures to neurotoxins on feeding behavior. This assay may provide a practical link between changes at the biochemical and population level.

APPENDIX

Table A-1

Time (mean ± SE) it took hybrid striped bass to capture each of four consecutive prey (fathead minnows) during a 6-day diazinon exposure followed by a 6-day recovery period. Control, low, medium and high treatments correspond to diazinon concentrations of (mean ± SE) 0.7 ± 0.1, 19.6 ± 0.7, 62.9 ± 2.0, and 98.4 ± 1.4 µg/l, respectively. (Referenced on page 41)

		Time to capture prey (seconds)				
Prey	Treatment	Day 0	Day 3	Day 6	Day 9	Day 12
1	Control	4.09 (± 0.67)	4.63 (± 1.69)	1.79 (± 0.31)	5.71 (± 2.42)	4.00 (± 1.58)
	Low	6.74 (± 1.72)	2.11 (± 0.30)	2.14 (± 0.27)	2.44 (± 0.44)	1.50 (± 0.29)
	Medium	14.45 (± 7.35)	4.42 (± 1.07)	582.14 (± 171.21)	307.20 (± 198.83)	49.00 (± 44.36)
	High	4.75 (± 1.93)	204.56 (± 126.71)	1024.17 (± 203.15)	567.75 (± 206.93)	398.25 (± 328.89)
2	Control	7.59 (± 1.71)	15.29 (± 5.35)	12.00 (± 6.65)	12.83 (± 9.44)	7.25 (± 1.89)
	Low	9.84 (± 1.91)	4.05 (± 0.33)	4.07 (± 0.47	8.67 (± 3.09)	5.00 (± 1.910
	Medium	21.00 (± 8.83)	264.10 (± 122.91)	931.00 (± 187.56)	362.33 (± 215.34)	168.25 (± 123.16)
	High	7.00 (± 1.97)	454.69 (± 159.11)	1269.50 (± 155.91)	803.25 (± 265.50)	435.75 (± 320.60)
3	Control	15.88 (± 3.85)	40.06 (± 16.46)	18.00 (± 9.61)	61.33 (± 56.34)	15.50 (± 5.98)
	Low	27.42 (± 7.30	12.11 (± 5.04)	13.00 (± 5.60)	14.11 (± 3.73)	10.00 (± 3.14)
	Medium	86.90 (± 50.27)	404.10 (± 144.98)	1172.47 (± 155.94)	998.70 (± 228.88)	842.80 (± 332.19)
	High	26.50 (± 18.92)	838.94 (± 175.12)	1383.17 (± 116.83)	1190.50 (± 219.83)	1129.50 (± 331.39)
4	Control	38.56 (± 7.85)	251.41 (± 118.65)	153.17 (± 123.06)	434.86 (± 275.02)	8.00 (± 1.00)
	Low	87.53 (± 23.94)	122.95 (± 79.05)	37.57 (± 16.25)	170.89 (± 101.87)	153.00 (± 143.35)
	Medium	165.45 (± 71.00)	841.55 (± 163.45)	1243.13 (± 137.58)	1067.70 (± 232.19)	1188.40 (± 246.20)
	High	42.94 (± 24.78)	1080.63 (± 163.01)	1495.92 (± 4.08)	1448.57 (± 51.43)	1444.50 (± 646.00)

Table A-2

Time (mean ± SE) it took hybrid striped bass to capture each of four consecutive prey (fathead minnows) during a 6-day fluoxetine exposure followed by a 6-day recovery period. Low, medium and high treatments correspond to diazinon concentrations of (mean ± SE) 23.2 ± 6.6, 51.4 ± 10.9, and 100.9 ± 18.6 µg/l, respectively.
(Referenced on page 67)

		Time to capture prey (seconds)				
Prey	Treatment	Day 0	Day 3	Day 6	Day 9	Day 12
1	Control	7.98 (± 3.45)	13.00 (± 7.14)	16.29 (± 8.56)	3.00 (± 0.95)	2.80 (± 1.11)
	Low	11.27 (± 2.88)	50.79 (± 19.28)	397.00 (± 146.62)	63.58 (± 17.97)	31.60 (± 18.59)
	Medium	1.57 (± 4.22)	329.67 (± 128.1)	465.56 (± 158.31)	432.18 (± 207.1)	176.80 (± 151.12)
	High	18.59 (± 8.37)	808.55 (± 152.63)	878.06 (± 163.62)	1129.00 (± 190.16)	1212.00 (± 288.00)
2	Control	26.09 (± 8.79)	23.29 (± 9.2)	44.64 (± 17.58)	6.60 (± 1.85)	6.80 (± 1.83)
	Low	37.45 (± 10.36)	414.00 (± 138.97)	575.25 (± 166.22)	732.27 (± 210.11)	328.60 (± 293.66)
	Medium	28.19 (± 9.81)	568.95 (± 150.49)	810.19 (± 179.77)	835.75 (± 252.93)	719.40 (± 310.87)
	High	37.00 (± 12.48)	1005.59 (± 139.4)	1147.94 (± 145.04)	1279.30 (± 141.39)	1216.00 (± 284.00)
3	Control	159.61 (± 122.84)	215.76 (± 117.6)	293.36 (±136.85)	25.70 (± 10.87)	180.00 (± 163.54)
	Low	129.86 (± 29.48)	772.00 (± 148.15)	1256.92 (± 131.49)	1125 (± 186.76)	537.40 (± 310.99)
	Medium	96.19 (± 31.55)	844.33 (± 156.6)	1250.00 (± 134.83)	1196.18 (± 174.39)	909.00 (± 361.93)
	High	146.36 (± 41.5)	1299.55 (± 93.94)	1353.07 (± 87.51)	1500.00 (± 0.00)	1255.20 (± 244.80)
4	Control	593.04 (± 128.13)	589.18 (± 152.29)	780.93 (± 186.80)	520.00 (± 216.34)	651.60 (± 347.00)
	Low	604.81 (± 126.88)	1249.95 (± 116.03)	1378.25 (± 102.29)	1144.36 (± 186.01)	1392.60 (± 107.40)
	Medium	502.10 (± 126.96)	1239.00 (± 111.92)	1361.13 (± 97.41)	1257.36 (± 163.00)	1059.20 (± 285.60)
	High	671.91 (± 133.37)	1419.64 (± 55.47)	1435.63 (± 64.38)	1500.00 (± 0.00)	1500.00 (± 0.00)

Table A-3

Time (mean ± SE) it took hybrid striped bass to capture each of four consecutive prey (fathead minnows) during a 27-day fluoxetine exposure. Low, medium and high treatments correspond to diazinon concentrations of (mean ± SE) 0.08 ± 0.02, 0.87 ± 0.12, and 9.44 ± 0.82 µg/l, respectively. (On next page)
(Referenced on page 95)

		Time to capture prey (seconds)									
Prey	Treatment	Day 0	Day 3	Day 6	Day 9	Day 12	Day 15	Day 18	Day 21	Day 24	Day 27
1	Control	7.80 (± 3.04)	2.13 (± 0.31)	2.68 (± 1.06)	3.18 (± 1.08)	3.89 (± 2.65)	1.00 (± 0.00)	1.25 (± 0.25)	4.13 (± 2.33)	1.64 (± 0.66)	7.60 (± 6.60)
	Low	9.33 (± 2.84)	5.25 (± 2.24)	7.95 (± 4.92)	6.63 (± 2.64)	20.40 (± 10.00)	2.25 (± 0.59)	1.55(± 0.28)	2.80 (± 1.69)	1.20 (± 0.20)	1.40 (± 0.40)
	Medium	6.20 (± 1.83)	13.40 (± 8.89)	14.37 (± 8.22)	4.46 (± 1.23)	15.38 (± 9.43)	6.33 (± 4.25)	2.72 (± 0.66)	17.00 (± 12.48)	5.05 (± 4.00)	3.20 (± 1.74)
	High	4.25 (± 1.12)	10.00 (± 7.18)	51.32 (± 25.30)	22.17 (± 12.76)	15.69 (± 9.85)	18.00 (± 15.24)	16.72 (± 14.18)	2.50 (± 1.02)	14.20 (± 11.96)	3.00 (± 2.00)
2	Control	9.75 (± 20.51)	9.26 (± 5.53)	6.05 (± 2.50)	13.50 (± 4.61)	10.21 (± 4.35)	2.43 (± 0.48)	5.38 (± 2.04)	7.00 (± 4.05)	12.90 (± 10.54)	12.00 (± 9.26)
	Low	26.25 (± 8.11)	15.74 (± 7.12)	12.89 (± 5.82)	36.40 (± 15.73)	45.00 (± 17.25)	6.40 (± 3.03)	6.90 (± 3.43)	9.10 (± 4.74)	2.80 (± 0.20)	4.60 (± 1.25)
	Medium	14.10 (± 4.97)	24.10 (± 11.85	22.26 (± 11.08)	69.00 (± 56.59)	77.54 (± 40.07)	31.11 (± 15.61)	50.81 (± 47.04)	25.33 (± 16.62)	33.10 (± 24.20)	19.80 (± 10.36)
	High	8.95 (± 1.97)	31.30 (± 14.76)	132.44 (± 83.01)	32.42 (± 13.35)	40.23 (± 20.08)	31.60 (± 20.25)	24.11 (± 19.75)	15.80 (± 9.36)	36.40 (± 21.52)	6.25 (± 3.28)
3	Control	39.45 (± 43.72)	98.58 (± 58.85)	11.21 (± 3.82)	80.36 (± 45.37)	46.29 (± 29.03)	4.00 (± 0.49)	31.00 (± 23.60)	79.88 (± 72.91)	14.60 (± 10.61)	64.00 (± 46.50)
	Low	56.60 (± 24.43)	78.50 (± 46.05)	66.16 (± 28.65)	185.13 (± 97.33)	195.27 (± 110.32)	12.70 (± 6.22)	22.00 (± 8.81)	20.20 (± 12.14)	7.80 (± 2.67)	173.25 (± 148.41)
	Medium	30.20 (± 8.91)	147.55 (± 79.27)	73.17 (± 29.89)	130.85 (± 114.22)	207.38 (± 114.30)	95.11 (± 52.84)	125.13 (± 118.43)	74.11 (± 38.94)	103.20 (± 71.44)	341.00 (± 290.39)
	High	48.15 (± 14.88)	217.85 (± 101.60)	294.22 (± 131.96)	177.42 (± 109.22)	232.23 (± 117.40)	154.00 (± 104.95)	49.56 (± 32.94)	105.00 (± 67.78)	101.60 (± 35.30)	64.25 (± 58.93)
4	Control	97.05 (± 62.91)	147.21 (± 102.46)	136.53 (± 80.37)	439.71 (± 177.51)	340.36 (± 174.43)	8.29 (± 2.71)	66.88 (± 37.28)	257.13 (± 181.60)	34.20 (± 24.51)	545.00 (± 332.05)
	Low	197.25 (± 87.83)	255.90 (± 99.24)	397.74 (± 139.34)	369.60 (± 130.11)	415.47 (± 153.11)	82.10 (± 64.65)	252.00 (± 157.42)	200.30 (± 149.24)	30.40 (± 11.73)	734.00 (± 299.37)
	Medium	260.05 (± 92.36)	484.05 (± 126.79)	343.16 (± 125.22)	249.62 (± 154.02)	451.46 (± 178.08)	279.33 (± 151.73)	201.88 (± 185.64)	230.33 (± 136.30)	196.20 (± 109.08)	447.60 (± 270.35)
	High	211.00 (± 79.04)	621.50 (± 151.25)	457.11 (± 144.09)	500.92 (± 154.83)	472.23 (± 149.24)	387.80 (± 188.00)	243.00 (± 160.54)	420.30 (± 200.17)	194.80 (± 80.55)	228.00 (± 212.04)