

ABSTRACT

Title of Dissertation: HARMFUL ALGAL BLOOM STRESSORS
ALTER BEHAVIOR AND BRAIN ACTIVITY
IN THE KILLIFISH, *FUNDULUS*
HETEROCLITUS.

James D. Salierno, Doctor of Philosophy, 2005

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Harmful algal bloom (HAB) events are increasing in severity and frequency worldwide, and are known to severely impact fish populations. Impacts of HABs on fish, as well as other organisms, occur through toxic and physical stress. Behavioral and central nervous system (CNS) alterations can have direct consequences to the fitness and survival of individuals and populations. This study investigated and characterized alterations in social and swimming behaviors and brain activity in mummichog (*Fundulus heteroclitus*) exposed to HAB stressors. The mummichog is an ecologically important estuarine fish species exposed to a variety of HAB events in the wild. A behavioral analysis system was developed to study swimming and social behavior of fish and an immunohistochemistry technique was used to investigate alterations in neuronal activity as evidenced by c-Fos protein expression. HAB stressors included excitatory (domoic acid, brevetoxin) and inhibitory (saxitoxin) neurotoxic agents as well as direct exposures to the dinoflagellate

Pfiesteria shumwayae and the diatom *Chaetoceros concavicornis*. *P. shumwayae* and *C. concavicornis* are HAB species that are known to induce mortality through physical trauma to fish. Brevetoxin exposure increased swimming and social behaviors whereas saxitoxin decreased these behaviors. The effects of saxitoxin on swimming and social behaviors were consistent with exposure to a fish anesthetic, MS-222. Similarly, it was found, through c-Fos expression, that the excitatory HAB neurotoxins brevetoxin and domoic acid, increased neuronal activity while saxitoxin decreased activity. Exposure to *P. shumwayae* and *C. concavicornis*, resulted in significant dose related increases in neuronal activity. Stressor-specific neuronal activity was greatest in the optic lobe, but was also found in the telencephalon with physical stressors increasing activity greater than chemical stressors. Results demonstrate that sublethal exposures to HAB neurotoxins can alter swimming and social behavior in mummichog and exposures to both neurotoxins and algae can alter neuronal activity. Alterations in brain activity, and knowledge of specific regions within the brain activated during stress, can provide insights into the control of fish behavior. Ultimately, HAB exposure related changes in neuronal signaling may alter behaviors, resulting in individual and population level alterations during HAB events.

HARMFUL ALGAL BLOOM STRESSORS ALTER BEHAVIOR AND BRAIN
ACTIVITY IN THE KILLIFISH, *FUNDULUS HETEROCLITUS*.

By

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Preface

Harmful algal blooms (HAB) in the Middle Atlantic States and associated fish morbidity and mortality have increased in frequency and severity over the past several decades. The ability to predict and characterize environmental effects of different HAB species are essential to HAB remediation and control. This study was designed to investigate the effects of low level (sublethal) HAB stressor exposure on fish swimming behavior and brain activity. The goal was to investigate and quantify possible alterations in mummichog (*Fundulus heteroclitus*) swimming behavior and neuronal activity resulting from HAB exposure, demonstrating that fish can be affected by low level HAB stress, which may then alter individual and population dynamics in the environment.

In order to accomplish this goal, a software program was developed for the analysis of schooling, shoaling, and individual behaviors in fish. The software system was then coupled to an existing videography system which allowed the capture of thirty minute video segments of schools and shoals of mummichog before, during, and after exposure to toxins. Additionally, predator avoidance and startle response behaviors were analyzed by designing a model predator (bird) and an auditory/vibratory stimulus. Predator avoidance and startle responses in mummichog were then tested before, during and after exposures.

An acclimation study was conducted in addition to an exposure to tricaine methanesulfonate (MS-222), a common fish anesthetic, in order to validate the behavioral quantification system. The acclimation study consisted of measuring

mummichog behavior over a 3 day period to obtain baseline activities and changes in behaviors over time. Exposure of fish to MS-222 was then conducted as a second validation and for comparison to HAB neurotoxins. Once the system was validated and data from the acclimation and MS-222 experiments analyzed, mummichog were then exposed to known HAB neurotoxins, brevetoxin (PbTx-2) and saxitoxin (STX).

Behaviors are initiated and regulated through the CNS and neuronal signaling is essential for the proper responses to different stimuli. As such, investigations into possible alterations in brain activity resulting from HAB sublethal stressor exposure in fish were conducted. *c-fos*, an immediate early gene and its protein product, c-Fos, are indicators of neuronal activity and stress in mammals. The procedure used to quantify c-Fos protein in mammals was acquired and adapted for use in mummichog brains. A transport stress experiment was then conducted with mummichog to test the ability to quantify changes in c-Fos protein expression in stressed fish. Once changes in c-Fos expression were quantified in different regions of mummichog brains, mummichogs were exposed to HAB neurotoxins PbTx-2, STX, domoic acid DA, and HAB species, *Pfiesteria shumwayae* and *Chaetoceros concavicornis*. In addition, different regions of the brain were investigated in an attempt to discern if stress-specific regional expression in c-Fos occurred.

Hypotheses:

Ha (1). The software system designed for the quantification of schooling and shoaling behaviors will accurately describe alterations in mummichog behavior over an acclimation period and exposure to a fish anesthetic, MS-

222. A video-based analysis system will be developed to quantify swimming behaviors, including schooling and shoaling, in groups of mummichog.

Computer digitizing hardware will be coupled with a specific software program developed for the analysis of fish movement. Thirty minute video segments will be compiled, digitized, transformed into x,y coordinate data, and converted into behavioral endpoints. In addition, predator avoidance and startle response behaviors will be measured through the development of a predator model and an auditory/vibratory system. Analysis of the acclimation and MS-222 behavioral data will be used to validate the behavior quantification system.

Ha (2). Exposure to sublethal concentrations of brevetoxin (PbTx-2) and saxitoxin (STX) will alter behaviors in groups of mummichog, *Fundulus heteroclitus*. The behavior quantification system will then be used to examine alterations in schooling and shoaling behaviors through time resulting from HAB neurotoxin exposure. The excitatory neurotoxin, PbTx-2 will increase schooling and shoaling behaviors and the paralytic neurotoxin, STX will decrease these

swimming behaviors. Exposure to these neurotoxins will also alter both startle response and predator avoidance behaviors.

Ha (3). Alterations in neuronal activity resulting from transport stress in mummichog will be quantified through c-Fos protein expression, measured by immunocytochemistry. Immunocytochemistry, an antibody labeling technique used in mammalian research to stain and quantify c-Fos expression, will be adapted for use in mummichog. A transport stress experiment will then be conducted to examine c-Fos expression in mummichog resulting from moderate stress exposure. Transport stressed mummichog will display increase c-Fos expression compared to control fish. This will provide a method to quantify alterations in CNS neuronal activity in the fish brain.

Ha (4). Exposure to sublethal concentrations of PbTx-2, domoic acid (DA), STX, and a range of densities of the dinoflagellate *P. shumwayae* and the diatom *C. concavicornis*, will alter CNS neuronal activity, evidenced by altered c-Fos protein expression. Mummichog will be exposed to HAB stress, including neurotoxins and HAB species in order to examine alteration in neuronal activity. Excitatory (PbTx-2 and DA) and physical stressors (transport stress, *P. shumwayae*, and *C. concavicornis*) will increase neuronal signaling and c-Fos expression, while paralytic neurotoxins (STX) should block neuronal signaling and decrease c-Fos signaling when compared to control fish.

Ha (5). Different types of HAB stress (toxins vs. physical stress) will induce region specific alterations neuronal signaling, i.e., different stressors will increase activity in different locations within the brain. The immunocytochemistry technique provides information regarding alterations in brain activity to the level of individual neurons. Therefore, different stressor types will induce c-Fos proteins in different regions of the mummichog brain.

Conclusions

Exposure to sublethal HAB stressors in the laboratory resulted in quantifiable alterations in both behavior and neuronal activity in groups of mummichog. Mummichog exposed to HAB toxins and the anesthetic, MS-222, displayed significant alterations in schooling and shoaling behaviors and neuronal activity. Exposure to STX and MS-222 significantly decreased interactions, and schooling and shoaling behaviors whereas exposure to PbTx-2 increased these behaviors. The composition of schools, shoals, and sedentary groups of exposed fish significantly decreased from groups of 5 to groups of 3 fish after exposure. Additionally, exposure to all three toxins altered startle response and predator response behaviors. Twenty-four hours after exposure, groups of fish exposed to all 3 toxins had significant decreases in activity and frequency of behaviors compared with pre-exposure activity. These results suggest that sublethal exposure to HAB toxins can have demonstrable effects on swimming and social behaviors, as well as on startle response and predator avoidance behaviors. These alterations can last for at least 24 hours, which can

negatively affect groups of mummichog by reducing their ability to maintain schooling, shoaling, and avoidance behaviors in the laboratory. This is the first study to quantify alterations in social behavior of fish exposed to HAB toxins. This data will provide a better understanding how environmentally realistic concentrations of HAB stressors affect fish behavior.

Exposure of mummichog to DA, STX, PbTx-2, *Pfiesteria shumwayae*, *Chaetoceros concavicornis*, and transport stress resulted in significant alterations in c-Fos expression, an indicator of neuronal activity. The excitatory neurotoxins, DA and PbTx-2, and transport stress, increased c-Fos expression while STX significantly inhibited c-Fos expression. In addition, exposure to *P. shumwayae* and *C. concavicornis* increased c-Fos expression in a density dependent relationship. c-Fos expression in these fish may be associated with neuronal stress and alteration in signaling. Stressor-specific c-Fos expression was observed, with *P. shumwayae* and *C. concavicornis* increasing c-Fos expression in the telencephalon and optic lobes, and physical transport and chemical stressors increasing expression in the optic lobes. This is the first study to quantify alterations in c-Fos expression in the telencephalon and mesencephalon of fish. These alterations in brain activity may ultimately be linked to behavioral changes such as those quantified in the laboratory. The quantification of alterations in behavior and neuronal activity resulting from sublethal HAB stressor exposure in the laboratory may further elucidate changes in individual and population dynamics of fish exposed to HAB events in the field.

Dedication

This dissertation is dedicated to my Father who has always believed and pushed me to be the person that I am.

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Chapter 1: Literature Review

1.1 Chapter Summary

The purpose of this study was to quantify alterations in schooling and shoaling behaviors and brain activity in groups of mummichog, *Fundulus heteroclitus*, exposed to harmful algal bloom stressors. Therefore, the following areas of literature have been reviewed: the use of behavior in toxicology studies, mechanisms and ecology of schooling and shoaling behaviors in fish, biology of harmful algal blooms (HABs) and effects of HABs on fish, and the use of c-Fos expression as a measure of stress exposure and neuronal activity in fish.

Behavioral toxicology can be used to determine low-level effects of stress exposure in organisms. Inclusion of behavioral measures in traditional toxicology studies provides additional, environmentally relevant, endpoints of exposure other than morbidity or mortality. Characterizing behavioral changes in fish is an important tool to study how fish respond to the many stressors they encounter in the wild.

Schooling and shoaling are essential, evolutionarily stable behaviors required by many fish species. Alterations in schooling and shoaling behaviors can have direct consequences on individual and group fitness. Quantification of schooling and shoaling behaviors can be described using mathematical models, which subsequently can quantify changes in behavior resulting from stressor exposure. The ability to quantify these

complex fish behaviors is important when considering effects of stress exposures and contaminant levels in the environment.

Fish are exposed to natural biological stress in addition to anthropogenic stressors. HAB events, which are naturally occurring but exacerbated by anthropogenic activities, are increasing in severity and frequency worldwide and are known to severely impact fish populations. Impacts of HABs on fish occur through neurotoxic and physical stress exposures, which include mortality, morbidity, and alterations in swimming behaviors. The HAB dinoflagellate species, *Karenia brevis* and *Alexandrium fundyense*, release the neurotoxins brevetoxin (PbTx) and saxitoxin (STX), which are responsible for fish kills and human illness. In addition, the dinoflagellate *P. shumwayae* and the diatom *C. concavicornis* are HAB species that are known to induce mortality through physical trauma to fish. Knowledge of alterations in behavior of fish exposed to these HAB stressors can aid in the assessment of HAB impacts on aquatic communities. The main physiological target for HAB neurotoxins is the control center for behavior, the central nervous system (CNS). These changes in CNS functioning may be responsible for changes observed in behavior. Alterations in brain activity resulting from HAB exposure may alter regional neuronal signaling, and the ability to detect and quantify these changes may describe a link between behavioral change and neuronal activity.

Behaviors are initiated and regulated through the CNS and neuronal signaling is essential for the proper responses to different stimuli. *c-fos*, an immediate early gene, and its protein product c-Fos, are indicators of neuronal activity and stress in mammals. In mammals, c-Fos expression, measured through immunocytochemistry (ICC) staining, has been used as a biomarker of neuronal stress and stimulation from stress exposure. In

fish, c-Fos protein expression can indicate areas within the fish brain that respond to stress. General alterations in brain activity, as well as knowledge of specific regions within the brain activated during stress, provide valuable insights into the neuronal control of fish behavior as well as sublethal effects of HAB stressors, chemical and physical, on neurons. Therefore, the use of c-Fos expression may link behavioral alterations resulting from HAB stressor exposure to changes in brain activity, providing novel information of how HAB stress effects behavioral and neurological mechanisms in fish.

1.2 Behavioral Toxicology

Behavior as an endpoint is pivotal to the field of toxicology since behavioral interactions link organisms to their surrounding environment. Any behavioral change resulting from toxicological exposure will directly affect the fitness of an organism by altering the response to environmental stimuli. Additionally, behavior can provide a unique perspective by linking the physiology and ecology of an organism with its environment (Little & Brewer, 2001). Ultimately, behavior is a sequence of actions operating through the central and peripheral nervous systems, resulting from the manifestation of genetic, biochemical, and physiologic processes essential to life, such as feeding, reproduction and predator avoidance (Kleerekoper et al., 1972). In addition, behavior allows an organism to adjust to external and internal stimuli in order to satisfy the challenge of surviving in a dynamic environment. Behavioral alterations can be measured as endpoints for sublethal toxicity, serve as a tool for environmental risk

assessment, and analysis of toxicological impact. As a result, behavioral endpoints have been used to study the effects of chemicals and drugs on humans and other mammals in toxicology for thousands of years (Kane et al., 2005).

Integration of behavioral endpoints into aquatic toxicology has been hindered because, until recently, there was a poor understanding of how alterations in behavior may be related to ecologically-relevant issues such as predation avoidance, prey capture, growth, stress resistance, reproduction and longevity (Kane et al., 2005). Basic knowledge of exposure-related behavioral alterations relevant for ecotoxicological assays remain scarce, and systems that have the ability to link toxicology data with swimming and avoidance behaviors are needed (Vogl et al., 1999). Further, the ability to attain repeatable, quantifiable data from a large number of animals or exposures has been challenging. Lack of test standards, homogeneity of samples, variation in measured endpoints, and small sample sizes have hindered the progress of behavioral toxicology as a consensus-based discipline. Sound experimental design combined with proper statistical analyses are essential for developing approaches using fish movement for the bioindication of stressors (Vogl et al., 1999).

Quantifiable behavioral changes in organisms associated with stress and toxicant exposure provide novel information that cannot be gained from traditional toxicological methods (Little et al., 1990; Henry & Atchison, 1986; Bridges, 1997; Saglio & Trijasse, 1998). One of the first comprehensive reviews on aquatic behavioral toxicology was published by Rand (1985). Over the past 20 years, the field of behavioral toxicology has grown, in part, because of increased interest in the number of species used, endpoints measured, and methods to collect and interpret data. Numerous reviews have traced

these advancements in the field of behavioral toxicology (Little & Finger, 1990; Gray, 1990; Doving, 1991; Webber & Spieler, 1994; Little & Brewer, 2001; Kane et al., 2004). However, the recognition of behavioral toxicology as an important tool in aquatic toxicology is most clearly seen in the acceptance of behavioral endpoints in federal regulations. In 1986, the U.S. government accepted avoidance behavior as legal evidence of injury for Natural Resource Damage Assessments under proceedings of the Comprehensive Environmental Response, Compensation, and Liability Act of 1980 (NRDA, 1986). The additional tools provided by behavioral observation can support toxicological investigation with endpoints other than traditional fifty-percent lethal concentration determination studies (LC50), and may aid in determining new no observed effect levels (NOELs) and lowest observed effect levels (LOELs). Behavioral toxicology may ultimately provide sensitive, non-invasive, and broadly applicable endpoints for the description of integrated, whole animal responses associated with exposure to a wide variety of stressors (Rand, 1985; Kane et al., 2005).

Fish are ideal sentinels and test organisms for behavioral assays of various stress and chemical exposures due to their ecological relevance in many natural systems (Little et al., 1990). In addition, small fish species may bioaccumulate various contaminants and/or play a role in food web biomagnification (Lefebvre et al., 2001; Scholin et al., 2000). The development of behavioral endpoints in fish, as well as other organisms, serves as valuable tools to evaluate effects of environmental stress exposure. These endpoints of exposure are important because they can integrate endogenous and exogenous factors that can link biochemical and physiological processes, and can provide insights into individual- and community-level effects of environmental contamination

(Vogl et al., 1999; Brewer et al., 2001). Ecological relevance of such exposures may stem from altered vigilance, startle responses, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Little & Finger, 1990; Zhou & Weis, 1998). Changes in behavior may also alter juvenile recruitment, thereby disrupting population, community, and demographic dynamics over time (Bridges, 1997).

Behavioral endpoints used to evaluate alterations resulting from stressor exposure include: swimming activity and performance, feeding capacity, vulnerability as prey, rheotaxis, velocity, angular change, water column position, total distance traveled, acceleration, and surfacing (Little & Finger, 1990; Kato et al., 1996; Saglio & Trijasse, 1998; Weis & Weis, 1998; Zhou & Weis, 1998; Beauvais et al., 2000; Brewer et al., 2001; Gipson, 2001). Additionally, more complex behaviors including path tortuosity, fractal dimension (D), ratio of net to gross movement, chemoreception, optomotor responses, respiration, aggression, and social interactions have also been investigated (Little & Finger, 1990; Kato et al., 1996; Saglio & Trijasse, 1998; Weis & Weis, 1998; Zhou & Weis, 1998; Beauvais et al., 2000; Brewer et al., 2001; Gipson, 2001; Kane et al., 2004). Development of methods and techniques to quantify alterations in behavioral endpoints has proven challenging yet valuable.

A variety of methods have been developed to quantify movement data, including hand scoring through direct observation (Hansen et al., 1999; Bjerselius et al., 2001; Teather et al., 2001; Wibe et al., 2001), telemetry (Westcott & Graham, 2000), and computer digitization into x,y coordinate data (Vogl et al., 1999; Beauvais et al., 2000; Suzuki et al., 2003). Several computer tracking hardware and software systems have been developed for the purpose of behavioral analysis (Vogl et al., 1999; Beauvais et al.,

2000; Suzuki et al., 2003; Kato et al., 1996). More recently, the Motion Analysis NP110[©] system with Expert Vision software (Dodson et al., 1995; Brewer et al., 2001), BehavioQuant[©] object tracing system (Baganz et al., 1998; Vogl et al., 1999; Steinberg et al., 1995), and Image-Pro Plus[©] (Media Cybernetics, Silver Spring, MD) have been developed for motion analysis. Investigators must balance the cost of behavioral quantification systems with the amount of information that can be gained from such systems. This usually translates into specific systems designed by investigators involving in-house software programs and videography equipment. The overall goal is the ability to produce a system that will provide accurate descriptions of behavior sensitive enough to detect subtle alterations resulting from stress exposure.

The data collected by behavioral quantification systems are a useful indicator of sublethal contamination because behavioral endpoints frequently occur at concentrations that are chronically sublethal and also at concentrations lower than those known to affect growth (Little et al., 1993; Cleveland et al., 1991). Thus, behavior is a selective response that is constantly adapting through direct interactions with physical, chemical, social, and physiological aspects of the environment. Selective evolutionary processes have conserved stable behavioral patterns in concert with morphologic and physiologic adaptations. This stability provides the best opportunity for survival and reproductive success by enabling organisms to efficiently exploit resources and define suitable habitats (Little & Brewer, 2001). Alterations in stable behavioral patterns, such as schooling and shoaling in fish, can provide novel information into how stress exposure alters group dynamics of fish. These resulting alterations in group dynamics can directly affect predator avoidance, foraging, and habitat utilization.

1.3 Effects of Exposure on Schooling and Shoaling Behavior

Behavioral alterations in fish are valuable tools to discern and evaluate effects of environmental stress. Fluctuations in school structure and density can be caused by individual differences in motivation, physiology, and abiotic and biotic factors of the environment. Even if fish are not overtly harmed through exposure to a chemical, their ability to function in an ecological context may be altered (Scott & Sloman, 2004). As a result, exposure to anthropogenic pollutants can alter schooling and shoaling behaviors in fish. These alterations in turn, may be associated with reduced fitness and survival, resulting in adverse consequences at the population level (Bridges, 1997). Inappropriate response to environmental and physiological stimuli due to toxicant exposure can have severe implications for survival (Webber & Spieler, 1994). Changes in behavior may also alter juvenile recruitment, thereby disrupting population, community, and demographic dynamics over time (Bridges, 1997).

Behavior is a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival, and behavioral endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors. Basic knowledge of exposure related behavioral alterations relevant for ecotoxicological assays remain scarce, and systems that have the ability to link toxicology data with swimming and avoidance behaviors are needed (Vogl et al., 1999). Historically, toxicology studies typically focus on exposures of single fish in the laboratory, when in reality, many

species tend to congregate in groups and interact with many other components of their environment.

Using group behaviors as endpoints in toxicology studies enables the use of complex behaviors in bioassay testing, providing conclusions that can extrapolate more accurately to what occurs in nature. Recently, research has begun to investigate more complex behaviors that occur in the wild, such as foraging, predator avoidance, and social hierarchies, which may be more important ecologically than simple responses like total distance traveled and swimming performance (Scott & Sloman, 2004). Fish are an excellent model for studying ecological environmental pollutant effects since many behaviors are easily observed and quantified in a laboratory setting (Scott & Sloman, 2004). In addition, these behaviors have predictable structures, shapes, and responses to threats and environmental fluctuations.

It has been demonstrated, through the use of shoal choice experiments and frame capture, that pesticide exposures can alter shoaling and schooling behaviors. Atlantic silverside (*Menidia menidia*) exposed to an acetyl cholinesterase inhibiting insecticide, carbaryl, displayed alterations in parallel orientation and increased distances between fish when compared to controls (Weis & Weis, 1974). Similarly, schooling declined following exposure of yearling common carp (*Cyprinus carpio*) to 0.05 mg L⁻¹ DDT and of fathead minnows (*Pimephales promelas*) to 7.43 mg L⁻¹ of the herbicide 2,4-dinitrophenol at a pH of 7.57 (Holcombe et al., 1980). In addition, swimming orientation in schools of threespine stickleback (*Gasterosteus aculeatus*) was disturbed following exposure to the organotin bis(tributyltin)oxide (Wibe et al., 2001). Exposures to increased water temperatures decreased polarization, increased interactions and

disorientation, and retarded schooling development in larval sand smelt, *Atherina mochon* (Williams & Coutant, 2003).

Quantifiable behavioral changes in organisms associated with stress and toxicant exposure provide novel information that cannot be gained from traditional toxicological methods, including short-term and sublethal exposure effects, and the potential for mortality (Little & Finger, 1990; Bridges, 1997; Henry & Atchison, 1986; Saglio & Trijasse, 1998). Ecological relevance of such exposures may stem from altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Zhou & Weis, 1998; Little & Finger, 1990). In order to better understand alterations resulting from exposure, an understanding of the mechanics, ecology, and evolutionary significance of schooling and shoaling behaviors must be known.

1.4 Biology, Ecology, and the Evolution of Social Aggregations in Fish

The majority of organisms on this planet, including humans, live in social aggregations for many reasons. Living in groups can provide increased foraging success and efficiency (e.g., bees), reproduction and parental care, and probability of predator detection while also offering decreases in predation pressure. The resulting relationship between individuals can range from loose associations to strict eusocial hierarchies, and can have lasting beneficial effects at the individual, population, and community level of organization while providing increased survival and overall fitness. Social aggregations in animals are multi-level biological phenomena that depend upon the species,

ontogenetic stage, and physiological state (Radakov, 1973). In addition, aggregations can be comprised of closely related kin, distant relatives, or non-related individuals. Living in groups can also reduce the detrimental effects of environmental fluctuation and provide stability (Pavlov & Kasumyan, 2000). Solitary organisms that migrate can take advantage of social organization during migratory events to reduce energy expenditure and predation pressure (Radakov, 1973). Two disadvantages, however, are the increase in disease and parasite transmission combined with intraspecific competition for resources. Living as a part of a social group may occur as either an instinctual force or as a response to external factors in the environment (Aoki, 1980). Social aggregations of fish, in schools or shoals, are essential life history traits that can determine the fitness and survival of individuals and the propagation of a species.

Fish are the largest and most taxonomically diverse group of vertebrates known, with twenty five percent of all species forming schools or shoals and fifty percent doing so during larval and juvenile stages (Radakov, 1973; Pavlov & Kasumyan, 2000). Schools and shoals of fish are distinct from other vertebrate social groups due to their equipotentiality, (i.e., having no leader or hierarchal structure) (Radakov, 1973). Such schools and shoals with no dominant leader will form when resources and refugia are scarce, predation rates are high, and when individuals have no defensive morphology (e.g., spines, poisons, coloration, postures) (Radakov, 1973; Pavlov & Kasumyan, 2000; Grunbaum, 1998). The result is hundreds of individual fish gliding in perfect unison, appearing like a single organism, where the entire group is the leader (Niwa, 1996).

Teleost fishes utilize schooling and shoaling behaviors to detect, confuse, and escape predators as well as to locate mates, resources, and for migration (Masuda &

Tsukamoto, 1998; Radakov, 1973). The majority of schooling and shoaling species are small in body size with groups forming temporarily for short time periods or during specific life stages (Partridge, 1982). Furthermore, fluctuations in group behaviors may occur due to temporal and spatial changes in environmental conditions such as light, oxygen, temperature, hydrodynamics, salinity, and predator/prey abundance. Some species of herring (Clupeidae) have the inability to live a solitary life and will die when kept alone under laboratory conditions, illustrating the importance of schooling behavior as a life history characteristic in some species (Gerasimov, 1962; Radakov, 1973). In addition, schooling and shoaling can serve as an adaptive characteristic that can change between species, individuals of different ages and sizes, and motivational and physiological states. Schooling and shoaling behaviors have a high biological significance in nature, the success of which relies upon the individuals in the group, and as a result has been a research focus of ichthyologists for nearly a century.

The first scientific observations of schooling behavior were conducted by A.E. Par (1927) and C.M. Breder (1929), who investigated the mechanics and structure of fish schools. The interest in studying fish schools lies within the biological complexity, flexibility, and overall intrigue of the school itself (Radakov, 1973). Since then, mechanical, physical, biological, and ecological aspects of schooling behavior have been investigated by numerous researchers worldwide (Radakov, 1973; Partridge, 1982; Pavlov & Kasumyan, 2000; Aoki, 1980). As a result, social aggregations in fish are classified into two types, schooling and shoaling. Although definitions of schooling and shoaling have been provided by several researchers Radakov (1973), Pavlov and

Kasumyan (2000), and Aoki (1980), particularly clear definitions and explanations are provided by Pavlov and Kasumyan (2000):

Fish school – All individuals are oriented in the same direction situated at a certain distance from each other, and are unitary in all movements (i.e., polarized).

Fish shoal – A simple, spatial aggregation of fish attracted by a stimulus occurring independently of each other; no unitary movement exists between individuals (i.e., non-polarized).

In addition, Radakov (1973) stated that a school must contain individuals of the same ontogenetic stage, have no leader, maintain close contact, and exhibit actions beneficial to the entire group. In contrast, Gallego and Heath (1994) referred to a schooling event as two or more fish that encounter each other and swim together for a certain period of time. Obviously, definitions are highly subjective and depend upon the species in question and investigators. Further distinctions have been made for fish species exhibiting polarized schooling behavior. These include obligate and facultative schools (Breder, 1967):

Obligate school – Species that cannot survive alone and display a stable, constant structure. Obligate schooling species break apart infrequently and only due to a large stimulus. Examples include herring, menhaden, and Atlantic silverside.

Facultative school – Species that only school in response to external stimuli or during certain periods of the year. Facultative schooling species can survive alone and school only when beneficial to the individuals. Examples include, mummichog, minnows, and three-spine stickleback.

Distinctions between obligate and facultative schooling species are difficult due to the large variation in form and structure among different species, combined with ontogenetic, physiological, and environmental factors that may alter the time spent schooling (Radakov, 1973). Although differences exist between schooling and shoaling behavior and between obligate and facultative schooling species, the underlying mechanisms and physics of these behaviors are similar.

1.4.1 Mechanisms of Schooling and Shoaling Behavior

Schooling and shoaling behavior, in their simplest form, are a balance of attractive and repulsive forces between individuals. All bodies in space have attractive and repulsive forces acting upon them. Newton's second law of motion states that the total force is equal to the sum of all individual vector forces, $F = ma$. When bodies are in motion and traveling in the same direction, these forces are referred to as ponderomotor forces (Pavlov & Kasumyan, 2000). If the angle of direction between the centers of two moving individuals is 54.4 degrees, the attractive and repulsive forces are equal and cancel each other. Any angle greater or less than 54.4 degrees will alter the forces of

attraction and repulsion, causing one force to be greater than the other (Pavlov & Kasumyan, 2000). In addition, fish do not swim directly behind each other due to the creation of turbulent micro whirls of water created directly behind a swimming fish. An isometric school is one in which all individuals are 54.4 degrees in angular deviation apart and distributed in a shifted, chess board configuration (Radakov, 1973). This isometric structure may reduce friction and drag between individuals while maximizing efficiency by reducing forces. The structure of schools may also be a result of hydrodynamic conditions in the habitat combined with individual species energetic capabilities (Pavlov & Kasumyan, 2000). In a well-polarized school, all individuals have the same velocity and horizontal distance apart while maintaining parallel orientation to each other, with the force of attraction at the center of the school (Aoki, 1980). This resulting arrangement of individuals (angle, distance, and force) will determine the overall speed and direction of the school, as well as communication flow and response to stimuli (Aoki, 1980). These qualitative and quantitative constructs of schools have led to mathematical models and descriptions of schooling and shoaling structure.

When schooling and shoaling behaviors are simplified into vector forces, mathematical values and theoretical models can be quantified using calculus and differential equations. For a more comprehensive explanation of the mathematical modeling of fish schools, refer to Niwa (1994; 1996a; 1996b) and Pavlov and Kasumyan (2000). Using Newton's second law of motion, in conjunction with observations of fish schools, the following mathematical conclusions and numerical constructs have been published to describe the optimal fish school. The nearest neighbor angle (NNA) between fish varies with the direction of the total force of the school, with an optimal

angle of 54.4 degrees (Niwa, 1996). The nearest neighbor distance (NND) should be between sixteen and twenty-five percent of the mean body length and is statistically normally distributed (Niwa, 1994). The optimal NND index was calculated to be 2.44, which is the ratio of nearest neighbor distances divided by the average fish length (Pavlov & Kasumyan, 2000). However, Aoki (1980) concluded that the optimal NND is one-half the fish length with fish alignment in front or behind neighbors as opposed to at the sides. Ultimately, the velocities of the individuals determine the NND, with velocity increasing as school polarity increases (Breder, 1954). School areas are ellipsoid in shape with length to depth ratio of 1.3-1.7, 1.3 for obligatory schools and 1.7 for facultative schools (Aoki, 1980). In a polarized school, the slope of the individual versus group velocity and the volume to surface area of the school should be small, illustrating the coordination between the individuals and the school. The volume occupied by the school in the water column is equal to $1/6 \pi Lh$, where L equals school length and h equals school height (Pavlov & Kasumyan, 2000). The development of these mathematical models and values provides valuable insight and a way to compare and contrast different types of natural fish aggregations.

Through the use of mathematical constructs and models combined with observations over the past twenty years, several general characteristics of fish schools have been derived. As stated by Darwin (1859), natural selection acts upon the individual and in “social animals it will adapt the structure of each individual for the benefit of the community if each in consequence profits by the selected change”. Indeed, schooling and shoaling behavior appears to have developed to increase the fitness of all members in the school (Partridge, 1982). Schools have been adapted over time in such a way that the

school is the leader with each individual maintaining and following the school with no dominance pattern (Partridge, 1982; Radakov, 1973).

Schools and shoals however, have temporary leaders, remaining only for a few minutes, which play an integral part in the construction of informational flow through the individual members of the group. Schools with individuals that remain as leaders for at least 2 seconds have higher polarization because it provides time for all members to receive the locomotory information (Aoki, 1980). The most integral pieces of information received from the leader are the direction and parallel arrangement of the school. Fish in the middle of the school can mimic individuals on the outer edge and the leader, reaping the benefits of reduced drag, friction, and hydrodynamic forces. As a result of the increased efficiency in the center of the school, individual fish will rotate as the leader to allow all individuals to take advantage of its properties (Niwa, 1994; Pavlov & Kasumyan, 2000).

Schools also maintain individuals at different depths to increase vigilance and visibility for foraging, whereas smaller groups will swim at the same depth and behave similarly to each other (Partridge et al., 1980; Pavlov & Kasumyan, 2000). It was determined that groups of fish between four to six individuals are well integrated and exhibit schooling behaviors representative of larger groups (Partridge et al., 1980; Partridge, 1982; Pavlov & Kasumyan, 2000; Radakov, 1973). Independent of size, the resultant shape of a school is constantly in a state of fluctuation, with constant parallel orientation and arrangement of members (Pavlov & Kasumyan, 2000). Schools and aggregations of fish behaving as a single unitary organism tend to be temporary with

separation and combination of individuals occurring depending on environmental conditions.

1.4.2 Abiotic and Biotic Factors Influencing Schooling and Shoaling

In order to achieve a high degree of polarization, fish must respond to environmental stimuli and biological status of the individuals. Schools and shoals require external forces in order to polarize with schooling density and structure depending on individual activities and environmental conditions. Environmental changes over both temporal and spatial scales alter schooling and shoaling structures (Aoki, 1980). Visual, chemical, acoustic, and physiological cues (e.g., oxygen, temperature, salinity) are essential components to schooling synchronization. Alterations in these physical and biological cues will result in a repulsive response, dissolving schools and shoals (Radakov, 1973). In addition, different habitats produce different schooling structures, with increasing polarization of the school as homogeneity of the habitat increases. Schools of fish exhibit high polarization in the open ocean due to decrease in refugia and alter their NNA depending on surrounding habitats (Aoki, 1980). The health of individuals is also integral; parasites, hunger, and illness can alter locations, leadership, and motivation of individuals (Pavlov & Kasumyan, 2000). The overall success of a school depends on the ability of individuals to use abiotic and biotic cues, process the information efficiently, and relate it back to the school.

Once environmental cues are processed, vision and the lateral line are the major systems used for regulation and maintenance of schools. Visual cues are the basis of

schooling behavior and are best demonstrated by the high degree of schooling during daylight hours. Visual cues are used for orientation, maneuvering, and polarization of the school. In addition, the color and position of spots on the body help to maintain position of fish in schools (Pavlov & Kasumyan, 2000). The attractive force between individuals is produced by visual cues; while the lateral line provides the repulsive force, keeping fish from getting too close (Partridge, 1982). It is agreed that vision is more important than the lateral line system for schooling, however both systems are needed, allowing fish to compensate if they lose either of the two senses (Pitcher, 1979).

The lateral line system is used for school stability, reliability of contact, assessment of position, and coordination during maneuvers (Pavlov & Kasumyan, 2000). The lateral line system accomplishes this through a canal system integrated into the integument with pressure sensitive goblet cells relaying information directly to the brain. Control for both visual and lateral line structures are found in the optic tectum and the telencephalon, respectively (Pavlov & Kasumyan, 2000). The optic tectum is a region known to play a role in the integration of motor and visual stimuli in fish and is key to the development of schooling (Ishizaki et al., 2001). This integration of visual and lateral line systems allows individuals to perceive and react to environmental and school stimuli, making schooling behavior an evolutionarily adaptive trait for many fish species.

1.4.3 Adaptive Significance and Evolutionary Benefits to Schooling Behavior

Schooling and shoaling behavior provides an evolutionary benefit and is a significant adaptive trait in individuals (Pavlov & Kasumyan, 2000). This requires the

appropriate allocation of resources between maintenance, growth, and reproduction. The result of such energy allocation is life history strategies designed to transmit the most genetic material as possible to the next generation (Horn & Rubenstein, 1984). In addition, energy allocation is not stable, and must be altered depending on environmental, social, and individual conditions. Investigation of costs and benefits associated with group living behavior indicates an increase in fitness and a positive influence on individuals (Pulliam & Caraco, 1984). The benefits to schooling and shoaling behavior outweigh the costs, resulting in the majority of fish species spending time living in social groups which benefit both the individuals and species as a whole. In addition, since fish schools have no permanent leader or hierarchical structure, all individuals of the school can attain increased benefits.

Schooling and shoaling behaviors increase inclusive fitness in fish through the reduction of predation pressure while increasing foraging, swimming, reproductive, and migratory efficiency (Pavlov & Kasumyan, 2000). Schools and shoals of fish can increase vigilance and reduce overall predation rates, providing a defense against predation. Certain types of predators will not attack schools and shoals due to the decrease in success rate. As a result, schools and shoals create a pseudo refuge for fish in desolate or homogenous habitats (Radakov, 1973). Schooling and shoaling formations can change shape in the presence of a threat, providing collective mimicry and making it difficult for predators to single out individuals (Pavlov & Kasumyan, 2000). Schools and shoals can also detect potential predators at greater distances, increasing escape time for individuals (Radakov, 1973). The overall success of a school depends on the number of individuals comprising the school (dilution effect) and the ability to detect and confuse

predators (Pavlov & Kasumyan, 2000). Fish, unlike mammalian species, have no group scouts or lookouts, but do contain alarm chemicals released into the water when the skin is broken, warning others. Schools will scatter apart, dive, and perform a fountain effect in order to evade predators and reduce capture (Partridge, 1982). Individuals within a school or shoal that are compromised in some way (parasitized, different size, hungry, or near the edge) tend to be eaten at a higher rate than other individuals (Pavlov & Kasumyan, 2000). In addition, schools and shoals tend to lose form and structure as temperature increases and photoperiod decreases, resulting in an increase in predator efficiency. The success of schools or shoals for defensive purposes depends on the physical state of the individuals, the density of the school, and the ecology of the predators and prey (Radakov, 1973).

In addition to defensive properties, schools and shoals can increase foraging and migratory efficiency. Schools and shoals have the capability to locate food and resources faster, especially when patchily distributed over different environmental gradients (Pavlov & Kasumyan, 2000; Grunbaum, 1998). These group structures also allow for range expansion while reducing variance in searching success due to a reduction of drag and conservation of energy reserves (Pavlov & Kasumyan, 2000; Grunbaum, 1998). However, changes in individual physiology and intraspecific competition will alter school structure, with satiated fish in the center and hungry fish located on the periphery or leading for longer periods of time (Pavlov & Kasumyan, 2000). Schooling and shoaling behavior increases migration success and efficiency through a reduction in predation pressure, and increases energy conservation due to the reduction in hydrodynamic forces (Radakov, 1973). Fish schools and shoals can take advantage of

increased foraging and migratory success as long as the benefits, in terms of fitness, outweigh the costs of intraspecific competition with other members for resources.

1.4.4 Ontogeny of Schooling and Shoaling Behaviors

Considering the evolutionary advantages to schooling and shoaling behaviors, development of these behaviors begins early in development for fish. Schooling coordination begins once individuals are able to feed on their own and shortly after developing (or smolting in salmonids) into juveniles (Radakov, 1973). Mutual attraction between individuals needs to develop along with imitation behavior and optomotor response, which maintains orientation, unity, and shape of individuals in the school during periods of rapid maneuvers (Pavlov & Kasumyan, 2000). Development of schooling behavior is tightly linked to visual and lateral line systems, which are dependant upon CNS development. Visual and forebrain regions and their connections in the brain need to be developed in order to initiate the attraction and imitation behaviors required for schooling and shoaling (Pavlov & Kasumyan, 2000). In addition, individual fish need to maintain contact with each other. Young fish, when raised deprived of social contact, developed abnormal behavior (Pitcher, 1979). Herring (*Clupeidae*) exhibit schooling behavior at 35mm, Atlantic menhaden (*Brevoortia tyrannus*) at 19 mm, and northern anchovies (*Engraulis mordax*) and Atlantic silversides (*Menidia medidia*) at 13 mm (Gallego & Heath, 1994; Higgs & Fuiman, 1996; Masuda & Tsukamoto, 1998). All of these species require the development and maturation of visual and lateral line systems, and are able to school shortly after metamorphosis into juveniles. Schooling

and shoaling behavior occurs through a genetic mechanism dependant upon life stage, but a certain amount of social interaction and environmental cues are required for final development. The ability to quantify these complex fish behaviors is important when considering effects of stress exposures and contaminant levels in the environment.

1.5 Biology and Ecology of Harmful Algal Blooms (HABs)

HAB events have been occurring since the origin of life and have been recorded as far back as biblical times (Hallegraeff, 1993). More recently, HAB events have increased in frequency, range, and virulence on a global scale, resulting in research on toxin releasing dinoflagellates (Burkholder, 1999). In addition, with the majority of the world's food supply and essential biotic and abiotic cycling occurring in the ocean, effects of HABs need to be evaluated and solutions determined. HABs tend to occur in shallow, tidal, un-stratified estuaries with decreased flushing and increased salinity (Bricelj & Lonsdale, 1997). HABs can persist for long periods of time if the water column and favorable environmental conditions remain stable (Pelley, 1998). As a result, The Harmful Algal Bloom and Hypoxia Research and Control Act (P.L. 105-383) was passed by Congress with aims to provide "an assessment which examines the ecology and methods for reducing, mitigating, and controlling harmful algal blooms, and the social and economic costs and benefits of such alternatives" (CENR, 2000). HABs can have deleterious economic effects in addition to negatively effecting biological systems, reducing fish consumption, tourism, and industry. Algal blooms are also the leading cause of hypoxia, periods of low oxygen in marine and estuarine systems, due to

increased aerobic respiration (Hallegraeff, 1993). HAB exposure can result in human illness and death, as well as mortalities of wild and farmed fishes, birds, and marine mammals, and altered food webs and trophic dynamics (Landsberg, 2002; Steidinger et al., 1973). The increase in HAB events worldwide and the enactment of the Harmful Algal Bloom and Hypoxia Research and Control Act, have made detection, containment, and remedy of these events a priority.

There are several dozen species worldwide that are responsible for HAB events and capable of producing toxins, with the major taxa being dinoflagellates with diatoms slightly less common (Anderson, 2000). Additionally, some 5000 algal species have been described as harmful, which includes species known to produce harm, indirectly or directly, to aquatic animal health, human health, and ecosystem integrity (Landsberg, 2002; Hallegraeff, 1993). These organisms reproduce asexually and rapidly proliferate under favorable environmental conditions, creating a “bloom” of phytoplankton in the water column and can release toxins when consumed or crushed by waves. Algal bloom events occur across most of the continental U.S. and have been increasing in range over the past 25 years (CENR, 2000). Range expansion can be attributed to ballast water transfer, changes in competitive abilities and trophic structure, dispersal from currents and storm events (e.g., El Niño), and anthropogenic changes in coastal communities (CENR, 2000; Anderson, 1997). In addition, increases in HAB incidence may be due to the recent trend of mild winters, allowing for increased growth periods and decreased winter die-offs. However, even with unfavorable and harsh environmental conditions, many HAB organisms can “hibernate” as a dormant cyst life cycle stage, ensuring survival (CENR, 2000; Anderson, 1997).

Recently, with advances in detection technology, scientists have been able to locate and classify HAB events on a more consistent basis. Examples include the range expansion of *Karenia brevis* (dinoflagellate) from Florida to North Carolina by Gulf Stream transport, *Alexandrium tamarense* (dinoflagellate) expansion due to hurricanes, and blooms of *Pseudo-nitzschia australis* (diatom) in California transported to coastal regions during upwelling events (Anderson et al., 2002; Anderson, 1997; Tester & Steidinger, 1997). In North America, algal bloom occurrences follow seasonal trends with blooms usually occurring in late summer and early fall (3-4 month period), and can affect regions up to 30,000 km² (CENR, 2000). When optimal conditions are present, algal blooms can have deleterious effects on humans and entire ecosystems.

The increasing incidence of algal blooms on a global scale is a result, in part, of anthropogenic activities. Phytoplankton bloom dynamics rely on synergistic interactions of physical, chemical, and biotic processes (Paerl, 1988; Paerl, 1997). An increase in greenhouse gasses, global warming, coastal pollution, and nutrient runoff increases favorable environmental conditions for these opportunistic algal species. Increases in iron, selenium, and chelating agents have been found to increase growth for *Alexandrium* spp. (brown tides) (Bricelj & Lonsdale, 1997). The role of nutrient loading into estuarine systems on HAB events is currently unclear. Burkholder (1999) stated that eutrophication of estuarine systems as a result of excess nitrogen and phosphorous from agricultural runoff can provide the foundation for algal blooms to occur. However, Bricelj and Lonsdale (Bricelj & Lonsdale, 1997) determined that eutrophication was not the most important factor for the growth and proliferation of brown tides. Hence, regulation of coastal pollution and decreasing nutrient enrichment may not reduce all

HAB events. In addition, El Niño and storm events, oceanic current flux, and ballast water release, increase the range of algal species and their effects on habitats (Burkholder, 1999; Anderson, 1997). Fluctuations in flow, tides, currents, salinity, cation concentration, and dissolved organics can increase algal bloom occurrence on a global scale (Anderson, 1997; Bricelj & Lonsdale, 1997).

Increases in anthropogenic activity may alter fundamental structures of coastal ecosystems and allow HAB species to thrive. HABs change ecosystem community interactions through the alteration of competitive levels with other plankters, predation patterns of zooplankton and bivalves, and attenuation of light (Bricelj & Lonsdale, 1997). Native phytoplankton species may be unable to adapt to high nutrient conditions, and therefore be out-competed by HAB species (CENR, 2000). HAB events can also occur if predation rates by zooplankton and benthic filter feeders can no longer control bloom proliferations. Attenuation of light into the water column by HAB proliferation deprives submerged aquatic vegetation, phytoplankton, and periphyton of energy needed for photosynthesis (Anderson, 1997). This attenuation can kill submerged aquatic vegetation and reduce refugia for larval and juvenile fish, altering the status of fisheries (CENR, 2000). The presence of algal blooms may alter energy flow through trophic systems by changing fish migration and juvenile foraging patterns (Burkholder, 1999). To better understand the dynamics and effects of HABs on humans and other organisms, the natural history, cycling, and toxins of HAB species needs to be extensively understood.

1.5.1 Effects of HAB Events on Fish Species

Fish mortality is one of the most frequent consequences of HAB events (CENR, 2000). *Karenia* spp. were the direct cause of millions of fish deaths between 1997 and 1998 in Texas, North Carolina, and Florida (CENR, 2000; Steidinger et al., 1998). Fish mortality events associated with brevetoxin (PbTx) exposure from *Karenia* spp. are common, widespread, and affect hundreds of species (Steidinger et al., 1973; Landsberg, 2002). Fish kills of this magnitude can collapse fisheries, decrease tourism, recreation, fish and shellfish consumption, as well as drive communities into bankruptcy. In addition, HAB events can kill benthic filter feeding bivalves, altering benthic-pelagic coupling and species composition (Bricelj & Lonsdale, 1997). Fish kills (100 tons per day) from PbTx exposure have occurred frequently during the summer and autumn months in Florida and symptoms associated with morbidity and mortality include lethargy, violent convulsions, edema, hemorrhaging, and respiratory failure (Lewis, 1992; Steidinger et al., 1998). Sublethal behavioral symptoms include, labored breathing, convulsions, excess mucus production, loss of balance and righting combined with twisting, corkscrewing, and uncoordinated swimming behavior (Steidinger et al., 1998). PbTx can kill marine organisms through direct exposure, or accumulate in their tissues, poisoning organisms at higher trophic levels. PbTxs from *K. brevis* can be absorbed directly into the bloodstream across the gill lamellae in fish, causing a direct and immediate effect (Trainer & Baden, 1999). In addition, a second route of exposure

through ingestion of PbTx laden copepods has been documented in pinfish (*Lagodon rhomboides*) and spot (*Leiostomus xanthurus*) (Tester et al., 2000).

STX is another important HAB biotoxin produced by 11 different *Alexandrium* species, as well as other species of dinoflagellates and cyanobacteria (Landsberg, 2002). In contrast to gill uptake of PbTx in fish, ingestion is the main route of exposure of STX (Landsberg, 2002). Winter flounder, *Pseudopleuronectes americanus*, fed *A. tamarensis* at densities above 100 cells/ml had greater mortality than fish fed control diets (Mills & Klein-MacPhee, 1985). Larval Japanese anchovy, *Engraulis japonica*, fed STX exposed sea bream larvae, demonstrated high mortality and behavioral alterations (White et al., 1989). Similarly, behavioral alterations, including loss of equilibrium and irregular swimming were observed in Atlantic herring, *Clupea harengus harengus*, fed crude extracts of *A. tamarensis* (White, 1977). STX exposure was implicated in mortalities of chub mackerel, *Scomber japonicus*, in Argentina (Montoya et al., 1996). Dying fish were seen gasping, swimming upside down and on their sides at the surface. Gut contents of dead mackerel contained STX laden salps as well as *A. tamarensis* cells (Montoya et al., 1996).

Milkfish, *Chanos chanos*, exposed to pure STX as well as the algal cells displayed edema, hyperplasia, and necrosis of the secondary lamellae, and was not proportional to concentration (Chen & Chou, 2001). Further, exposure resulted in a decrease in O₂ consumption and cause of death was asphyxiation due the alteration in gill function (Chen & Chou, 2001). In larval zebrafish, STX induced abnormalities in the eyes, pericardial cavity, and yolk sac. In addition, the air bladder failed to inflate and temporary paralysis was observed in exposed zebrafish. STX induced significant effects

on physiology, growth, and survival of larvae including a rapid loss of sensory function (Lefebvre et al., 2004). Temporary paralysis may lead to possible predator avoidance and prey capture effects, a secondary effect of exposure.

Pfiesteria shumwayae and *Chaetoceros concavicornis* are HAB species that play a role in fish kills along the United States coastline. *P. shumwayae*, a member of the toxic *Pfiesteria* complex, is associated with ulcerative lesions in fish along the Mid-Atlantic of the United States (Burkholder & Glasgow, 1995; Glasgow et al., 1995; Lovko et al., 2003). Fish kills in North Carolina and Maryland estuaries, co-occurring with the presence of *Pfiesteria* complex and related organisms, have brought HABs to the forefront of public attention in these coastal communities (Burkholder et al., 1992; Law, 2001; Magnien, 2001; Noga et al., 1996). In addition, *Pfiesteria* species have been discovered in other estuaries on the East Coast including New York and South Carolina, as well as the Gulf Coast and in Europe (Glasgow et al., 2001b; Jakobsen et al., 2002; Law, 2001; Rublee et al., 1999). In the Chesapeake Bay region, *Pfiesteria* and *Pfiesteria* like organisms (PLOs) were implicated in fish kills and human health issues in the Pocomoke, Kings Creek, and Chicamacomico river systems in the summer and fall of 1997 (Magnien, 2001). As a result of the widespread distribution of the HAB species and public interest, research into the mechanisms of fish mortality and human illness remains ongoing.

It is currently unclear whether all strains of *Pfiesteria* complex organisms are toxic to fish, but knowledge regarding physical attraction and attachment to fish, through the use of a peduncular organ, resulting in epidermal damage, is well understood. *P. shumwayae* (CCMP 2089) directly attaches to fish skin, gill tissue, olfactory organs, and

oral mucosa, causing extensive tissue damage and mortality in laboratory exposures (Vogelbein et al., 2001; Vogelbein et al., 2002; Berry et al., 2002). Physical damage to fish, resulting from exposure to *P. shumwayae* (CCMP 2089), leads to degradation of the mucal coat, necrosis, and sloughing of epidermal cells, leading to a complete loss of epidermal and respiratory epithelium (Lovko et al., 2003; Vogelbein et al., 2001). Similarly, Burkholder et al. (2001a) noted sloughing of epithelial tissue resulting from physical attack in a strain of *P. shumwayae* from the Neuse River, North Carolina. Strains of *P. piscicida* and *P. shumwayae* from the Neuse estuary displayed a strong positive attraction to fish excreta and mucus as well as digestion of cellular components (Cancellieri et al., 2001).

Physical exposure to *P. piscicida* and *P. shumwayae* concurrently creates damage to gill lamellae, multi-focal epidermal erosion, and lesions (Burkholder & Glasgow, 1997; Glasgow et al., 2001a; Noga et al., 1996). Fish mortality resulting from *Pfiesteria* complex exposure is well documented in the laboratory, however, less is known regarding fish kills or adverse health effects in the field due to the complex assemblage of organisms present. Fish exposed to sublethal concentrations in the laboratory and then allowed to recover developed bacterial and fungal infections post-exposure (Burkholder & Glasgow, 1997; Glasgow et al., 2001a; Noga et al., 1996). Exposure to fungal, bacterial, and *Pfiesteria* complex organisms, combined with immune system activation, may play synergistic roles in fish mortality in the wild (Law, 2001; Dykstra & Kane, 2000; Vogelbein et al., 2001).

Behavioral alterations have been observed and documented in fish from the wild and the laboratory. Menhaden were observed swimming erratically near the surface, and

exhibiting distress in the Maryland *Pfiesteria* bloom of 1997 (Magnien, 2001). During the 1991 blooms in North Carolina, fish ‘walks’ were observed, which describe writhing at the surface, actively trying to avoid bloom conditions (Burkholder et al., 1995). In the laboratory, exposure to *Pfiesteria* complex organisms caused a loss of equilibrium, disorientation; lethargy combined with periods of hyperactivity, general depression, decreased respiration, wavering, fin twitching, and settling to the bottom of the aquaria (Burkholder et al., 1995; Berry et al., 2002; Gordon et al., 2002; Lewitus et al., 1995; Noga et al., 1996). These documented alterations are observational only, at concentrations lethal to fish, with the mechanism(s) of action driving these behaviors unknown.

Similarly to *Pfiesteria* spp., *Chaetoceros* spp., have been shown to kill fish through direct physical contact. *Chaetoceros* is a harmful diatom genus commonly occurring in many temperate coastal waters which can cause finfish mortalities at concentrations as low as 5 cells/ml in salmonids species (Yang & Albright, 1992; Bell et al., 1974). Several *Chaetoceros* species can form long chains of bullet shaped cells that have hollow spines (i.e., setae). These spines are further studded with even smaller spines (spinules) along their length (Yang & Albright, 1992). Fish mortality has been attributed to microbial infections due to damaged gill tissue, gill hemorrhage, or suffocation from excess mucus production (Yang & Albright, 1994; Bell, 1961). Yang and Albright (1992) described *Chaetoceros* spines and spinules penetrating secondary lamellae of the gill, leading to hyperplasia, hypertrophy, and partial or complete fusion of secondary lamellae. In addition, *Chaetoceros* exposure affects physiological parameters, including decreases in partial pressure of oxygen, ventilation volume, and oxygen

consumption, with increases in respiration frequency, haematocrit, lactate, and glucose values. There is a general trend of increased mucus production followed by hyperplasia, then hypertrophy in fish exposed to sublethal concentrations of *Chaetoceros* (Yang & Albright, 1992). The limitation on gill function leads to hypoxic conditions in the fish.

Mortality in salmonids exposed to blooms of *Chaetoceros* is caused by a combination of stressors and not the algal bloom alone. *Chaetoceros* blooms exacerbate mortality due to other diseases such as vibriosis and bacterial renal disease. Exposure to 4-52 *Chaetoceros* cells/ml, suppresses neutrophils, lymphocytes, and thrombocytes of the immune system of salmon, with 15 cells/ml killing juvenile salmon (Yang & Albright, 1994). In addition, aquaculture farms noted high mortality due to vibriosis and bacterial kidney disease (BKD) of penned salmon in the presence of *Chaetoceros concavicornis* blooms (Albright et al., 1993). Vibriosis was the major cause of death and they found no death with exposure to other bloom species. This depression of the immunoprotective system in *Chaetoceros*-exposed fish was exacerbated by hypoxic conditions, with exposed fish more susceptible to vibriosis and BKD than healthy fish (Albright et al., 1993). Neurotoxins, in addition to physical stressors, can induce deleterious effects in fish populations.

1.5.2 Ecological Roles of Domoic Acid (DA), Brevetoxin (PbTx), and Saxitoxin (STX)

HABs can have deleterious effects on many levels within marine habitats. Such effects can include hypoxia, altered food web dynamics, contamination of shellfish and

fish with neurotoxins, and direct neurotoxicity of higher-level consumers through bioaccumulation and trophic transfer (Landsberg, 2002; Lefebvre, 2001; Lefebvre et al., 2002a; Scholin et al., 2000; Tester et al., 2000). The toxins produced by these phytoplankton species, however, are metabolically costly to produce and must provide an advantage to the organisms other than toxicity to fish, marine mammals and humans. As a result, there must be other hypotheses as to why these biologically expensive toxins are derived and retained through evolutionary history (Cembella, 1998). Several different hypotheses have been suggested by plankton biologists that include; a reservoir for nitrogen, a product of nucleic acid synthesis, bioluminescence, chemical defense (allelopathy), ion transporter systems, a way to reduce nutrient stress, a chemical signal to conspecifics, and for anti-bacterial and fungal functions (Cembella, 1998). *Karenia brevis* and *Pseudo-nitzschia* spp. produce toxins in times of nutrient stress during late growth stages, which suggests these compounds may be used to scavenge nutrients from the environment (Bates, 1998; Wright & Cembella, 1998). Currently, there have been many theories, but no real concrete conclusions regarding the ecological use of toxins by their phytoplankton producers.

DA is an amino acid (311 mw) that is an analog of kanic and glutamic acid, and is an excitatory neurotransmitter (affinity is 2 fold higher for kanic acid and 10 fold higher for glutamic acid) (Bates et al., 1998). Eight species of *Pseudo-nitzschia* worldwide produce DA, with toxicity varying between species. The largest concern ecologically is the trophic transfer of DA from fish to seals, whales, pelicans, and humans. DA is not produced by *Pseudo-nitzschia* until several days to weeks after the bloom has been initiated, and then can be detected in zooplankton, mussels, and fish (Bates et al., 1998;

Pan et al., 1998). Bloom initiation and maintenance are linked to light, salinity, temperature, nutrients (N, P, Si, Fe, Mg), and water column stratification. Once these conditions become suboptimal, DA production within cells is increased. Once population growth begins to decline towards a stationary phase, DA is produced. This can be attributed to nutrient stress in *Pseudo-nitzschia*, specifically, Si and P limitation (Pan et al., 1998). Si is needed for frustule formation and DNA synthesis while P is needed for cellular membranes, lipids, and energy metabolism. DA increases coincide with alkaline phosphatase, an enzyme used for P sequestering activity and which is an indicator of stress. Once growth decreases due to Si limitation, available energy is allocated for DA production. This shift in allocation may function to scavenge limiting nutrients from the environment. Rue and Bruland (2001) demonstrated that DA functions as a siderophore to scavenge iron, and as a chelator for copper at toxic concentrations. DA can increase iron levels when they are limiting and decrease copper concentrations, which may extend the life of the cell. Whether DA can scavenge Si or not is unknown at his time. Bates (1998) determined that DA was not used for growth, osmoregulation, allelopathy, anti-feeding, or for antibiotic or antifungal purposes. DA is only produced during stress as a function to promote survival and propagation of the cell.

In contrast, PbTx, a monocyclic ladder frame polyether is derived from the acetate cycle during the entire growth period. There are up to 9 different species of brevetoxins in class B, the most commonly quantified from the environment are PbTx-1, 2, and 3 (Dickey et al., 1999). In some species, these toxins have potent antifungal and antibacterial activity, and may increase during stressful events (Cembella, 1998). In addition, PbTx can decrease copepod feeding rates, inducing paralysis, regurgitation, and

low fecundity. This allows *K. brevis* blooms to persist in the environment by deterring grazing pressure. Grazers, as a response, increase predation on other algal species, increasing nutrients for *K. brevis* (Cembella, 1998). As a result, *K. brevis* blooms can monopolize the nutrients, which may be an explanation as for how they can persist for up to 18 months (Steidinger et al., 1998). In addition, *K. brevis* is an unarmored dinoflagellate, and PbTx can be released into the water easily upon cell lysing, providing a possible measure of antifungal or antibacterial protection for other cells. Doucette et al. (1999) illustrated that 2 species of algicidal bacteria can significantly reduce *K. brevis* populations. Currently, PbTx is used by *K. brevis* to reduce grazing pressure, which reduces competitor pressure, allowing blooms to utilize more nutrients and sustain for longer periods of time (Cembella, 1998).

Brevetoxins maintain a specific binding affinity for voltage-sensitive sodium channels (VSSC) in excitable membranes (Deshpande et al., 1993). The binding of PbTx-*n* to site 5 inhibits channel inactivation, increases the VSSC open time 2 fold, and shifts activation to more negative membrane potentials (Jeglitsch et al., 1998). PbTx increases the likelihood of channel opening, increases sodium ion influx, release of excitatory amino acids, activates N-methyl-D-aspartate (NMDA) receptors, and increases Ca²⁺ influx through the voltage gated calcium channel (VGCC) (Berman & Murray, 1999). This resultant signaling cascade promotes neuronal depolarization, leading to hyper-excitability, and may reduce the number of channels available for impulse conduction depending on the tissue (Franz & Leclaire, 1989; Deshpande et al., 1993).

Brevetoxin exposure has been linked to several types of physiological and behavioral problems in mammals and humans. Subsequently, symptoms associated with

PbTx exposure have been grouped as neurotoxic shellfish poisoning (NSP). NSP was designated a reportable disease by the Department of Health for the state of Florida in 1999 (Flemming & Baden, 2000). In mice, Baden and Mende (1982) found labored breathing, loss of appetite and motor function, and exhibited SLUD syndrome (salivation, lacrimation, urination, and defecation) when exposed to PbTx. Cause of death from high doses was respiratory failure (LD = 100 g/kg), however, mice recovered in two to three days from sublethal doses. More recently, studies on rats exposed to PbTx displayed behaviors such as head bobbing, loss of righting and reflexes, and an increase in convulsive movements (Templeton et al., 1989). Respiratory paralysis is due to PbTx inhibiting diaphragm skeletal muscle through the depolarization of the phrenic nerve (Deshpande et al., 1993). In humans, prolonged respiratory exposure can lead to dizziness, tunnel vision, and skin rashes. Respiratory effects can be reversed by leaving the effected area, however, chronic effects have been reported in the elderly and in asthmatics (Flemming & Baden, 2000). Oral exposure results from the ingestion of shellfish that have accumulated PbTx in their tissues. Common symptoms include cranial nerve dysfunction, sensory abnormalities, reflex and muscle coordination problems, and gastrointestinal abnormalities (Franz & Leclaire, 1989).

Similar to *Karenia* spp. and PbTx, STX is produced by *Alexandrium* spp., a species with a global distribution and a threat to human health, wildlife, and local economies (Velez et al., 2001). STX is also produced by several other species of dinoflagellates and cyanobacteria (Landsberg, 2002). These resultant symptoms of exposure have been grouped together and named paralytic shellfish poisoning (PSP). PSP is a recurrent problem associated with blooms of the harmful dinoflagellate

Alexandrium. STX produced by these algae are accumulated in shellfish and other grazers, which is then passed on to humans through ingestion. Symptoms include tingling, numbness, headache, dizziness, nausea, vomiting, rapid pain, and anuria (Bower et al., 1981; Kao, 1993). There is no loss of consciousness and the reflexes are unaltered except for pupillary size and sight may be temporarily lost. In severe cases, these symptoms spread to the extremities with respiratory difficulty, difficulty swallowing, sense of throat constriction, speech incoherence or complete loss of speech, as well as brain stem dysfunction. In very severe cases, within 2-12 hours, there is complete paralysis and death from respiratory failure in absence of ventilatory support. If victims survive for 12 hours post exposure, regardless of severity, victims start to recover gradually and are without any residual symptoms within a few days (Bower et al., 1981; Kao, 1993).

Toxic blooms of *Alexandrium* occur on the East coast of the U.S. in ME, MA, and Georges Bank (Anderson, 1997). In semi-continuous cultures of *A. tamarense*, STX increased as growth rate increased, with highest abundance of STX abundance in mid-exponential growth phase (Anderson et al., 1990). In contrast to DA and PbTx, STX concentration increases with cellular growth, with maximum toxin concentration at the exponential growth phase. STX is one of the deadliest poisons known, with poisoning in humans resulting from consumption of bivalves or fish containing bioaccumulated STX (Lefebvre et al., 2004).

Symptoms of both NSP and PSP include mild to moderate intoxication, general malaise, facial paralysis, asthenia, dysphagia, headache and GI disturbance (Valenti et al., 1979; Chang et al., 1993). Higher exposures can lead to severe intoxication and reduced

cardio-respiratory performance. Lethality from STX exposure in humans has been connected to paralysis of respiratory musculature (Garcia et al., 2004). In guinea pigs and cats, depression in breathing and blood pressure with cardio-vascular collapse was documented in conjunction with initial declines in heart rate and respiration rate (Andrinolo et al., 1999; Chang & Benton, 1993). Death was attributed to myocardial failure following apnea, with central and peripheral cardio-respiratory components compromised. The functional integrity of the diaphragm was seriously compromised affecting the central respiratory rhythmogenic network. In cats, concentrations of 2.7 mg/kg will cause respiratory arrest but not death outright. However, 10 mg/kg will dramatically decrease blood pressure with irreversible arrhythmia and cardiac arrest within minutes (Andrinolo et al., 1999). In addition, STX appears to cross the blood brain barrier, increases gamma-amino butyric acid (GABA) and decreases glutamate (Andrinolo et al., 1999; Chang et al., 1993).

1.5.3 Neurotoxic Mechanisms of Action of PbTx, STX, and DA

Voltage sensitive sodium channels (VSSCs) are transmembrane polypeptides bound in cellular membranes that control sodium influx into cells (Catterall & Gainer, 1985). This is accomplished through voltage dependant increases in sodium permeability that initiate action potential signaling, thereby regulating the depolarization associated influx of sodium ions and depolarizing the cell. Once the cell membrane potential has been depolarized and an action potential signal generated, the sodium channel closes and the resting potential is restored through the action of the sodium-potassium pump. This

sequence is directly regulated through the alpha subunit and its 3 auxiliary subunits, betas 1-3 (Catterall, 1992; Cestele & Catterall, 2000). The alpha subunit consists of four homologous domains (I-IV) each with 6 segments (S1-S6). The membrane loops between S5 and S6 form the ion selectivity filter and outer region of the pore (Terlau et al., 1991). The result is constant control of signaling between neurons in the central and peripheral nervous systems. Sodium channels are located in all cells but are concentrated in skeletal muscle and neural tissue. As such, many types of neurotoxins strongly bind to specific receptor sites on sodium channels, altering channel function. Six different receptor sites have been identified; STX acts at receptor site 1 and PbTx at site 5 (Cestele & Catterall, 2000).

Brevetoxin is hydrophobic, binds to site 5 on the voltage sensitive sodium channel (VSSC), and mechanism of action is opposite of STX. PbTx molecules have a high affinity to bind with sodium channels in excitable membranes, and disrupt the flow of sodium ions by maintaining the channel in an open configuration, preventing repolarization of the membrane (Deshpande et al., 1993; Rodriguez et al., 1994). This disruption shifts action potentials to levels 2 fold more negative, elongating the action potential and period of depolarization (Trainer & Baden, 1999; Dechraoui et al., 1999). In addition, PbTx inactivates the sodium-potassium pump, preventing energy-dependent sodium efflux and reducing intracellular pH and oxygen concentrations.

STX is a water soluble heterocyclic guanidine and the C8 guanidinium needs to be protonated in order for STX to bind to the sodium channel (Strichartz, 1984). STX acts at the extra-cellular side of the protein and through interacting with receptor site one, STX blocks sodium conductance through binding monovalent cations. STX and

tetrodotoxin (TTX), the classical sodium channel blocker, bind at different receptor domains at site 1 (Narahashi et al., 1964; Hille, 1975). Similar to TTX, STX eliminates ion current, rendering channels impermeable. The binding is reversible. If STX is injected into the cells, no effects are seen and STX does not seem to reach the outside of the nerve, demonstrating the affinity for the external pore of the Na channel (Henderson et al., 1973).

In contrast to PbTx and STX, DA is a NMDA/glutamate channel agonist. DA binds to alpha-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA)/kinate receptors as well as NMDA receptors and the L-type voltage sensitive calcium channels (VSCC). DA increases intracellular calcium concentrations, reversing the operation of the Na/Ca exchanger, creating alterations in neuronal signaling and survival (Berman et al., 2002). DA can induce brain lesions, seizures, memory loss, and neuronal death (Todd, 1993). In mice, DA concentrations between 0.5mg/kg and 4 mg/kg have been found to induce scratching, seizures, convulsions, and death (Peng & Ramsdell, 1996). Therefore, DA is affecting signal conductance further down the pathway, allowing an influx of Ca^{2+} but resulting in a similar effect, depolarization and alterations in signaling. Although the strategies differ, the ecological roles for DA (increase nutrient uptake) and PbTx and STX (reduce grazing and competitor pressure) are to increase and maintain population density during bloom conditions. Alterations in brain activity resulting from HAB stressor exposure may alter regional neuronal signaling, and the ability to detect and quantify these changes may form a link between behavioral change and neuronal activity.

1.6 The Role of c-Fos Expression and Brain Activity in Fish

Behavioral changes in organisms can result from complex alterations at the biochemical and physiological level of organization. Knowledge of the underlying biochemical and physiological processes that control behavior is crucial. In the CNS, the command center of behavior, organisms possess mechanisms within neurons that provide biochemical responses to extracellular stimulation. These mechanisms can induce cellular changes, either by adapting to stimuli or through repair and regeneration, which are essential for survival. In many instances, these adaptations and repairs require alterations in gene expression. Adaptation of the body to environmental conditions is only possible due to the rapid response of brain structures associated with intracellular signal transduction and genetic regulation (Rybnikova et al., 2003).

Immediate early genes (IEGs), located within the nuclei of neurons, are induced as a result of neuronal stimulation (electrical or second messenger) requiring no synthesis of transcription factors. As a result, IEGs can be induced rapidly, with activation observed within 15-30 minutes of stimulus application (Herdegen & Leah, 1998). IEG activation then codes for new proteins (cytokines, enzymes, HSP70, oxygenase) as well as transcription factors to activate other genes. IEG encode transcription factors that lead to expression of late genes that are responsible for phenotypic changes in cells (Herrera & Robertson, 1996). *c-fos* is an IEG that codes for the transcription factor, c-Fos. Neuronal excitation leads to an increase in c-Fos mRNA, which then leads to an increased AP-1 protein DNA binding, and regulation of gene expression (Sonnenberg et al., 1989; Zhang et al., 2002). The synthesized transcription factor by *c-fos*, c-Fos, can then regulate gene

activation to elicit a response. These newly synthesized proteins and cellular products resulting from the gene expression then initiate, regulate, or modify neuronal cellular mechanisms in response to stimuli.

Induction of IEG expression in the brain occurs under a variety of conditions and is believed to be a link between external stimuli and long-term alterations in phenotypes (Thiriet et al., 2001). The response of *c-fos* to extracellular stimuli functions as a transcription factor network to couple extracellular stimuli and gene expression (Bakin & Curran, 1999). This increase in genetic activity leads to a prolonged change in neurons, which can occur through alterations in depolarization, neurochemistry, and synaptic transmission (Hunt et al., 1987). IEGs have been implicated in the induction of specific genes responsible for changes in cellular architecture and their products function as nuclear mediators that couple external stimuli to long term changes in gene expression in the cell (Morgan & Curran, 1989). It has been demonstrated that the *c-fos* pathway can regulate neuronal excitability and ultimately survival (Zhang et al., 2002).

1.6.1 *c-fos* Induction Cascade

Herdegen and Leah (1998) provide an extensive review regarding the specifics of the *c-fos* initiation cascade. In order for *c-fos* to be activated and to synthesize transcription factors, an external stimulus is required. The stimulus can occur either electrically or chemically, however, the majority of signals that induce *c-fos* are derived from second messenger systems, cyclic adenosine monophosphate (cAMP) or calmodulin kinase. Once a signal stimulus has activated cAMP from adenylate cyclase, calmodulin

kinase, or by membrane depolarization, kinases are activated and translocated to the nucleus via the nuclear pore complex (NPC) bound to HuR, a shuttle protein (Gallouzi & Steitz, 2001). These kinases include the mitogen-activated protein (MAP) kinase pathway, ERK, JNK, SAPK, and PKA (induced by cAMP) and CRE (induced by calmodulin kinase). Once in the nucleus, kinases phosphorylate constitutive transcription factors (e.g., CREB, ATF, SRF, TCF) bound to the DNA, activating transcription of *c-Fos* mRNA. The *c-Fos* mRNA is then translocated back to the cytoplasm through the NPC with HuR for protein and transcription factor synthesis. At this stage, the IEG *c-Fos* mRNA synthesizes the transcription factor *c-Fos*. Once in the nucleus, *c-Fos* forms a heterodimer with other factors (*c-Jun*, *JunB*), creating an AP-1 protein complex. Once activated, the AP-1 complex then binds to regulatory sites on DNA, regulating expression (Herdegen & Leah, 1998).

1.6.2 Examples of *c-Fos* Induction

The result of the *c-fos*, *c-Fos*, and AP-1 complex cascade is a regulation of cell excitability and survival (Zhang et al., 2002). As a result, *c-fos* induction and resulting protein expression of *c-Fos*, can be used as a biomarker of neuronal stress and to assess the brain areas that are activated when animals are exposed to different types of stressful stimuli (Rybnikova et al., 2003; Martinez et al., 2002). Basal *c-Fos* expression is usually very low in mammals, and stress causes a rapid increase in expression that provides information about neuronal circuits involved in behavioral, autonomic, and neuroendocrine responses to stress (Martinez et al., 2002). However, *c-Fos* activation is

a general integrative stress response involved in adaptive responses to stimuli and memory, but currently no direct interpretation is possible because knowledge of which genes operate in the brain for specific circumstances is not known (Martinez et al., 2002). Despite some limitations to using c-Fos as a measure of neuronal activity (generalized response, and lack of cellular *c-fos* induction does not necessarily mean lack of activation), this technique has been a powerful tool in the functional mapping of neural circuitry mediating many stimuli, including stress (Figueiredo et al., 2003). As a result, *c-fos* and c-Fos expression have been sensitive methods to screen for enhanced neuronal activity in response to stimulations and changes in the environment over the past 2 decades (Hoffman & Lyo, 2002; Sadananda & Bischof, 2002).

Studies have been conducted in rats to investigate the role of *c-fos* in the regulation of downstream genes, or in behavioral and physiological responses associated with *c-fos* activation (Hansson et al., 2003). Previous studies have shown that *c-fos* can be induced in rats through glutamate receptor agonists, ion channel flux, light, magnetism, dioxins, spinal cord injury, traumatic stress, and the psychoactive drugs haloperidol and clozapine (Abraham & Brewer, 2001; Ceccatelli et al., 1989; Cheng et al., 2002; Morgan & Curran, 1986; Morris et al., 1998; Murphy & Feldon, 2001; Nemeč et al., 2001; Sonnenberg et al., 1989). Zhang et al. (2002) described how induction of *c-fos* decreased seizures through down regulation of NMDA receptors in the presence of increased calcium concentrations. In addition, *c-fos* induced genetic changes can allow habituation to substances and increase long-term memory (Kaufer et al., 1998; Herdegen & Leah, 1998; Tischmeyer & Grimm, 1999). Correlations between locomotion behaviors

and c-Fos expression have been demonstrated in rats, with a relationship shown between a set of behaviors within walking and c-Fos expression (Espana et al., 2003).

In addition, alterations in cellular ionic balance can result in c-Fos expression. As ionic potassium concentrations are increased in the presence of ionic calcium, the calmodulin pathway is initiated, resulting in increased c-Fos expression. The neurotoxin, tetrodotoxin, (TTX) can abolish this effect through the blocking of the potassium gradient and thereby restoring intracellular voltage (Morgan & Curran, 1986). Scorpion venom, BmK, is a VSSC channel neurotoxin that partially blocks VSSC channels and significantly inhibits c-Fos expression (Zhang et al., 2003). Pesticides such as the carbamate acetylcholinesterase inhibitors (pyridostigmine) and pyrethroids (permethrin) induce c-Fos mRNA levels and neuronal excitability (Friedman et al., 1996; Imamura et al., 2002). Antisense blockade of c-Fos in rat hippocampus attenuated excitatory amino acid toxicity and seizure-induced heat shock protein hsp70 expression (Baille et al., 1997; Lu et al., 1997). The use of c-Fos expression as an indicator in neuronal activity resulting from stressor exposure is well documented in rats, however, mechanisms of c-Fos expression in other taxa remain less understood.

1.6.3 c-Fos Expression in Fish

Currently, research involving expression of c-Fos is mostly conducted on rats in an attempt to better understand human brain function. However, fish are exposed to many of the same stressors (xenobiotics, neurotoxins, and environmental estrogens) in the environment, and c-Fos induction may serve as an important measure of exposure.

The *c-fos* gene maintains a strong homology between species and is highly conserved among fish species (Bosch et al., 1995). In fish, the nature of the c-Fos protein has not yet been elucidated (Bosch et al., 1995). Immunohistochemistry (IHC) has been used to detect c-Fos protein as a marker of neuronal activity and to map neuronal pathways activated by certain stimuli. In addition, IHC has been used to compare and differentiate activated and inactivated neurons in specific regions of the fish brain in relation to startle response and chemical stressors (Bosch et al., 1995; Bosch et al., 2001; Matsuoka et al., 1998).

Advantages of quantification of c-Fos expression using IHC compared to other methods (e.g., 2-deoxyglucose, 2DG) are increased resolution and detection of regional and cellular activity. c-Fos expression does not provide the same information as the 2DG method. c-Fos and 2DG provide different aspects of neuronal activation, with c-Fos activated in areas where plastic change is occurring with activation, rather than activation without change (Sadananda & Bischof, 2002). Thus, c-Fos activation provides better resolution when investigating adaptive alterations in neurons resulting from stress exposure. However some cells can express c-Fos with no stress exposure while others respond to stress without c-Fos expression (Bosch et al., 1995; Figueiredo et al., 2003). Bosch et al. (1995) found 30% of control fish expressed small amounts of c-Fos (1-2 cells/region) while expression in experimental fish was several fold greater (Bosch et al., 1995). However, this method can only provide relevant results when a clear control can be designed and established. Vibratory startle response resulted in increases in c-Fos expression when compared to controls, including the Mauthner cells in the medulla of rainbow trout (Bosch et al., 2001). c-Fos expression can serve as a general biomarker of

neuronal stimulation. However, with adequate control groups, c-Fos expression can provide a specific, detailed label of the mechanism and location of action of toxins and other stressors in the brain at the cellular level.

1.6.4 Potential for c-Fos Expression Resulting from Harmful Algal Bloom Stressors

The neurotoxins, PbTx, DA, STX, *Pfiesteria*, and *Chaetoceros*, given their mechanisms of action, should induce *c-fos* activity, leading to cellular repair and reduction of stress. PbTx is a sodium channel agonist that binds to site 5 on the channel, increasing depolarization of the cell by holding the channel in the open configuration, and allowing an excess of sodium ions. This depolarization causes alterations in neural excitability and signaling, which leads to cellular death by acidosis and decreased oxygen levels. PbTx can cause seizures, muscle spasms, ataxia, and block neuronal end plate potentials, leading to death by respiratory failure (Baden & Mende, 1982). *c-fos* can be induced by PbTx exposure through two routes. First, the alteration in ion flux generated by PbTx (increased sodium concentration) would activate the adenylate cyclase and cAMP second messenger system, leading to *c-fos* induction. *c-fos* could then possibly down-regulate sodium channel synthesis or inhibit signal-inducing mechanisms. Second, as the action potential propagates from axon to dendrite, the depolarization will trigger the opening of kinate/AMPA/NMDA receptors, thereby increasing calcium uptake. The increase of intracellular calcium will then activate the camodulin kinase system, leading to *c-fos* induction. Most likely however, in order to initiate *c-fos* transcription with PbTx, both depolarization and intracellular calcium may be needed. Ciguatoxin, chemically

similar to PbTx, induces c-Fos mRNA in the rat brain and increases immunohistochemical expression of c-Fos (Peng et al., 1995).

STX blocks VSSCs, eliminates ion current, and renders sodium channels impermeable (Henderson et al., 1973). STX mediates toxicity by obstructing the ion pore of the VSSC, thereby stopping flow of sodium into excitable cells and resulting in nerve dysfunction (Anderson, 1997). This inhibition of signaling can lead to paralysis and rapid loss of sensory function in fish (Lefebvre et al., 2004). A STX induced block of neuronal signaling may lead to a reduction in brain activity and reduce *c-fos* induction. Tetrodotoxin (TTX), a neurotoxin with the same mechanism of action as STX, has been demonstrated to inhibit c-Fos expression in the rat cortex, visual systems, and striatum (Hausmann et al., 2001; LaHoste et al., 2000; Lu et al., 2001). Fish exposed to sublethal concentrations STX may reduce *c-fos* induction and hinder neuronal activity.

DA, in contrast, is a glutamate channel agonist and maintains a high affinity for kinate, NMDA, and AMPA receptors (10 fold higher than glutamate). DA can induce brain lesions, seizures, memory loss, and neuronal death. The loss of neurons is believed to be the cause of memory loss. Similar to PbTx, once DA increases intracellular calcium concentrations through kinate, NMDA, and AMPA receptors, cAMP and calmodulin will initiate the cascade to induce *c-fos* (Zhang et al., 2002). DA has been proven to induce the expression of c-Fos in the rat brain through the activation of NMDA and AMPA receptors, which increases intracellular Ca^{2+} concentrations (Peng & Ramsdell, 1996). This increase in calcium alters ionic flux within neurons, resulting in *c-fos* induction in rats at concentrations as low as 0.5 mg kg^{-1} . Two mg kg^{-1} is required for alterations in behavior, illustrating that the use of c-Fos is a more sensitive biomarker for

DA exposure in rats (Peng & Ramsdell, 1996). *c-fos* induction as a result of DA can decrease seizures and down regulate the number of kainate and AMPA receptors to decrease the effects and mortality on neurons. In contrast, the physical irritants, *Pfiesteria* and *Chaetoceros*, may induce c-Fos in different areas of the brain in comparison to the neurotoxins DA and PbTx-2, or not at all.

1.7 Conclusions

Schooling and shoaling behaviors are complex social behaviors utilized by a wide diversity of fish species to increase individual fitness and propagate their genes to the next generation, requiring a balance of energy allocation. These behaviors have predictable structures, shapes, and responses to threats and environmental fluctuations. Through the use of integral mathematics, numerical values of schooling characteristics and models quantifying schools have been developed. Schools and shoals provide defense from predation while increasing reproductive, foraging and migration efficiency. The reduction of hydrodynamic forces (friction and drag) by a school allows for energy conservation while swimming and the ability to negotiate differences in environmental gradients and habitats. In addition, these behaviors are intimately tied to and regulated by the visual and lateral line systems in conjunction with the CNS, and are developed as soon as fish are able to swim and feed. Alterations in school structure and density can be caused by individual differences in motivation, physiology, abiotic and biotic environmental factors, and anthropogenic pollutants. The benefits of schooling and shoaling behaviors to reduce stress and effects of their surrounding environment has

proven to outweigh the associated costs, and has been adapted as a life history characteristic in many fish species.

HAB events have been occurring for centuries and recently HAB events have increased in frequency, range, and virulence on a global scale. This increase in incidence of algal blooms on a global scale may be a result of anthropogenic activities. HABs can have deleterious economic effects in addition to harmful effects on biological systems, reducing fish consumption, tourism, and industry. In addition, algal blooms are also the leading cause of hypoxia, periods of low oxygen in marine and estuarine systems, due to increased photosynthetic aerobic respiration. Many HAB organisms can directly kill marine organisms through direct exposure, or accumulate in their tissues, poisoning organisms at higher trophic levels. Brevetoxin, saxitoxin, and domoic acid are potent neurotoxins synthesized by the HAB species, which are known to harm organisms through direct exposure or by bioaccumulation. *Pfiesteria shumwayae* and *Chaetoceros concavicornis* are HAB species that play a role in fish kills along the United States coastline. As a result, knowledge regarding how these HAB species and toxins effect fish populations and behavior is an important step into learning the environmental effects of HAB-related stress on individual organisms.

Behavioral changes in fish can result from complex alterations at the biochemical and physiological level of organization. Knowledge of the underlying biochemical and physiological processes that control behavior is crucial. In the CNS, the command center of behavior, organisms possess mechanisms within neurons that provide biochemical responses to extra cellular stimulation. *c-fos*, an immediate early gene (IEG), is located within the nucleus of neurons and is induced as a result of neuronal stimulation.

Induction of *c-fos* leads to c-Fos protein expression in the brain which occurs under a variety of conditions and believed to be a link between external stimuli and long-term alterations in phenotypes. Therefore, c-Fos protein expression in the brain can serve as an indication of exposure to a variety of stressful stimuli. Currently, research involving expression of c-Fos is mostly conducted on rats in an attempt to better understand human brain function. However, fish are exposed to many of the same stressors (xenobiotics, neurotoxins, and environmental estrogens) in the environment, and c-Fos expression may serve as an important measure of exposure. In addition, many HAB species release neurotoxins known to disrupt neuronal signaling and ionic balance. Therefore, the use of c-Fos expression may link behavioral alterations resulting from HAB stressor exposure to changes in brain activity, providing novel information on how HAB stress affects behavioral and neurological mechanisms in fish.

Chapter 2: A Novel Software System to Quantify Social Behavior in the Mummichog, *Fundulus heteroclitus*

2.1 Abstract

Group living and social interaction between conspecifics have proven to be an evolutionary stable strategy for many species. The study of group behavior, specifically schooling and shoaling in fish, has been a focus of research for several decades. However, quantification of schooling and shoaling has been difficult due to the complex nature of the behaviors and substantial computational time. This paper describes the design of a novel software program used for the analysis of schooling, shoaling, and individual behaviors in the estuarine killifish, *Fundulus heteroclitus*. When the software system is coupled with an existing videography system, we are able to capture video of schools and shoals of fish at up to 30 frames per second for up to 1 hour continuously. The ultimate application of this system is to quantify changes in aggregative behavior in fish resulting from exposure to environmental and anthropogenic stressors. In addition to describing the program software, an analysis of behavioral alterations over an acclimation period is presented. Significant decreases in schooling, shoaling, individual velocity, and number of interactions were observed. In addition, there was an increase in nearest neighbor angle (NNA) and distance over time. These results describe decreased social interactions and polarization (degree of unity in movement) over time. No changes in group behaviors were observed during different observation periods after a 24 hour arena

acclimation. The use of this system to study alterations in group swimming behavior resulting from stress exposure has offers a valuable tool in the field of behavioral ecology.

2.2 Introduction

Aggregations and group living have beneficial effects at the individual and community levels of organization by providing an increase in survival and fitness (Hamilton, 1971). Social aggregations are multi-level biological phenomena that differ between species, ontogenetic stage, and physiological state (Radakov, 1973).

Aggregations can be comprised of closely related kin, distant relatives, or non-related individuals. Living in groups provides increased foraging success and efficiency, reproduction, parental care, and predator vigilance, while decreasing predation pressure through dilution effects (Alexander, 1974). Group living can also reduce detrimental effects of environmental fluctuation, thus providing stability to individuals and populations (Grunbaum, 1998). As a result, group living is witnessed throughout many vertebrate taxa and occurs in many forms, ranging from loose aggregations to strict hierarchal societies.

Fish are the largest and most taxonomically diverse group of vertebrates known, with twenty five percent of species forming schools or shoals, and fifty percent doing so during larval and juvenile stages (Radakov, 1973; Pavlov & Kasumyan, 2000; Aoki, 1980). The majority of schooling and shoaling species are small, with aggregations forming temporarily for short time periods or during specific life cycles and stages

(Partridge, 1982). Teleost fishes utilize schooling and shoaling behaviors to detect, confuse, and escape predators as well as to locate mates and resources, and for migration (Radakov, 1973; Masuda & Tsukamoto, 1998). Schools and shoals of fish are distinct from other vertebrate social groups due to their equipotentiality, or having no permanent leader or hierarchal structure (Radakov, 1973). Such schools and shoals with no dominant leader tend to form when resources and refugia are scarce, predation rates are high, or when individuals have no defensive morphology such as, spines, poisons, coloration, and postures (Radakov, 1973; Pavlov & Kasumyan, 2000; Grunbaum, 1998). Due to the high biological significance in nature, schooling and shoaling have been a research focus for nearly a century.

Qualitative analysis of fish schools began in the late 1920's with investigations into the mechanics and structure of fish schools (Parr, 1927; Breder, 1929). Since then, mechanical, physical, biological, and ecological aspects of schooling behavior have been investigated worldwide, and definitions of schooling and shoaling have been provided by several researchers (Radakov, 1973; Pavlov & Kasumyan, 2000; Partridge, 1982; Aoki, 1980). As a result, social aggregations in fish are classified into two types: schooling and shoaling. Pavlov and Kasumyan (2000) define a fish school as having all individuals oriented in the same direction, situated at a certain distance from each other, and unitary in all movements (i.e., polarized). Shoaling, in contrast, is a spatial aggregation of fish typically attracted by a stimulus, with no uniformity of movement between individuals (i.e., non-polarized). Shoaling behavior of fish occurs for social reasons, where the structure of the group is not important (Wibe et al., 2002). Therefore, different species of

fish may display schooling and shoaling behaviors based on the inherent attraction between individuals of the species as well as environmental stimuli.

Schooling and shoaling behaviors have been historically difficult to quantify due to the complexity of the behaviors. Kotrschal and Essler (1995) provide an extensive review of the use of fish locomotion analyses with respect to swimming, search, sensory, and schooling and shoaling behaviors, as well as ontogenic development. Tracking multiple fish over an extended period of time with computers is memory and computationally intensive, and many studies have resorted to investigating a small number of video frames and manual plotting fish coordinates for calculation of parameters (Fuiman & Webb, 1988; Gallego & Heath, 1994; Koltes, 1985; Masuda & Tsukamoto, 1998; Partridge, 1980; Rehnberg & Smith, 1988; Suzuki et al., 2003). Many software programs can analyze schooling endpoints, which follow strict criteria of polarization. Shoaling, in contrast, is more difficult to analyze due to the loose definition used to define aggregations of individuals, swimming or not. In addition, current software programs analyze a very small number of frames due to the complexity and large file sizes of the data. As technology advances, quantification of schooling and shoaling behaviors should become more efficient and accurate.

This chapter describes a software system designed and developed to quantify group behaviors, using a killifish model, for extended periods of time, in up to 1 hour increments. This software, in conjunction with a videography system, can quantify schooling, shoaling, and swimming behaviors of groups of up to ten fish concurrently. The ultimate goal is to utilize this system to quantify alterations in aggregative and interactive behaviors due to exposure to stressors such as contaminants, toxins, and

environmental fluctuations. The goal of this study was to examine fluctuations in social behaviors over temporal acclimation and diurnal periods in experimental arenas using the killifish, *Fundulus heteroclitus*, as a model. Alterations in group mummichog behaviors over time in experimental laboratory arenas were quantified and the results are discussed.

2.3 Methods

2.3.1 Test Species

The mummichog, *Fundulus heteroclitus*, is an essential species for the success and productivity of estuarine marsh environments. These small cyprinidontid teleosts regulate the trophic structure of marsh systems through control of invertebrate populations (crustaceans and annelids), as well as serving as prey for larger predators such as blue crabs, striped bass, and shore birds. Mummichog are eurythermal and euryhaline fish that thrive in a variety of salt, estuarine, and brackish marshes, and maintain a cosmopolitan distribution along the East Coast of the United States. Due to their extensive range, wide tolerances, and ecological importance, mummichog have gained status as an important species for marine scientific research (Atz, 1986). In addition, mummichog have been used as biomarkers and sentinels for contaminant exposure in estuarine systems (Weis et al., 2001; Ownby et al., 2002; Eisler, 1986). Mummichog are year round-residents in most estuarine systems with a narrow home range, providing an excellent model in which to measure aggregative and congregative behaviors between individuals (Fritz et al., 1975; Smith & Able, 1994). Fish used in the

present study were collected from a reference site in Solomons, MD, treated for ectoparasites, and laboratory-acclimated for 4 weeks prior to experimentation. Fish were acclimated to a 14:10 hour light cycle, fed three times a week (Finfish Silver, 38% protein, 1.5 mm slow sinking pellets, Ziegler Bros., Gardner, PA), and fasted 48 hours prior to testing. Temperature, pH, and salinity of the experimental arenas were maintained at the same values as holding tanks.

2.3.2 System Design

The physical experimental design of the exposure arenas has been described previously (Kane et al., 2004) (Fig. 1). Briefly, ten 10-liter exposure arenas were designed and constructed with water flow electronically controlled by multi-channel, digital peristaltic pumps (Masterflex L/S, Cole-Parmer, Vernon Hills, IL). The exposure arenas maintained 5 liters of exposure water and were placed on a 14:10 L:D photoperiod combined with a computer controlled dusk and dawn cycle. Twelve color CCD cameras with manual iris and focus control were mounted above respective arenas, and were connected to dedicated VCR decks for recording. The VCR decks were connected to a single multiplexer to support real-time display for observation of all arenas. VCR recording and stop functions were activated and synchronized remotely using computer based X-10 technology. Once exposures and observations began, there were no human activity in the room.

Analog video data were digitized at 3 frames per second using a Macintosh platform (G4, 900 MHz, 850 Mb SDRAM). Data were digitized in real time, cut in

Adobe Premiere 5.0[©], and imported into a commercial tracking program, Videoscript Professional, version. 2.2.13[©]. A least squares estimator method was applied to the coordinate data to minimize the least square of total distance between each frame in order to estimate individual fish paths. The tracking program then converted the digital output into x,y coordinate data with the use of a custom algorithm designed for tracking the movement of multiple fish. The x,y coordinate data was then analyzed using software designed at the University of Maryland Aquatic Pathobiology Center to obtain the desired endpoints. These endpoints are defined in Table 1.

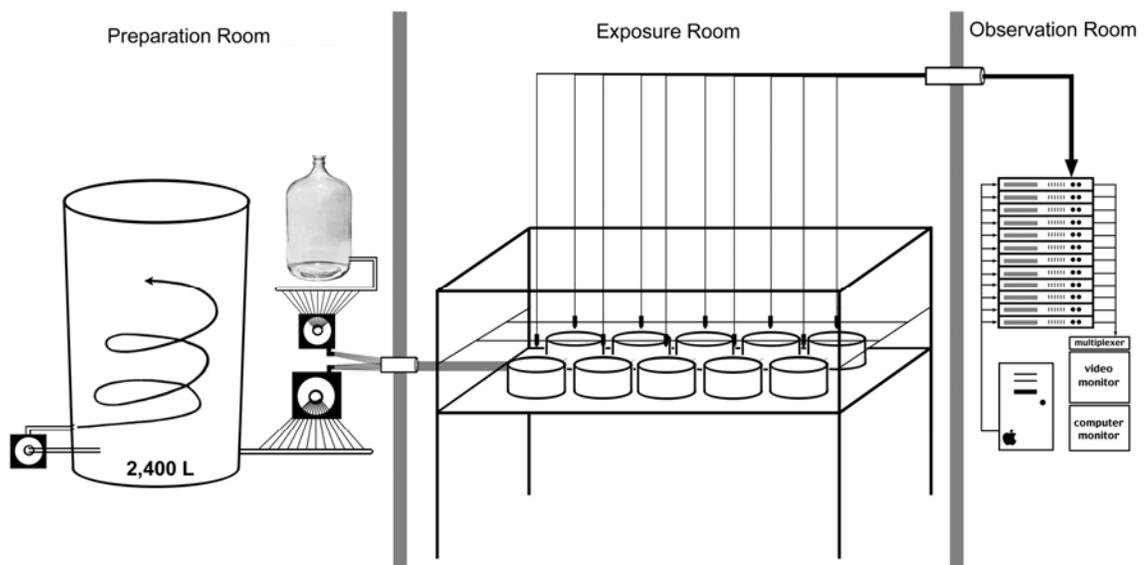


Figure 1 Behavioral analysis experimental system. Schematic diagram showing water preparation room, exposure room and remote computer/video control systems. Analog CCD cameras are mounted above respective exposure vessels. Fluorescent tubes create shadowless illumination. Video signals are recorded on VCR decks that are controlled by X-10 computer software and hardware. A video multiplexer and display monitor permit simultaneous viewing of animals in real time during an experiment. Reproduced from Kane et al., 2004.

Table 1 Group behavioral endpoints.

BEHAVIORAL ENDPOINT:	DEFINITION:
1. Movement Behaviors:	
Aggregative Swimming:	Three or more fish; group organized
Percent Shoaling	Number of frames satisfying shoaling criteria divided by the total number of frames multiplied by 100
Shoal NNA	Angle of trajectory between 2 fish in a shoal; must be greater than 45°
Shoal NND	Average distance to nearest neighbor for each fish in a shoal (minimum of 3 fish)
Percent Schooling	Number of frames satisfying schooling criteria divided by the total number of frames multiplied by 100
School NNA	Angle of trajectory between 2 neighboring fish in a shoal; must be less than or equal to 45°
School NND	Average distance to nearest neighbor for each fish in a shoal (minimum of 3 fish)
Velocity	Speed of school calculated in centimeters per second
Solitary Swimming:	Individual swimming; no organized group
Percent Solitary	Number of frames not satisfying shoaling or schooling criteria divided by the total number of frames
Solitary NND	Average distance to nearest neighbor for individual fish
2. Interactions	The number of times two fish swim within 0.1 body lengths of each other (irrespective of satisfying movement criteria)

2.3.3 Group Endpoint Determination

The endpoints (Table 1) calculated by the software program used the following criteria. The first criterion was to delineate between schools, shoals, and solitary behaviors. A minimum of 3 out of the 5 fish per arena was required by the software to calculate schooling or shoaling behavior. Fish body length used for the calculations was 50 pixels, equal to 71mm. Movement of fish was defined as one-half body length per second (35mm/sec) and was calculated on a frame-by-frame basis at 3 frames per second. A shoaling event was calculated when movement of at least three fish was satisfied and

the distance of a fish from its nearest and second nearest neighbor were 0.5 and 1.0 fish lengths, respectively, with no angular criteria. For reference fish, the Eurasian minnow *Phoxinus phoxinus*, and *Gnathopogon elongatus*, schooling NNDs have been determined to be 0.5-0.9 and 1.1 body lengths respectively (Partridge, 1980; Aoki, 1980). A schooling event met the criteria of a shoal and had a nearest neighbor angle (NNA) less than or equal to 45° for a minimum of 2 seconds (6 frames). If schooling occurred for 5 frames or less, it was considered shoaling and calculated as such. Individual data was calculated when a frame did not meet the criteria for schooling or shoaling and always included all 5 fish. Once the software recognized a group or solitary behavior, the number of interactions, velocity, nearest neighbor distance (NND) and angle (NNA), and the percent time spent in the configuration (school, shoal, or solitary) were calculated.

An interaction between 2 fish was defined as one fish being within one tenth of a body length or less (equal to or less than 5 pixels, 7.1 mm) from its nearest neighbor. Velocity was calculated as displacement (cm) per unit time (sec) and NND was calculated as the average distance between each individual and its nearest neighbor for each fish in the group. NNA was calculated as the average angle between the trajectories of each fish and its nearest neighbor (Higgs & Fuiman, 1996; Masuda & Tsukamoto, 1998). NNA was constrained between 0°-180°, with 0° corresponding to 2 fish swimming parallel and in the same direction, and 180° if parallel and swimming in opposite directions. We used direction of movement as an indicator since the orientation between the snouts of the two fish was not calculated. Percentage of time spent schooling, shoaling, and solitary, were the number of frames schooling, shoaling, and solitary divided by the total number of frames.

2.3.4 Experimental Procedure

An acclimation experiment was conducted over 3 days to examine the necessary time required for killifish to acclimate to the experimental arenas, i.e., no significant change in recorded behaviors over time. In addition, on the final day, a diurnal experiment was conducted to investigate any diurnal fluctuations in behaviors. Ten groups of 5 fish (60-83 mm), were randomly selected from the laboratory population, fasted for 48 hours, and placed in the arenas (5 fish per arena, at least 2 of each sex). It has been demonstrated that groups of fish larger than 3 exhibit schooling and shoaling behaviors, with 4-6 individuals having the highest integration (Partridge, 1980; Partridge, 1982). Flow in the exposure arenas was maintained at 7ml/min, with 2 exchanges/day in order to ensure adequate water quality over the 72 hour observation period. The cameras and VCRs automatically recorded 30 minute data segments beginning at noon for 3 days: day 0 (30 minutes after introduction to the vessel) day 1, day 2, and day 3 (24, 48, and 72 hours post introduction, respectively). On day 3, four equally spaced 30-minute clips at 0900, 1200, 1500, and 1800 hours were recorded to investigate differences in behaviors during the 10-hour recording period. From each arena, seven 30-min clips over the course of the two experiments, totaling of 70 half-hour recordings from all arenas were generated. Video clips were digitized at 3 fps, 5400 frames/clip analyzed per time period (37,800 frames total). Water quality data were collected each day from 2 identical surrogate vessels containing fish dedicated for water quality determination and not used for behavioral observation (Table 2). Differences in behavioral endpoints between the 3

days, and the four 30 minute periods within the last day, were then analyzed for statistical differences.

Table 2 Water quality parameter values (N = 2) recorded each day and during both experiments. BDL = below detectible limit (< 0.001 mg/L).

Water Quality Parameter	Day 0	Day 1	Day 2	Day 3
pH	8.1	8.1	8.1	8.1
Salinity, PSU	5.0	5.0	5.0	5.0
Temperature, °C	23.0	23.5	23.5	23.5
Unionized Ammonia, mg/L	BDL	BDL	BDL	BDL
Dissolved oxygen, mg/L	5.9	5.3	5.2	4.9

2.3.5 Statistical Analyses

An experimental unit consisted of the group of 5 fish in each arena. A completely randomized statistical design was used with behavior as the response (Table 1) and day (0, 1, 2, & 3) as the categorical variable. Non-normal data were transformed to meet the assumptions of the ANOVA procedure. Data that could not be transformed to meet the assumptions were ranked prior to analysis. A repeated measures 1-way ANOVA (PROC MIXED, repeated, SAS, vs. 8.1, Cary, N.C.) was used to compare behavioral endpoints over time for both experiments. In addition, several covariance structures were investigated in an effort to discern a best fit structure for both sets of data. The ante-dependence -1 (ANTE-1) structure provided the best fit and was used for both sets of data. A Tukey-Kramer *post-hoc* mean comparison test was used to evaluate differences ($\alpha \leq 0.10$) between time periods in the event of a significant F statistic.

2.4 Results

Four fish out of the 50 tested died during the course of the 72-hour acclimation; behavioral measures collected prior to death were included and analyzed. A low frequency of schooling was observed with a significant decrease in percent schooling during the acclimation period ($p = 0.0001$, Figure 2). No changes in schooling NND, NNA, or velocity occurred due to the decrease in schooling behaviors observed over time ($p > 0.10$, data not shown). Shoaling and individual endpoints, in contrast, displayed several significant changes over the 72-hour experimental period. The number of interactions between individuals and shoal velocity significantly decreased within the first 24 hours and then remained constant over the remaining 2 time periods (Figures 3 and 4, $p = 0.0021$ & 0.001 , respectively). Shoal NNA significantly increased within the first 24 hours and then remained constant over the following 2 time periods (Figure 5, $p = 0.0008$). Shoaling percentage and NND, in contrast, displayed highly significant changes after 24 hours with less significant changes on day 3. The percentage of time spent shoaling significantly decreased from 85 to 45 percent (Figure 6, $p = 0.0002$) after 24 hours and from 50 to 30 percent on day 3 (Figure 6, $p = 0.01$). Similarly, shoal NND significantly increased after 24 hours (Figure 7, $p = 0.0013$) and further increased by 72 hours (Figure 7, $p = 0.04$).

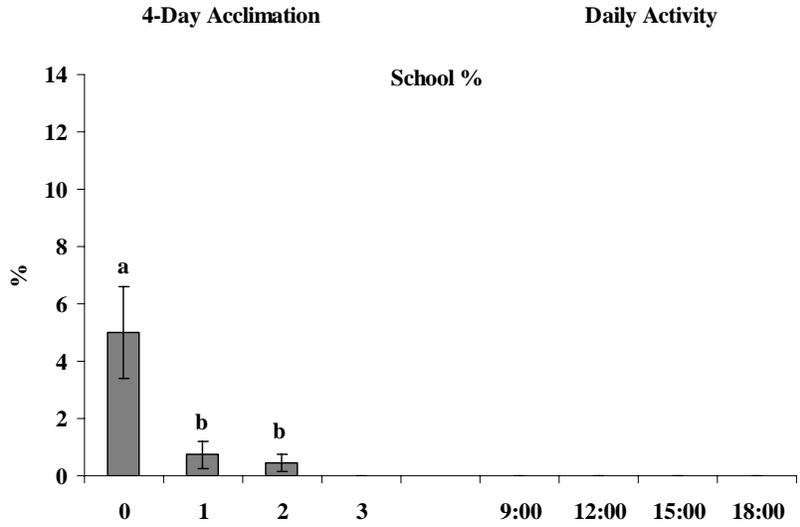


Figure 2 Percentage of time schooling in groups of mummichog from the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

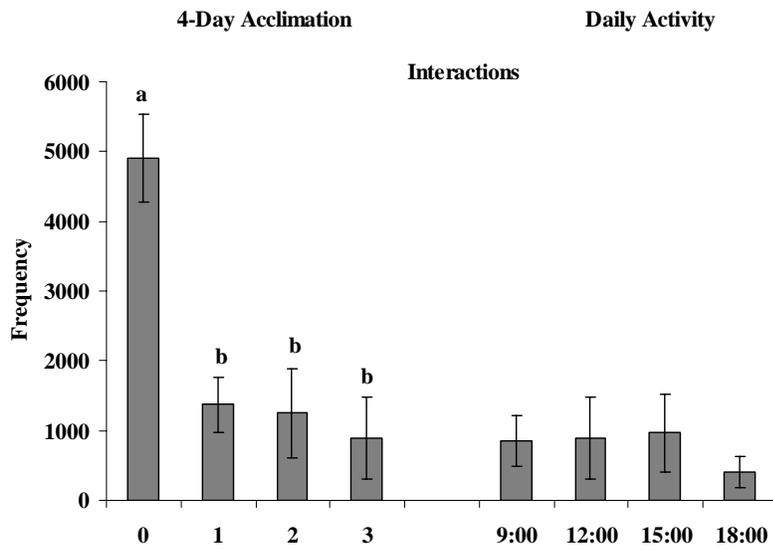


Figure 3 Frequency of interactions in groups of mummichog from the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

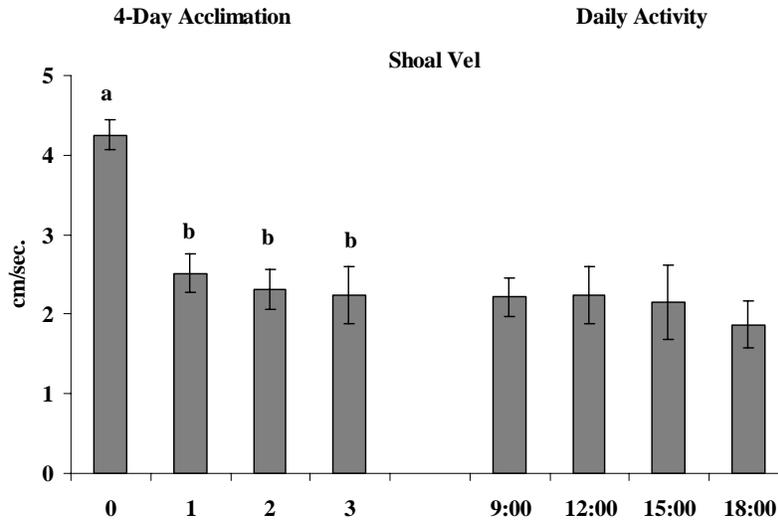


Figure 4 Shoaling velocity in groups of mummichog over the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

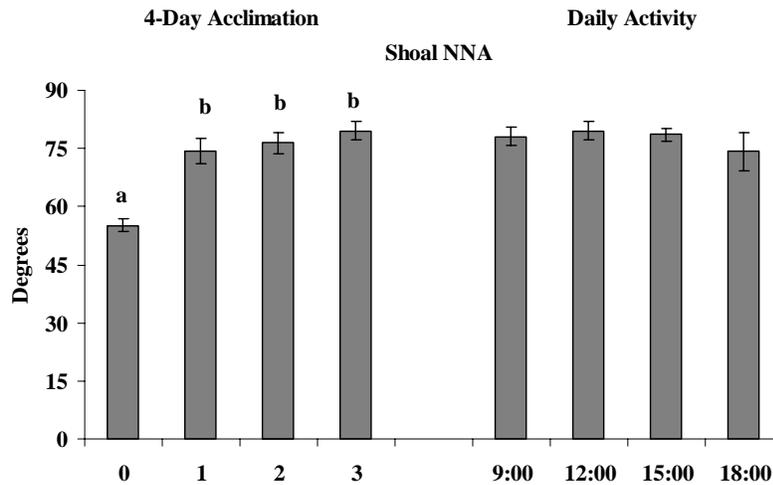


Figure 5 Shoaling nearest neighbor angle (NNA) in groups of mummichog over the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

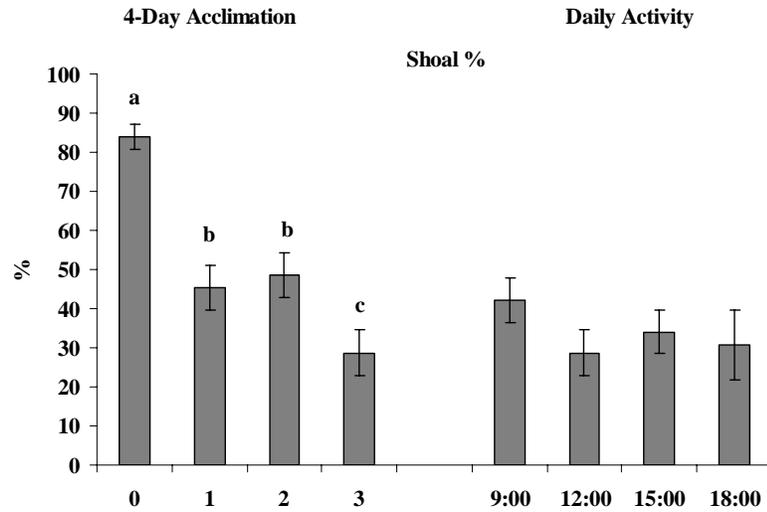


Figure 6 Percentage of time shoaling for mummichog over the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

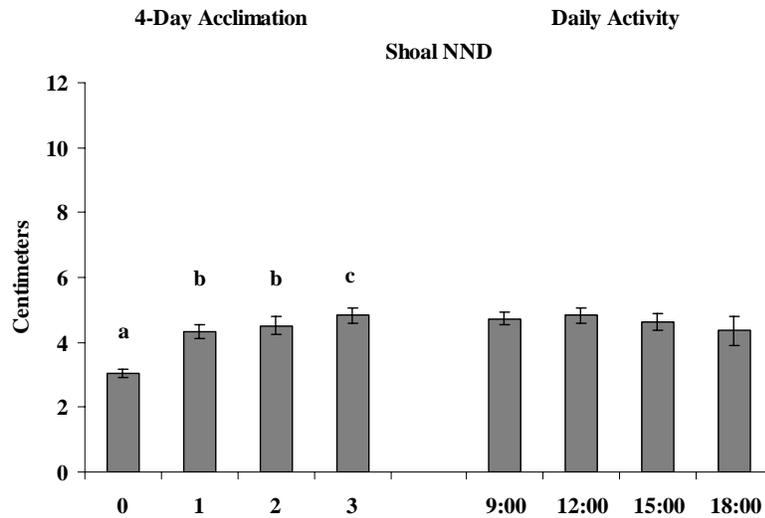


Figure 7 Shoaling nearest neighbor distance (NND) in groups of mummichog over the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

Alterations in individual endpoints mirrored the changes observed in the shoaling endpoints. Individual velocity significantly decreased after 24 hours and remained constant for the remainder of the experiment (Figure 8, $p = 0.0002$). Similar to shoaling percentage and NND, time spent solitary and solitary NND displayed notable changes after 24 and less significant changes on day 3. Time spent solitary significantly increased and NND significantly increased after 24 hours (Figures 9 and 10, $p = 0.0001$ & 0.0004 , respectively) with further increases and decreases on day 3 (Figures 9 and 10, $p = 0.003$ and 0.03 , respectively). These significant changes in the endpoints observed post 72 hours were 1 to 2 orders of magnitude less than the post 24-hour values. In addition, no significant differences were observed in any of the group or individual endpoints between the 4 observation periods during the final day (Figures 2 - 10, $p > 0.10$).

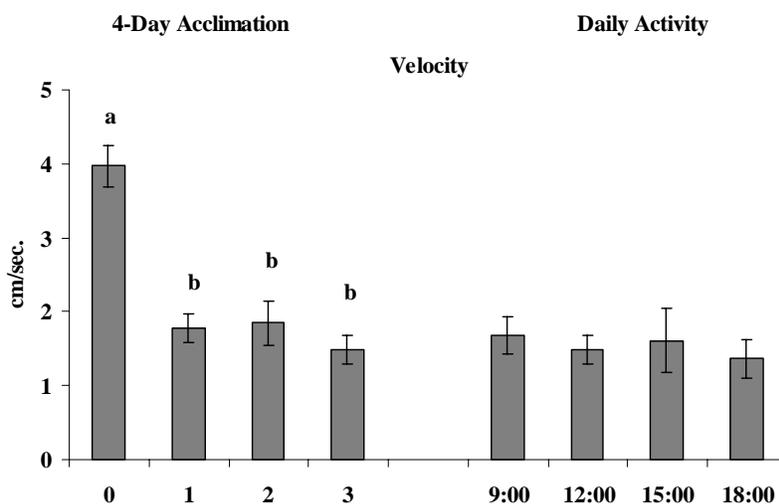


Figure 8 Individual velocity from the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

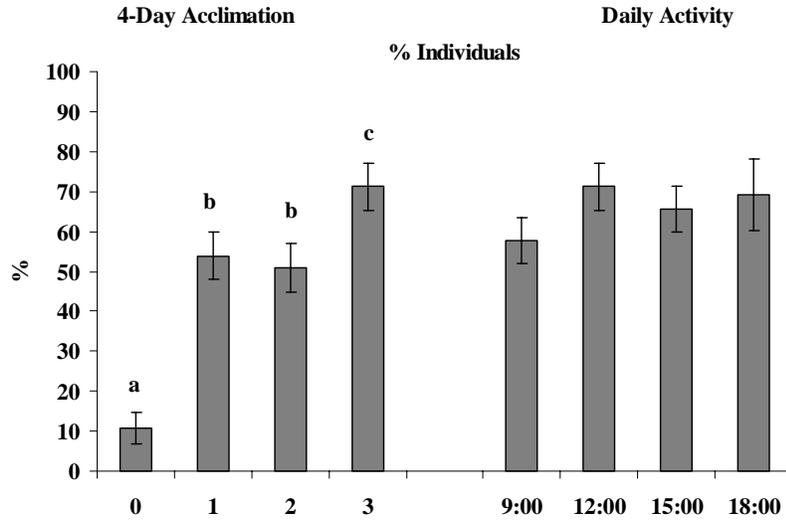


Figure 9 Percentages of time fish were solitary during the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

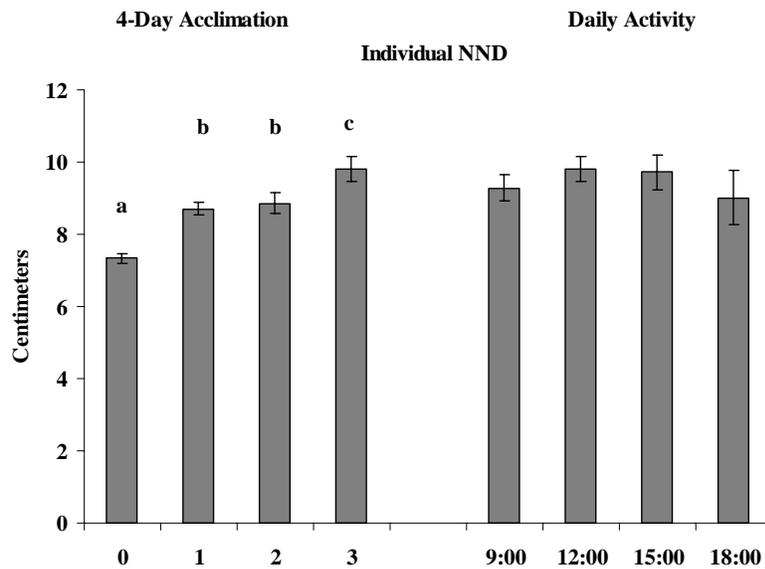


Figure 10 Individual nearest neighbor distances during the 3 day acclimation (Day 0, 1, 2, and 3) and the final day (0900, 1200, 1500, and 1800) experiments. Data are means \pm S.E. Times with different letters are significantly different at the $\alpha \leq 0.10$ level.

2.5 Discussion

Schooling and shoaling behaviors have attracted considerable attention as model systems in an attempt to unravel the functions of group living (Hoare et al., 2000). To investigate group swimming behaviors in mummichog, mathematical criteria were selected based on existing literature and preliminary observations, to measure both schooling and shoaling behaviors. Since schooling behaviors were infrequent, conclusions and comparisons only to shoaling behavior were drawn.

Mummichog travel and reside in aggregations, are non-migratory, and maintain a year-round association with conspecifics in the population. Appropriately, the common name of this species, mummichog, is a Native American word meaning “going in crowds” (Nichols & Breder, 1927). Results of this study confirm that, under laboratory conditions, mummichog exhibited a higher frequency of shoaling than schooling behaviors.

The mortality of 4 fish (8%) during the 72-hr observation period was atypical of other similar exposures conducted with mummichog in our laboratory. This mortality rate for control animals is higher than desired, but is considered within acceptable limits for toxicity assays as described by the U.S. EPA (Peltier & Weber, 1985). These fish most likely succumbed due to a combination of stressors including fasting, net transfer, confinement, aggression by dominant fish, and possibly individual predisposition. However, the resultant data from this study describes and confirms our predictions for acclimation of groups of mummichog to experimental arenas. We expected and observed

compact group swimming around the arenas in an exploratory fashion followed by decreases in speed and inter-individual association as acclimation time progressed.

The decrease in schooling and shoaling, and number of interactions between individuals, corresponds with increases in NNA and NND, describing a general disassociation over time. Initial NNA was 54.5 degrees, which is similar to theoretical schooling values described previously (Radakov, 1973). However, after 1 day, NNA increased to 80.0 degrees, illustrating a decrease in polarization. This agrees with the expected value for random orientation of 90 degrees as defined by Masuda and Tsukamoto (Masuda & Tsukamoto, 1998). There is, however, no defined NNA value for shoaling, and given the nature of shoaling, we used NND only. In addition, there are no generally accepted values for NNA and NND for schooling fish (Higgs & Fuiman, 1996). Decreased group and solitary velocity further illustrate acclimation over time and describe a general reduction in activity as acclimation progresses. As shoaling behaviors decreased over time, increased solitary behavior further confirm the disassociation of individuals. Increases were observed in the percentage of time spent solitary and NND with decreases in velocity. Evaluation of the various endpoints described in this study confirmed a shift in behavior over time (72-hr) consistent with a group of fish familiarizing themselves with a new surrounding.

Relatively minor changes in behavior on the final day of the experiment do not influence our conclusions that groups of mummichog appear acclimated after 24 hours of introduction to the arenas. Nearest neighbor distance and percent shoaling displayed minor decreases post 24 hours. However, these are endpoints that have no locomotion component and may play a less important role in acclimation than velocity or frequency

of interactions. Additionally, a three-day acclimation period is logistically too long for experimentation and would introduce further stressors, such as starvation, into the system. The experimental time period for future experiments will occur between 24 and 48 hours post introduction into the arenas, a period at which no significant changes in any of the endpoints were observed. During this time period, mummichog shoaled, on average, 50% of the time with an average angle of 75 degrees separated by 4.5 centimeters. We believe that 24 hours is a sufficient amount of acclimation time for this species prior to experimentation, as previously reported with individual mummichog in the same arenas (Kane et al., 2004).

Behavior is the ultimate result of complex biochemical and physiological factors, and has the ability to fluctuate depending on endogenous and exogenous stimuli. Group organization is ultimately determined by the social interactions between individuals. However, environmental changes over temporal and spatial scales are known to alter schooling and shoaling structures (Aoki, 1980). Visual, chemical, acoustic, and physiological cues (e.g., oxygen, temperature, salinity) are essential components to schooling synchronization. Alterations in these physical and biological cues due to toxin or stress exposure may result in a repulsive response, dissolving schools and shoals (Radakov, 1973). Group responses to changes in environmental factors, such as alterations in water quality parameters, toxin exposure, light and chemosensory stimuli can be measured and quantified. As toxicology and ecology continue to examine the responses and movement patterns of fish, a behavioral record will be required to answer many questions observed in response to toxicants as well as environmental fluctuations. Standard toxicological methods tend to study the effects of stressors within an individual,

rarely *between* individuals, which is what occurs in a natural setting. Several examples include exposure of goldfish, *Carassius auratus* to herbicides and Atlantic silversides, *Menidia menidia*, to Carbaryl decreased grouping and schooling behavior, respectively (Weis & Weis, 1974; Saglio & Trijasse, 1998). In contrast, exposure of Atlantic silversides to sublethal concentrations of copper resulted in increased schooling polarization (Koltes, 1985).

This software system can investigate alterations in group behavior in response to stress exposure. The system has the flexibility to analyze up to 10 fish per arena at once and the computer program can be adapted to analyze any number of individuals in a school or shoal, with the only limitation being computation time. One of the advantages of this system is that it can record and analyze up to 1 hour of time at 30 fps, which is an improvement over previous systems (Fuiman & Webb, 1988; Gallego & Heath, 1994; Hartwell et al., 1991; Higgs & Fuiman, 1996; Masuda & Tsukamoto, 1998; Partridge, 1980; Rehnberg & Smith, 1988; Suzuki et al., 2003; Hassan et al., 1992). The ability to look into longer periods of time is more relevant when comparing interactions between fish and changes in social structure due to stressor exposure. Additional endpoints that require further consideration are relatedness, size structure, gender, and shoal size. Many species prefer to shoal with conspecifics, which has been demonstrated in both laboratory and field populations of fish groups, in addition to shoaling with similar sized individuals (Barber & Ruxton, 2000; Barber & Wright, 2001; Griffiths & Magurran, 1999; Hoare et al., 2000; Krause et al., 1996; Krause et al., 2000; Ward & Krause, 2001).

This system has the ability to investigate changes in structure and function of group dynamics as well as movement patterns in fish through time. The endpoints used

by this system are environmentally relevant social and movement behaviors that occur on a continuous basis in nature, which can directly affect individual fitness. This quantification of group behaviors is useful when investigating how environmental fluctuations or other stressors may alter the ability of fish to interact with other individuals of the population. Exposure to stressor may negatively alter group behaviors involved in reproduction, competition for resources, predator avoidance, and habitat utilization. Ultimately, data generated from this system have the ability to foster development of models that can relate stressor-induced changes within individual and population interactions and dynamics, relating overall community and trophic level interactions in environmental systems.

Chapter 3: Behavioral Responses of Individual and Groups of Killifish, *Fundulus heteroclitus*, Exposed to a Predation Threat.

3.1 Abstract

The ability for fish to actively evade predation is essential to the success of the individual. In order to accomplish this, fish must maintain a suite of behaviors to deal with predation pressure. Further, these behaviors must be adaptive to accurately respond to fluctuations in predation pressure. We examined predator response behaviors of individual and groups of killifish, *Fundulus heteroclitus*, to a simulated visual aerial predator, to investigate if response behaviors remain similar within individuals and groups after repeated simulated “threats,” and if responses vary based on whether they are alone or in a group. The killifish is a good model for the investigation of predator avoidance behaviors because they live in shallow estuarine habitats and are vulnerable to aerial predation threats. We used a computer-based video tracking system designed to record 15 seconds of baseline movement before, and 15 seconds of movement after, presentation of the visual “fly-by.” The stimulus consisted of wood bird silhouettes that “flew” over the exposure arenas. The visual “fly-by” stimulus elicited a significant response whereby 96% of the individual fish and 95% of the groups of fish exhibited a startle response followed by cessation of movement as a result of the simulated predator presentation ($p < 0.001$). This cessation of movement lasted for $12.4 (\pm 0.9)$ seconds for individual fish and $8.2 (\pm 0.3)$ seconds for groups of fish, which were significantly

different ($p < 0.01$). Velocity and percent movement for groups of fish before and after the simulated predator presentation were consistently higher than individuals. Groups of fish responded with significant decreases in average velocity, shoal velocity, interactions, percent movement, and percentage shoaling, along with significant increases in percentage of aggregation. Groups of fish displayed a higher degree of overall movement and initiated swimming behaviors before individuals, describing the ability for killifish to alter predator response behaviors based on social dynamics. As a result, these eminently repeatable and quantifiable responses could serve as endpoints for the investigation of alterations in predator avoidance behaviors due to stress exposure.

3.2 Introduction

The ability to evade predation is essential to the success of an organism. An animal captured and consumed by a predator has its cumulative fitness abruptly terminated, and therefore, strong selective pressure exists to evolve escape mechanisms to mitigate predation (Ryer & Olla, 1998). As a result, organisms need to maintain a constant and consistent suite of predator avoidance tactics in order to react to threats and to survive. However, these behaviors must also be adaptive and maintain the ability to change depending on the environmental conditions, predator and threat type, and social organization among and between species. Predation pressure may vary little over ecological time, but the risk of being preyed upon may vary greatly on a seasonal, daily, or even momentary basis (Lima & Dill, 1990). Different fishes have evolved over

evolutionary time to live in aggregations, shoals, and schools as an attempt to increase fitness and survival.

Fish are the largest and most taxonomically diverse group of vertebrates known, with twenty five percent of species forming schools or shoals during their life and fifty percent doing so during larval and juvenile stages (Radakov, 1973; Pavlov & Kasumyan, 2000; Aoki, 1980). Teleost fish species utilize schooling and shoaling behaviors to detect, confuse, and escape predators as well as locate mates, resources, and for aid in migration (Radakov, 1973; Masuda & Tsukamoto, 1998). An individual fish in a school has a lower risk of predation through a reduced probability of attack in an encounter with a predator (Pitcher & Parrish, 1993).

Fish respond to an attack with a suite of behaviors that minimizes contact with the predator and maximizes predator confusion (Litvak, 1993). Social groups tend to become more compact to reduce the amount of staggering in the presence of a predator (Lima & Dill, 1990; Magurran & Pitcher, 1987; Pitcher, 1983; Ryer & Olla, 1998; Sogard & Olla, 1997). Schools and shoals enjoy a number of anti-predator advantages not shared by solitary fish. For example, large schools detect predators at a greater distance, and as a consequence achieve a better compromise between vigilance and foraging (Magurran & Pitcher, 1987). Groups of golden shiners (*Notemigonus crysoleucas*) startled, increased speed, total distance traveled, shoal depth, and nearest neighbor distance (NND) when presented with an overhead bird (Litvak, 1993). Groups of walleye pollock (*Theragra chalcogramma*) decreased NND in the presence of a predation threat (Ryer & Olla, 1998). However, some predators may be attracted to schools and less towards individuals, thereby increasing the probability of predation, with aggregative fish having

higher mortality than solitary fish of the same species (Connell & Gillanders, 1997). As a result, groups of fish require modifications in schooling and shoaling behaviors depending on predation pressure and threat type.

In order to respond to alterations in predation pressure, fish must actively adapt avoidance strategies. Natural selection would presumably favor individuals capable of recognizing the degree of threat posed by the predator (Helfman, 1989). This would avoid expending more energy than required for a possible threat, keeping energy allocation in balance. It has been demonstrated that fish can adapt predator avoidance behaviors as predation level changes (Helfman, 1989). This phenotypical plasticity allows fish to vary inter-individual distances, allowing different degrees of group cohesiveness to balance relative advantages and disadvantages of schooling behavior (Nemtsov, 1994; Sogard & Olla, 1997).

We tested the predator response behavior of individuals and groups of fish to a simulated visual aerial predator to determine (1) if individual responses are similar to group responses and (2) if fish response behaviors vary based on whether they are alone or in a group. Our model for this study was the estuarine killifish, *Fundulus heteroclitus*. This species is essential for the success and productivity of many mid-Atlantic estuarine marsh environments. These small cyprinidontid teleosts regulate the trophic structure of marsh systems through control of invertebrate populations (crustaceans and annelids), as well as serving as prey for larger predators such as blue crab, striped bass, and shore birds. Therefore, killifish provide a good model for the investigation of predator avoidance behavior because they live in shallow estuarine habitats and are vulnerable to aerial predation threats.

3.3 Methods

Adult male and female killifish were captured from a reference site, transported to the Aquatic Pathobiology Laboratory, treated for ectoparasites, and acclimated to laboratory conditions for 4 weeks prior to experimentation. Laboratory conditions consisted of a 10:14 L:D regime with temperature of 20-22° C and 6-8 PSU. Fish (N = 72) were randomly selected and acclimated in PVC exposure arenas for 60 minutes prior to experimentation (Kane et al., 2004).

A computer-based video tracking system designed for fish behavioral recording (Kane et al., 2004) was used to record 15 seconds of baseline movement before, and 15 seconds of movement after presentation, of the visual “fly-by” stimulus. The stimulus consisted of wood bird silhouettes that "flew" over the exposure arenas via a system of monofilament lines and pulleys. In order to test for habituation to the stimulus, the predation threat was presented to individuals (N = 12) and groups (N = 12 groups of 5) of fish 4 times with 5 minutes between each presentation. Behavioral endpoints, pre- and post-predator threats, were then quantified using custom software designed for the study of fish movement (refer to Chapter 2). Data from the first presentation was analyzed for alterations in individual and group behaviors resulting from presentation of the stimulus. The sequence of 4 repeated presentations was analyzed for habituation to the stimulus. Individual fish response variables included percentage of fish exhibiting a startle response, immobility duration, percent time fish remained motionless, percentage of time moving, and velocity. Group fish response variables included immobility duration,

percent stopping, group percent movement, shoaling velocity, percent startle, shoaling and aggregation, and nearest neighbor distance and angle (NND and NNA). Individual and group data were then analyzed separately and compared for differences at $\alpha \leq 0.10$. Wilcoxon 1-tail signed rank sum tests were used to evaluate differences in behaviors and habituation pre- and post-stimulus presentation.

3.4 Results

3.4.1 Habituation to Repeated Visual Threats

Repeated “fly-by” stimuli for both the individual and groups of fish resulted in significant reductions in both percent movement and velocity. For all four repeated trials of individuals, percent movement ($p < 0.003$) and velocity ($p < 0.05$) were significantly reduced for each trial (Figures 11 and 12). Groups of fish demonstrated significantly reduced percent movement in all 4 trials ($p < 0.002$, Fig 11) and velocity and in 3 out of the 4 trials ($p < 0.01$, Fig. 12). The consistent response over repeated trials demonstrates that fish did not habituate to the predator stimulus over time. However, for each trial with groups of fish, pre- and post-velocity and percent movement for group fish were consistently higher than for individuals. These results demonstrate that a simulated aerial predation threat can achieve the same response over repeated presentations.

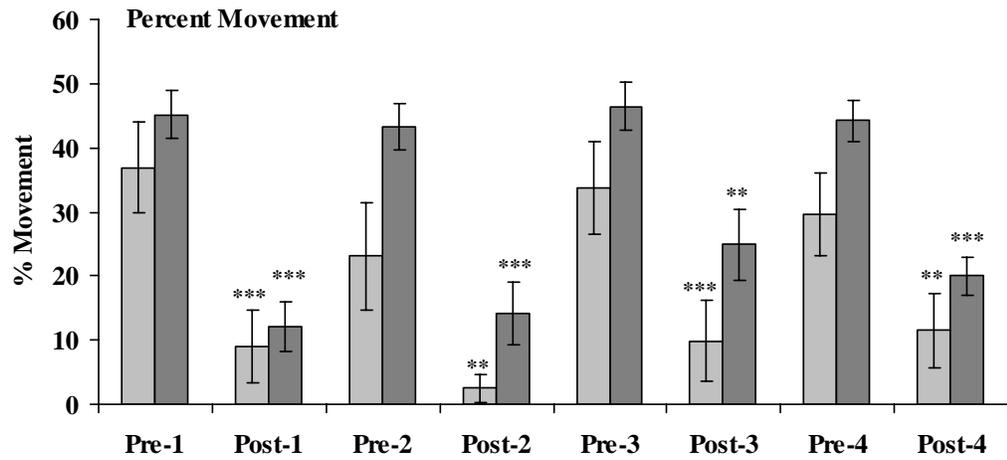


Figure 11 Alterations in the percent movement in individuals (light gray bars) and groups (dark gray bars) of mummichog before (pre) and after (post) a simulated predator stimulus. Trials were run four consecutive times separated by 5 minutes in order to test repeatability of the response. **, $p = 0.01$ and ***, $p = 0.001$.

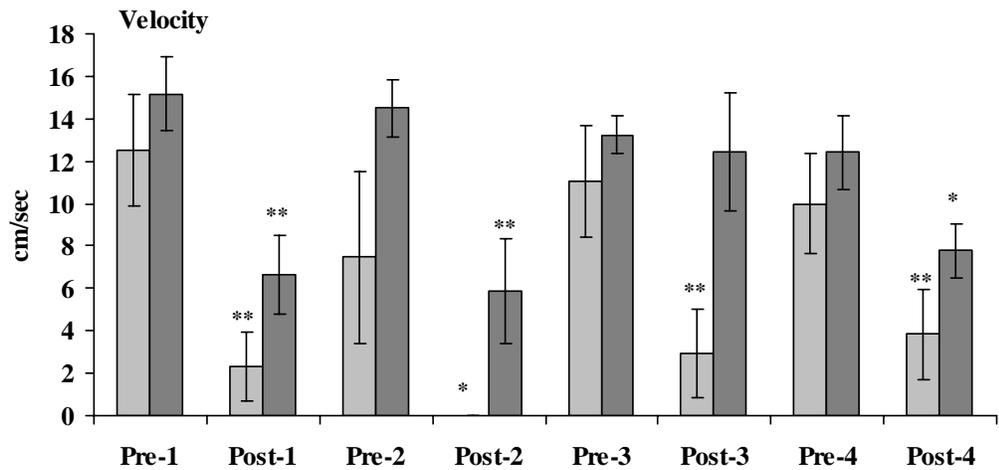


Figure 12 Alterations in the individual (light gray bars) and groups (dark gray bars) velocities of mummichog before (pre) and after (post) a simulated predator stimulus. Trials were run four consecutive times separated by 5 minutes in order to test repeatability of the response. *, $p = 0.05$, and **, $p = 0.01$.

3.4.2 Single and Groups of Fish Responses to a Simulated Visual Threat

The visual “fly-by” stimulus elicited a significant ($p \leq 0.10$) response whereby 96% of the individual fish (Fig. 13) and 95% of the groups of fish, stopped moving. This cessation of movement lasted for $12.4 (\pm 0.9)$ seconds for individual fish and $8.2 (\pm 0.3)$ seconds for groups of fish, which were significantly different from each other ($p < 0.01$). Figure 13 graphically depicts cessation of movement of 12 individual fish through time. Prior to cessation of movement, 29% of individuals and 41% of groups evoked a startle response similar to a flash expansion behavior (Pitcher & Parrish, 1993). Velocity and percent movement significantly decreased in individual fish after the visual threat was presented.

Groups of fish responded with significant decreases in shoal velocity, interactions, percentage shoaling, and shoaling NND, along with significant increases in percentage of aggregation ($p \leq 0.10$, Table 3). No significant differences in NNA between groups of fish were observed. Overall, groups of fish tended to slow down and become less active and organized, i.e., there was a shift from shoaling to aggregating, with fewer interactions. However, in cases where fish are shoaling, a significant increase in polarization was observed (shoaling NND, $p = 0.013$, Table 3). These results demonstrate in groups of killifish, that movement and shoaling behavior decreased significantly when exposed to a simulated predator.

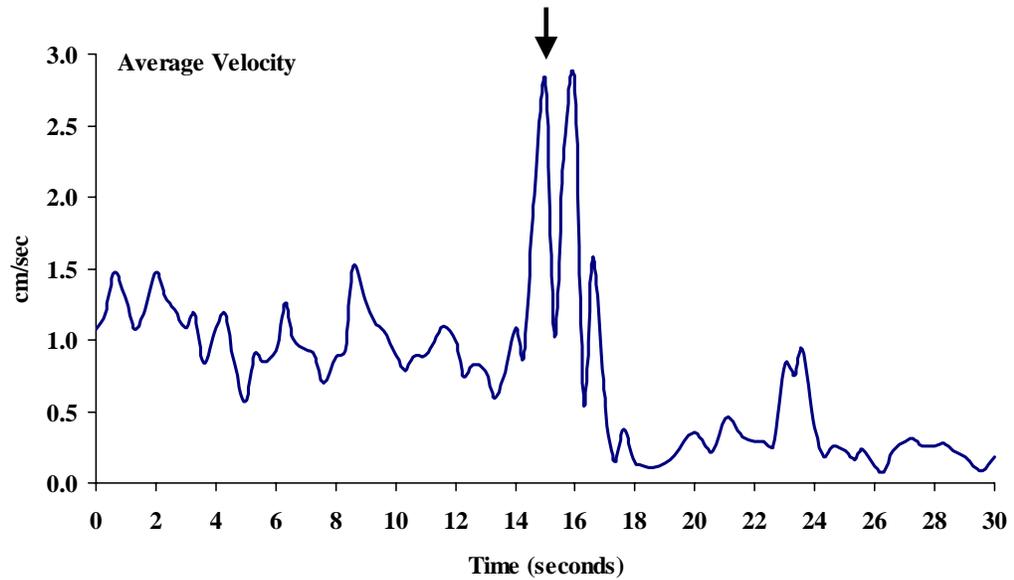


Figure 13 Average velocity of individual mummichog during the 30 second recording period (N = 12). The simulated predator “flies” over the experimental vessels at 15 seconds (arrow), note the peak in velocity (startle response) followed by a cessation of movement compared with pre-stimulus velocity.

Table 3 Alterations in group and shoaling behaviors pre- and post-predator stimulus

Behavioral Endpoint	Pre-stimulus mean (\pmS.E.)	Post-stimulus mean (\pmS.E.)	P value
Shoal Velocity, cm/sec	7.8 (\pm 0.56)	4.8 (\pm 1.8)	0.097
Interactions, #	37.9 (\pm 3.75)	23.9 (\pm 4.03)	0.010
Percent Shoaling	32.0 (\pm 4.45)	5.30 (\pm 2.59)	0.0017
Shoaling NND, cm	17.9 (\pm 1.86)	7.98 (\pm 3.08)	0.013
Percent Aggregated	50.0 (\pm 7.23)	72.3 (\pm 10.8)	0.006

3.4.3 Single vs. Group Behaviors

Individuals and groups of killifish significantly decreased movement behaviors when exposed to a simulated predator. As mentioned previously, groups of fish displayed a higher degree of overall movement, expressed in percent movement and velocity, before and after presentation of the stimulus. In addition, duration of immobility was significantly different ($p < 0.001$) between individuals (12.4 ± 0.9) and groups (8.2 ± 0.3), with groups initiating swimming behaviors before individuals.

3.5 Discussion

Punctuated predation is characteristic of natural systems where contact between predator and prey is episodic, such as fish and avian predators (Ryer & Olla, 1998). Killifish reside in shallow, tidal, estuarine habitats and are always subject to avian predators. Predator response behaviors of killifish observed in this study, associated with the visualization of an “avian predator,” was quantitatively assessed using our system hardware and software. It is unclear that experimental fish discerned the wood figures as avian predators. However, startle response in fish is a unique, innate escape response to adverse visual or acoustic stimuli and, as evidenced in the present study, is clearly associated with the presentation of a shadowless, overhead visual stimulus. All fish tested in this study, upon presentation of the simulated predation threat, notably reduced activity as a response. An animal is more visible when moving than when stationary, and

decreasing activity lowers predation rate (Magnhagen, 1988). Regardless of whether the fish is alone or in a group, reduction in activity was apparent and quantifiable.

Differences in predator evasion behaviors exist between individuals and groups of killifish, with individuals being more “cautious” than groups. Solitary killifish remained motionless upon presentation of the simulated predator, whereas groups of killifish were more variable in their response. Startle responses to aerial models in fish have been documented previously, but differences in response to aerial models between individuals and groups have not been previously investigated (Litvak, 1993). Groups of killifish responded more often with a startle response, which resulted in a rapid dispersion of fish. In addition, groups of fish displayed higher swimming velocities and spent less time motionless after threat presentation. Shoal organization and overall polarization increased in groups of pollock, *Theragra chalcogramma*, that remained in shoals after threat presentation (Sogard & Olla, 1997).

Differences in killifish predation responses, depending upon being solitary or part of a group, supports the “dilution effect” and “selfish herd” hypotheses (Cresswell, 1994; Hamilton, 1971). These changes in behavior may be adaptive, with fish actively altering their predator response based on social organization (solitary vs. group). This increased vigilance is associated with increased group size (i.e., “more eyes to see with”) and faster predator detection (Bertram, 1978; Barnard, 1983; Magurran & Pitcher, 1987).

The degree of perceived threat may differ between groups and individuals, resulting in the behavioral differences observed. In addition, response of the prey may fluctuate depending on the motivation of the predator. Animals assess their risk of being preyed upon and incorporate this information into their decision-making (Lima & Dill,

1990). Damselfish, *Stegastes planifrons*, are a good example and are sensitive to model predator presentations that reflect the magnitude of the predatory threat they apparently perceive, where avoidance responses tend to increase as the degree of threat increases (Helfman, 1989). These adaptations in fish responses to fluctuating predation pressure has led to a proposed “threat-sensitivity hypothesis” (Helfman, 1989). This hypothesis states that animals balance predator avoidance against other fitness influencing activities, and that there is a graded response that reflects the magnitude of the predatory threat. Pollock can alter group cohesion depending on environmental influence and predation threat level (Sogard & Olla, 1997). Schooling golden shiners swim in the same horizontal plane in the absence of a predator, but then stagger into a more 3D arrangement in the presence of a predator (Abrahams & Colgan, 1985). Fish populations sympatric with specific piscine predators had increasing polarization as the level of predation increased (Pitcher, 1983). Ultimately, alterations in predator response behaviors resulting from exposure to environmental stressors may have detrimental effects on individual fitness and population dynamics in killifish. This study demonstrates that killifish living in groups may provide increased vigilance to avian predators considering the increased activity when compared to solitary fish.

Results of this study prove that visually-cued “predation avoidance” is a discernable fixed action pattern (FAP) in killifish. These data have formed the basis of a “visual fly-by” endpoint in our laboratory that can provide significant and reproducible results using the killifish model. This endpoint is an integral part of ongoing studies that investigate behavioral and movement alterations that are associated with contaminant and stress exposure in fish. Behavioral data from these controlled laboratory experiments are

important in developing a better understanding of low-level exposure effects on fish, and they help to bridge laboratory studies to field exposure scenarios. In addition, the use of ecologically relevant, behavioral endpoints provide quantitative data to show possible detrimental individual- and population-level effects other than mortality.

**Chapter 4: Alterations in Schooling, Shoaling, and Startle Response
Behaviors Resulting from Exposures to Harmful Algal Bloom
Neurotoxins, Brevetoxin and Saxitoxin in Mummichog, *Fundulus
heteroclitus***

4.1 Abstract

Harmful algal blooms (HABs) in the middle Atlantic states, and bloom-associated fish morbidity and mortality reports, have increased in frequency and severity over the past several decades. The ability to predict and characterize environmental effects of different HAB species are essential to HAB remediation and control. This study investigated the effects of low level HAB neurotoxin (brevetoxin, PbTx-2; and saxitoxin, STX) exposure on swimming behavior in groups of mummichog, *Fundulus heteroclitus*. In addition, fish were exposed to a common fish anesthetic, MS-222, for validation and comparison to HAB toxins. Thirty-minute video segments before, during, and after exposures were archived using a videography system, and analyzed using a software program designed for the analysis of schooling and shoaling behaviors. In addition, predator avoidance (bird model) and startle responses (auditory/vibratory) were compared before and after toxin exposure. Mummichog exposed to HAB toxins and MS-222 had significant alterations in schooling and shoaling behaviors. Exposure to STX and MS-222 significantly decreased ($p \leq 0.10$) fish interactions, and schooling and shoaling frequencies, whereas exposure to PbTx-2 increased ($p \leq 0.10$) these behaviors.

The number of sedentary groups of fish significantly ($p \leq 0.10$) increased in association to exposure to MS-222, and the frequency of solitary behaviors significantly ($p \leq 0.10$) increased with STX and PbTx-2 exposures. Compositions of schools, shoals, and sedentary groups significantly decreased from groups of 5 to groups of 3 after exposure to all three compounds ($p \leq 0.10$). Additionally, exposure to all three toxins altered startle response and predator response behaviors. Twenty-four hours after exposure, groups of fish exposed to all 3 toxins had significant decreases in activity and frequency of behaviors compared with pre-exposure activity. These results suggest that sublethal exposure to HAB toxins can have demonstrable effects on swimming and social behaviors, as well as on startle response and predator avoidance behaviors. This is the first study to quantify alterations in social behavior of fish exposed to HAB toxins. These data demonstrate how environmentally realistic concentrations of HAB toxins affect fish behavior.

4.2 Introduction

Behavioral alterations in fish can serve as valuable endpoints to discern and evaluate effects of environmental stress. Fluctuations in school structure and density can be caused by individual differences in motivation, physiology, and abiotic and biotic factors of the environment, including pollution. These alterations, in turn, may be associated with reduced fitness and survival, resulting in adverse consequences at the population level (Bridges, 1997). Changes in behavior may also alter juvenile recruitment, thereby disrupting population, community, and demographic dynamics over

time (Bridges, 1997). Inappropriate response to environmental and physiological stimuli due to toxicant exposure can have severe implications for survival (Webber & Spieler, 1994).

Selective evolutionary processes have conserved stable behavioral patterns in concert with morphologic and physiologic adaptations. This stability provides the best opportunity for survival and reproductive success by enabling organisms to efficiently exploit resources and define suitable habitats (Little & Brewer, 2001). Basic knowledge of exposure-related behavioral alterations relevant for ecotoxicological assays remains scarce, and systems that have the ability to link toxicology data with swimming and avoidance behaviors are needed (Vogl et al., 1999). Historically, toxicology studies focus on the exposure of single fish in the laboratory, when in reality, many species tend to congregate in groups and interact with many components of their environment.

The addition of group behavior endpoints in aquatic toxicology is highly relevant to fish biology, and the use of complex fish behaviors in bioassay testing may extrapolate more accurately to nature. Recently, research has expanded towards more complex behaviors that occur in the wild such as, foraging, predator avoidance, and social hierarchies which may be more important ecologically than simple responses like total distance traveled and swimming performance (Scott & Sloman, 2004). Fish are an excellent model for studying environmental pollutant effects since many behaviors can be easily observed in a laboratory setting (Scott & Sloman, 2004). In addition, these behaviors have predictable structures, shapes, and responses to threats and environmental fluctuations.

Quantifiable behavioral changes in organisms associated with stress and toxicant exposure provide novel information that cannot be gained from traditional toxicological methods, including short-term and sublethal exposure effects (Little & Finger, 1990; Henry & Atchison, 1986; Bridges, 1997; Saglio & Trijasse, 1998). Ecological relevance of such exposures may stem from altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Zhou & Weis, 1998; Little & Finger, 1990). Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment.

Although alterations in schooling and shoaling behaviors resulting from anthropogenic sources and contaminants have been documented, effects of naturally-occurring toxins on fish behavior remain less understood. Harmful algal blooms (HABs) are naturally occurring but have increased in frequency due to increased anthropogenic influx of nutrients into aquatic habitats. HABs can have deleterious effects on economies and biological systems, reducing fish consumption, tourism, and industry (CENR, 2000; Hallegraeff, 1993). HABs can have deleterious effects on many levels within aquatic habitats. Such effects include hypoxia, altered food web dynamics, contamination of shellfish and fish with neurotoxins, and direct neurotoxicity of higher-level consumers through bioaccumulation and trophic transfer (Landsberg, 2002; Paerl et al., 1998).

Two HAB species, *Karenia brevis* and *Alexandrium fundyense*, release the neurotoxins brevetoxin (PbTx) and saxitoxin (STX), which are responsible for fish kills and human illness (Landsberg, 2002). PbTx exposure has been linked to several types of physiological and behavioral problems in both marine mammals and humans (Steidinger

et al., 1973). Symptoms associated with brevetoxin exposure have been collectively termed neurotoxic shellfish poisoning (NSP). NSP was designated a reportable disease by the Department of Health for the state of Florida in 1999 (Flemming & Baden, 2000). In humans, prolonged respiratory exposure can lead to dizziness, tunnel vision, and skin rashes. Respiratory effects can be reversed by leaving the area, however, chronic effects have been reported in the elderly and asthmatics (Flemming & Baden, 2000).

STX is produced by approximately 11 *Alexandrium* species, a genus with a global distribution and a threat to human health, wildlife, and local economies (Landsberg, 2002; Velez et al., 2001). In addition, STX is produced by other dinoflagellate genera (*Gymnodinium* and *Pyrodinium*) as well as cyanobacteria (Landsberg, 2002). STX is one of the deadliest poisons known, with poisoning in humans resulting from consumption of bivalves containing bio-accumulated STX (Chang et al., 1993; Lefebvre et al., 2004). These resultant symptoms of STX exposure have been collectively named paralytic shellfish poisoning (PSP). PSP is a recurrent problem associated with blooms of the harmful dinoflagellate and cyanobacteria. Symptoms include tingling, numbness, headache, dizziness, nausea, vomiting, and rapid pain (Bower et al., 1981; Kao, 1993). Although a great deal of knowledge regarding the effects of brevetoxin and saxitoxin on marine mammals and humans is known, the effects of these neurotoxins on fish behavior are poorly understood.

The majority of HAB research has focused on the uptake and accumulation of toxins in various organisms as vectors of human exposure. Few studies, however, have investigated the effects of HAB neurotoxins on fish behavior. Changes in fish behavior may alter the ability to evade predation, thereby increasing transfer of toxins to higher

trophic levels (Lefebvre et al., 2001). This study quantified the effects of sublethal aqueous exposure of STX and PbTx on adult groups of an environmentally relevant fish species. Such sublethal exposures can be highly relevant to the bloom type exposures encountered in the wild of this species of fish. Investigating sublethal, short-term exposure in fish may simulate the effects of ephemeral HAB events or fish traveling through a bloom. Our software system was used to quantify exposure-related changes in fish social behavior over a 24-hour period (Chapter 2). In addition, we analyzed group responses to visual and auditory stimuli, pre- and post- exposure, in order to investigate alterations in predator avoidance and startle response resulting from toxin exposure.

4.3 Methods

Mummichog, *Fundulus heteroclitus*, were collected from a reference site (in MD), treated for ectoparasites, and laboratory-acclimated for 4 weeks prior to experimentation. Fish were acclimated to a 14:10 hour light cycle, fed three times a week (Finfish Silver, 38% protein, 1.5 mm slow sinking pellets, Ziegler Bros., Gardner, PA), and fasted 24 hours prior to placing fish in exposure arenas. Temperature, pH, and salinity of the experimental arenas were maintained at the same values as holding tanks. For each of the 3 experiments, 70 fish (5 fish per vessel, mean TL = 62 mm \pm 7mm) were randomly selected from the fasted laboratory-acclimated population and acclimated for 24 hours in the experimental arenas prior to exposure.

The physical experimental design of the exposure arenas has been described previously (Kane et al., 2004). Briefly, twelve 10-liter exposure arenas were designed

and constructed with water flow electronically controlled by multi-channel, digital peristaltic pumps (Masterflex L/S, Cole-Parmer, Vernon Hills, IL). The exposure arenas contained 5 liters of water and were placed on a 14:10 photoperiod combined with a computer controlled dusk and dawn cycle. Twelve color CCD cameras with manual iris and focus control were mounted above respective arenas and were connected to dedicated VCR decks for recording. Each VCR was connected to a single multiplexer to provide real-time display for observation. VCR recording and stop functions were activated and synchronized remotely using X-10 technology.

Analog video data were digitized at 3 frames per second using a Macintosh platform (G5, 2 GHz, 4 GB DDR SDRAM). Data were digitized in real time, cut in Adobe Premiere 6.5[©], and imported into a commercial tracking program, Videoscript Professional, version 3.1[©]. A least squares estimator method was applied to the coordinate data to minimize the least square of total distance between each frame in order to estimate individual fish paths. The tracking program then converted the digital output into x,y coordinate data with the use of a custom algorithm designed for tracking the movement of multiple fish. The x,y coordinate data was then analyzed using software designed at the Aquatic Pathobiology Center to obtain the desired endpoints (Tables 1 and 4).

Table 4 Definitions of behavioral endpoints derived from the analysis software.

BEHAVIORAL ENDPOINT:	DEFINITION:
1. Movement Behaviors:	
Aggregative Swimming:	Three or more fish; group organized
Percent Shoaling	Number of frames satisfying shoaling criteria divided by the total number of frames multiplied by 100
Shoal NNA	Angle of trajectory between 2 fish in a shoal; must be greater than 45°
Shoal NND	Average distance to nearest neighbor for each fish in a shoal (minimum of 3 fish)
Shoal Velocity	Speed of shoal calculated in centimeters per second
Shoal Composition	Percentage of time shoals, were comprised of 3, 4, or 5 individuals
Percent Schooling	Number of frames satisfying schooling criteria divided by the total number of frames multiplied by 100
School NNA	Angle of trajectory between 2 neighboring fish in a shoal; must be less than or equal to 45°
School NND	Average distance to nearest neighbor for each fish in a shoal (minimum of 3 fish)
School Velocity	Speed of school calculated in centimeters per second
School Composition	Percentage of time schools were comprised of 3, 4, or 5 individuals
Solitary Swimming:	Individual swimming; no organized group
Percent Solitary	Number of frames not satisfying shoaling or schooling criteria divided by the total number of frames
Solitary Velocity	Speed of fish calculated in centimeters per second when ungrouped
2. Sedentary Behaviors:	
	When 3 or more fish were within 1 body length from each other not swimming
Percent Sedentary	Number of frames when 3 or more fish were within 1 body length from each other not swimming multiplied by 100
Solitary NND	Distance between nearest and second nearest neighbor for each individual fish not in a shoal
Sedentary Composition	Percentage of time sedentary groups were comprised of 3, 4, or 5 individuals
3. Interactions	
	The number of times two fish swim within 0.1 body lengths of each other (irrespective of satisfying movement criteria)

In addition, the ability to detect and respond to both visual (predator avoidance) and vibratory (startle response) stimuli were examined pre- and post-exposure. The predator avoidance stimulus consisted of wood bird silhouettes that "flew" over the exposure arenas with the help of a system of motor and gear driven monofilament lines (Chapter 3). Thirty seconds of video were recorded before and after the simulated birds passed over the vessels. In contrast, the vibratory stimulus consisted of six mouse traps, affixed to the bottom of the exposure table at equal distance from each vessel, (1 for every 2 vessels) that were released simultaneously to produce both auditory and vibratory stimuli. Fifteen seconds of video were recorded before and after the traps were set off. The number of groups responding to the stimulus, number of fish responding per group, and immobility duration were calculated, in addition to the endpoints included in the group behavior software program.

4.3.1 Group Endpoint Software Determination

The endpoints (Table 4) calculated by the software program used the following criteria:

The first criterion was to delineate between schools, shoals, and solitary behaviors. A minimum of 3 out of the 5 fish was required for the software to calculate schooling or shoaling behavior.

Fish body length used for the calculations was 50 pixels, equal to 71 mm. Fish movement was defined as displacement of an individual by one-half body length per second (35mm/sec), and was calculated on a frame-by-frame basis at 3 fps.

A shoaling event was calculated when movement of at least three fish was satisfied and the distance of a fish from its nearest and second nearest neighbor were 0.5 and 1.0 fish lengths, respectively, with no angular criteria.

A schooling event met the criteria of a shoal but in addition had a nearest neighbor angle (NNA) less than or equal to 45° and needed to occur for a minimum of 2 seconds (6 frames). If schooling occurred for 5 frames or less, it was considered shoaling and calculated as such.

Solitary and sedentary group data were calculated when a frame did not meet the criteria for schooling or shoaling. A sedentary group was satisfied when at least 3 of 5 fish were 1 body length apart and not swimming. Once the software recognized a school, shoal, solitary, or sedentary behavior, the number of interactions, velocity, nearest neighbor distance (NND) and angle (NNA), and the percentage of time spent in the configuration (school, shoal, sedentary group, or solitary) were calculated.

An interaction between 2 fish was defined as one fish being within one tenth of a body length or less (equal to or less than 5 pixels, 7.1 mm) in distance from its nearest neighbor, irrespective of movement.

Velocity was calculated as displacement (cm) per unit time (sec) and NND was calculated as the average distance between each individual and its nearest neighbor for each fish in the group.

NNA was calculated as the average angle between the trajectories of each fish and its nearest neighbor (Higgs & Fuiman, 1996; Masuda & Tsukamoto, 1998) (Chapter 2). NNA was constrained between $0-180^\circ$, with 0° corresponding to 2 fish swimming parallel and in the same direction and 180° if parallel and swimming in opposite directions. The

fish movement vector was the indicator of trajectory since the orientation between the snouts of the two fish was not calculated.

Percentage of time spent schooling, shoaling, solitary, and sedentary were the number of frames schooling, shoaling, solitary, and sedentary divided by the total number of frames.

For the visual and auditory stimulus response data, percentage of groups responding was the number of groups per treatment in which at least one fish responded to the stimulus. Percentage of fish responding was the percentage of fish per treatment responding to the stimulus. Immobility duration was the amount of time in seconds the fish remained motionless after the stimuli.

4.3.2 Exposures

Three groups of 70 fish were randomly selected from the laboratory-acclimated population, fasted 24 hours, and placed in the arenas 24 hours prior to the exposure (5 fish, minimum 2 of each sex, per arena,). Flow in exposure arenas was maintained at 10 ml/min, 2.8 exchanges/day, in order to ensure adequate water quality over the experimental period. In three separate experiments, groups of fish were exposed to two concentrations of either brevetoxin (PbTx-2, low = 40 ppb, high = 55 ppb), saxitoxin (STX, low = 100 ppb, high = 150 ppb), or tricanemethanosulfonate (MS-222, low = 40 ppm, high = 60 ppm). Exposures were aqueous and preliminary LC50 studies were conducted to determine final exposure nominal concentrations (Gipson, 2001) (data not shown). A solvent carrier, Emulphor (EL-620, 0.001%) was added to the diluent water in

the PbTx exposure to increase solubility of PbTx. Water samples were collected at 45 min, 2 hr, and 24 hrs in order to analytically determine the amount of STX and PbTx in solution. Exposure concentrations of PbTx will be determined using a radioimmunosorbant assay (RIA) by Dr. Mark Poli (U.S. Army Medical Research Institute for Infectious Disease) (Poli et al., 1995; Gipson, 2001). Exposure concentrations of STX will be determined using a competitive receptor binding assay (Dr. Sherwood Hall, FDA) (Powell & Doucette, 1999). For the purposes of providing results and discussion in this dissertation, nominal concentrations will be presented. Compounds were mixed into solution and pumped into the arenas at 15 ml/min for 5 minutes. Dye experiments previously conducted in the arenas determined homogenous mixing occurred in 15 minutes (Gipson, 2001).

The cameras and VCRs automatically recorded a total of four, 35-minute data segments; one baseline clip, two exposure clips and a recovery 24 hours post-exposure clip. The 35-minute baseline period, including 5 minutes of initial diluent flow, was recorded after the 24 hour acclimation period and before the exposure began. Two 35-minute exposure periods were recorded. The first exposure period had initial 5 minutes of toxin introduction and the second exposure period, occurring 90 minutes later, had 5 minutes of diluent flow. Once the toxin was introduced into the vessels, flow was shut off to achieve a static exposure. After each 35 minute recording period, visual and vibratory stimulus tests were conducted on the groups of fish. After the second exposure period, 2 hours after the toxin introduction, flow was initiated and remained on throughout recovery.

Once the data had been recorded, the 35 minute video clips were digitized at 3 fps, 6300 frames/clip. For analysis, the first 5 minute period that contained either diluent or toxicant flow was deleted, resulting in a 30 minute clip (5400 frames). Four 30-minute clips (baseline, 2 toxicant exposures, and recovery) were analyzed for each vessel, 48 per experiment and 144 total (777,600 total frames analyzed). In addition, the visual (1-minute clip) and vibratory (30 second clip) tests were recorded and digitized at 3 fps. Each clip was then cut in half (pre- and post-stimulus) and each half was analyzed. Visual and vibratory/auditory clips were additionally scored for the percentage of groups responding, the number of fish per group responding, and the amount of time groups remained stationary after stimulus presentation. This yielded 192 visual and vibratory clips (96 of each) per experiment, 576 clips total. Water quality data were collected each day from 2 identical surrogate vessels dedicated for water quality determination and not used for behavioral observation (Table 5). Differences in behavioral endpoints between the four 30-minute periods, the pre- and post- visual and auditory/vibratory stimuli responses, and the scored behaviors within the last day, were then analyzed for statistical differences.

Table 5 Water quality parameter values (N = 2) recorded during the exposures.

Water Quality Parameter	MS-222, 4 hr	STX, 4 hr	PbTx-2, 4 hr
pH	6.78	6.86	7.91
Salinity, PSU	5.0	5.0	5.0
Temperature, °C	22.1	22.0	24.5
Unionized Ammonia, mg/L	0.0006	0.0006	0.0301

4.3.3 Statistical Analysis

An experimental unit consisted of the group of 5 fish in each arena. A completely randomized statistical design (CRD) was used with behavior as the response (Table 4.1) and exposure period (Baseline, Exposure 1, Exposure 2, and Recovery) as the categorical variable. Non-normal data were transformed to meet the assumptions of the ANOVA procedure. Data that could not be transformed to meet the assumptions were ranked prior to analysis. A repeated measures 1-way ANOVA (PROC MIXED, repeated, SAS, vs. 8.1, Cary, N.C.) was used to compare behavioral endpoints over time for both experiments. In addition, several covariance structures were investigated in an effort to discern a best fit structure for each set of data. The following covariance structures were used: compound symmetry, auto regressive, ante-dependence, heterogeneous compound symmetry, and heterogeneous auto-regressive (SAS, vs. 8.1, Cary, N.C.). A Tukey-Kramer *post-hoc* mean comparison test was used to evaluate differences ($\alpha \leq 0.10$) between time periods in the event of a significant F statistic.

The visual and vibratory/auditory stimulus data were analyzed using two different procedures. Data compiled by the software system, which compared behavioral endpoints pre- and post-stimulus presentation, were analyzed using a 2-tailed Wilcoxon signed rank test. Percentage of groups responding to the stimulus, percentage of fish responding per vessel, and immobility duration were ranked and analyzed using a repeated measures 1-way ANOVA (PROC MIXED, repeated, SAS, vs. 8.1, Cary, N.C.). The following covariance structures were used: compound symmetry, auto regressive,

and heterogeneous auto-regressive (SAS, vs. 8.1, Cary, N.C.). A Tukey-Kramer *post-hoc* mean comparison test was used to evaluate differences ($\alpha \leq 0.10$) between time periods in the event of a significant F statistic.

4.4 Results

Data from these experiments are presented textually according to the different behavioral endpoints followed by the different stress agents. This format provides easier conceptualization and fosters the comparison of results between stress agents. Graphical presentation of the data, located at the end of the results section, are also organized by behavioral endpoints with secondary organization by stress agent such that comparisons of behavioral endpoints across the different stressors can be made.

4.4.1 Aggregative Swimming Behaviors

No fish died in the control, MS-222, or STX exposed treatments. However, 17 % of fish died in the 55 ppb PbTx-2, and 10 % in the 40 ppb PbTx-2, 24 hours post-exposure. These fish most likely succumbed from the PbTx-2 exposure combined with 48 hours of fasting and vessel acclimation. Behavioral data collected from these fish prior to death were included in the analyses. Exposure to all three toxins resulted in significant alterations in several aggregative swimming behaviors as measured by the analysis software.

4.4.1.1 MS-222 Aggregative Swimming Behaviors

Aggregations of fish exposed to both MS-222 concentrations spent significantly less time schooling when exposed ($p = 0.011$ and 0.013 , Fig. 14 a), with no recovery 24 hours later ($p = 0.0809$ and 0.0169 , Fig. 14 a). Similarly to schooling, fish spent significantly less time shoaling when exposed to both 40 and 60 ppm MS-222 concentrations ($p = 0.0069$ and 0.0037 , respectively, Fig. 15 a), and did not recover 24 hours later ($p = 0.0897$ and 0.0027 , Fig. 15 a). Shoaling NNA in fish exposed to 60 ppm, but not 40 ppm, MS-222 had relatively minor but significant decreases after 90 minutes ($p = 0.109$, Fig. 16 a) with full recovery after 24 hrs. In addition, when fish were shoaling, NND significantly decreased from exposure to both MS-222 concentrations ($p = 0.0384$ and 0.0306 , Fig. 17 a) with recovery distances significantly greater than baseline in the 60 ppb ($p = 0.0125$, Fig. 17 a). Alterations in shoaling velocity occurred from MS-222 exposure, with significant increases 90 minutes after exposure ($p = 0.0496$ and 0.0001 , Fig. 18 a), and significant decreases 24 hours later in the 40 ppb ($p = 0.0539$, Fig. 18 a). Exposure to MS-222 had no effect on the percentage of time fish were not in a school or a shoal (Fig. 21 a). However, velocity of solitary fish significantly decreased after 90 minutes of exposure to both concentrations of MS-222, with subsequent recovery (40 and 60 ppm, $p = 0.0113$ and 0.0023 , Fig. 22 a).

Mummichog exposed to concentrations of MS-222 displayed alterations in group dynamics compared to baseline. Schools of fish exposed to 60 ppm MS-222 altered compositions by significantly decreasing time spent in schools of 4 fish ($p = 0.0174$, Figs

23 b). Shoal composition of mummichogs exposed to MS-222 significantly increased with regard to time spent in 3 fish shoals (40 ppm, $p = 0.0201$, Fig. 24 a), while significantly decreasing time spent in 5 fish shoals (40 and 60 ppm, $p = 0.0318$ and 0.0953 , Figs 24 a and b).

4.4.1.2 STX Aggregative Swimming Behaviors

Fish exposed to STX spent significantly less time schooling after 90 minutes of exposure to 100 ppb STX ($p = 0.095$, Fig. 14 b) with further significant decreases 24 hours later ($p = 0.0001$, Fig. 14 b). Significantly decreased shoaling also occurred in groups of fish exposed to 100 ppb STX after 90 minutes of exposure ($p = 0.0756$, Fig. 15 b) with further significant decreases in both concentrations 24 hours later ($p = 0.0018$ and 0.0122 , Fig. 15 b). In addition to decreased shoaling frequency, shoaling NNA and NND significantly increased after 90 minutes of exposure to STX (NNA, 100 ppb, $p = 0.0233$, and NND, 150 ppb $p = 0.0553$, Figs 16 b and 17 b), and significantly increased 24 hours later in both concentrations (NNA 100 ppb, $p = 0.0001$ and NND, $p = 0.0048$ 0.1017 , Figs 16 b and 17 b). No significant alterations in shoaling velocity occurred in groups of fish exposed to STX (Fig. 18 b).

Groups of fish exposed to STX increased solitary behaviors with exposure at both concentrations, with a significant increase in the 150 ppb group ($p = 0.0157$, Fig. 21 b). However, fish exposed to 100ppb STX significantly decreased velocity when not schooling or shoaling 24 hours post exposure ($p = 0.0003$, Fig. 22 b). Exposures of groups to STX did not alter schooling composition. However, in shoals of fish exposed

to both concentrations of STX, significant alterations in compositions were seen, with increases in 3 fish shoals ($p = 0.0888$ and 0.0552 , Fig. 25 a and b) and significant decreases in 4 (150 ppb, $p = 0.0764$, Fig. 25 b) and 5 fish shoals (100 and 150 ppb, $p = 0.0170$ and 0.0642 , Figs 25 a and b), with no recovery for either concentration ($p < 0.01$, Fig. 25 a and b).

4.4.1.3 PbTx-2 Aggregative Swimming Behaviors

Exposure to 40 ppb PbTx-2 after 30 minutes, in contrast to MS-222 and STX, significantly increased schooling with a similar non-significant trend in the 55 ppb ($p = 0.0805$, Fig. 14 c). Further, non-significant depression in schooling occurred following 90 minutes and during recovery (Fig. 14 c). Alterations in schooling from PbTx-2 exposure were less notable than the MS-222 or STX exposures. No alterations in schooling NND or NNA was observed in any exposures. Groups of fish exposed to both concentrations of PbTx-2 displayed non-significant increases in shoaling during exposure, with significant decreases in shoaling 24 hours later in the 55 ppb exposure ($p = 0.0475$, Fig. 15 c). Shoaling NND in PbTx-2 exposed groups significantly decreased upon exposure in the 55ppb ($p = 0.0151$, Fig. 17 c), followed by a significant increase 24 hours later ($p = 0.0034$, Fig. 17 c). No significant alterations in shoaling NNA or shoaling velocity occurred in groups of fish exposed to PbTx-2 (Figs 16 c and 18 c). Additionally, exposure to both concentrations of PbTx-2 significantly increased the time fish were not a part of a shoal or a school ($p = 0.0020$ and 0.0421 , Fig. 21 c) and fish did

not recover schooling or shoaling behaviors after 24 hours ($p = 0.0001$ and 0.0227 , Fig. 21 c).

Similarly, solitary velocity significantly decreased 24 hours after exposure in groups of fish exposed to 55ppb PbTx-2 ($p = 0.0233$, Fig. 22 c). Exposures of groups to PbTx-2 did not alter schooling composition. However, shoaling composition was significantly altered by PbTx-2 exposures, with significant decreases in time spent in 3 fish shoals ($p = 0.0432$ and 0.0453 , Figs 26 a and b) at 30 minutes and significant increases in time spent in 5 fish shoals ($p = 0.0023$ and 0.0100 , Fig. 26 a and b) in both concentrations during exposure. Further significant alterations occurred after 24 hours in the 40 ppb PbTx-2 exposure, with shoal compositions significantly increasing time spent in 3 ($p = 0.0688$, Fig. 26 a) and significantly decreasing time spent in 5 fish shoals ($p = 0.0110$, Fig. 26 a).

4.4.2 Interactions

4.4.2.1 MS-222 Interactions

Interactions between fish decreased from the baseline recording and the MS-222 exposure periods for both concentrations, with significant decreases between the baseline and recovery in the 40 ppm ($p = 0.0495$, Fig. 27 a). The frequency of interactions between fish remained significantly lower during recovery than baseline values in MS-222 exposed fish.

4.4.2.2 STX Interactions

Similar to MS-222, interactions between fish exposed to STX decreased from baseline for both concentrations, with a significant decrease in the 150ppb ($p = 0.0694$, Fig. 27 b) and a significant decrease between the baseline and recovery for both concentrations ($p = 0.001$ and 0.036 , Fig. 27 b). Frequency of interactions between STX exposed fish remained significantly lower during recovery than baseline values.

4.4.2.3 PbTx-2 Interactions

In contrast to the MS-222 and STX, exposure to both 40 and 55 ppb PbTx-2 resulted in the opposite trend, with significantly increased interactions ($p = 0.0805$ and 0.1050 , Fig. 27 c), followed by significantly decreased interactions 90 minutes post-exposure and 24 hours later ($p < 0.09$, Fig. 27 c).

4.4.3 Sedentary Behaviors

4.4.3.1 MS-222 Sedentary Behaviors

Exposure to 40 ppm MS-222 resulted in groups of fish significantly increasing the amount of time spent sedentary ($p = 0.0547$ and $p = 0.0053$, Fig. 19 a), with increased sedentary NND during exposure ($p = 0.0663$, Fig. 20 a). These increases in amount of time sedentary and sedentary NND remained significantly increased 24 hours later ($p =$

0.0849 and 0.0106, Figs 19 a and 20 a). Sedentary group compositions of fish exposed to MS-222 were significantly altered, with the occurrence of groups of 5 fish significantly decreasing (40 and 60 ppm, $p = 0.0018$ and 0.0062 , Fig. 28 a and b) and the occurrence of groups of 3 and 4 significantly increasing (60 ppm, $p = 0.0050$ and 0.00069 , Fig. 28 b). Sedentary groups of fish returned to baseline values 24 hours after exposure to 55 ppb MS-222, but did not recover after exposure to 40 ppb MS-222 (Fig. 28 a and b).

4.4.3.2 STX Sedentary Behaviors

In contrast to MS-222, time fish spent sedentary significantly decreased with exposure to both concentrations of STX ($p = 0.0081$ and 0.0243 , Fig. 19 b) after 30 minutes, but fish were able to recover (Fig. 19 b). In addition, NND between sedentary fish significantly increased during STX exposure to both concentrations ($p = 0.0737$ and $p = 0.0290$, Fig. 20 b) without recovery 24 hours later ($p = 0.0007$ and 0.0006 , Fig. 20 b). Sedentary group compositions of fish exposed to STX were significantly altered, with the groups of 5 fish decreasing (100 and 150 ppb, $p = 0.0392$ and 0.0171 , Fig. 29 a and b) and groups of 3 increasing (150 ppb, $p = 0.0642$, Fig. 29 b) without recovery (100 and 150 ppb $p = 0.0392$ and 0.0655 , Fig. 29 a and b).

4.4.3.3 PbTx-2 Sedentary Behaviors

Fish exposed to PbTx-2 displayed no changes in sedentary grouping during exposure (Fig. 19 c), with significant increases in sedentary behavior occurring 24 hours

later in the 55ppb ($p = 0.0283$, Fig. 19 c). However, significant decreases in sedentary NND occurred during exposure to 40 ppb PbTx-2 after 30 minutes ($p = 0.0365$, Fig. 20 c), followed by additional significant increases 24 hours later in both concentrations ($p = 0.0005$ and 0.0847 , Fig. 20 c). Similar alterations were observed in sedentary group compositions of fish exposed to PbTx-2, with significant decreases in groups of 5 fish after 90 minutes (40 and 55 ppb, $p = 0.0728$ and 0.0685 , Fig. 30 a and b). Sedentary groups exposed to 40 ppb PbTx-2 did not recover 24 hours after exposure, with significant increases in groups of 3 ($p = 0.0555$, Fig. 30 a) and 4 ($p = 0.0099$, Fig. 30 a) fish and a significant decrease in 5 fish groups ($p = 0.0144$, Fig. 30 a).

4.4.4 Visual Stimuli

When the simulated predator “flew” over the vessels during the baseline time period, fish responded with a startle response followed by a period of non-motion. For schooling and shoaling fish, the observed response was similar to a flash expansion (Pavlov & Kasumyan, 2000) followed by remaining motionless. Exposure to all three toxins resulted in significant alterations in the response of groups of fish to the simulated predator presentation.

4.4.4.1 MS-222 Visual Stimulus

The response observed and quantified during baseline stimulus presentation did not occur when fish were exposed to 40 and 60 ppm MS-222, with no group responding

to the simulated predator ($p < 0.001$, Fig. 31 a). However, 24 hours after exposure, the response observed during the baseline period returned ($p < 0.001$, Fig. 31 a). In addition to a decrease in the percent of groups exhibiting the response, the percent of fish in each group responding significantly decreased from 100 percent to 0 percent during exposure which then returned to baseline values 24 hours later (40 and 60 ppm, $p = 0.0001$, Fig. 31 b). There were no differences in the amount of time fish remained motionless after the stimulus due to the fact that the MS-222 exposed fish did not respond (Fig. 31 c).

When investigating changes in group software parameters before and after the visual stimulus, MS-222 exposure introduces variation in behaviors with effects seen mainly in the 60 ppm exposed fish. Interactions between fish increase after exposure, with a significant increase in interactions 24 hours after exposure in the 60 ppm ($p = 0.1060$, Fig. 37). Time spent shoaling significantly decreases after 90 minutes of exposure to both concentrations of MS-222, with recovery 24 hours later ($p = 0.0106$ and 0.0306 , Fig. 38). Significant increases in shoaling velocity post visual stimulus presentation is negated by the MS-222 exposure (40 and 60 ppm, $p = 0.0106$ and 0.0306 , Fig. 39 a). No alterations in time fish spent ungrouped in response to visual stimuli occurred with exposure to MS-222. Additionally, velocity of fish when not grouped increased during the baseline period at 40 ppm, which is abolished by exposure MS-222 and marginally recovers thereafter (Fig. 41 a). Significant increases in sedentary groups after visual stimulus presentation occurred during recovery from exposure to both 40 and 60 ppm MS-222 ($p = 0.0560$ and 0.0360 , Fig. 42 a).

4.4.4.2 STX Visual Stimulus

Exposure to STX did not affect the ability of fish to respond to the presentation of the simulated predator (Fig. 32). No alterations in the frequency of interactions in STX exposed groups were observed. Additionally, no deviations in shoaling behaviors were observed from exposure to STX. However, shoaling velocities in fish after 30 minutes of exposure to STX displayed significant increases when compared to baseline at 100 ppb, an opposite response to MS-222 exposure, which may describe an increase in activity due to exposure ($p = 0.105$, Fig. 39 b). In contrast, 24 hours later, a significant reversal in shoaling velocity was seen in the 100 ppb STX exposure ($p = 0.0306$, Fig. 39 b). No alterations in time fish spent ungrouped in response to visual stimuli occurred with exposure to STX. Significant decreases in ungrouped velocity resulting from visual stimuli were abolished by exposure to 150 ppb STX (Fig. 41 b). Exposure to STX significantly reduced the percentage of sedentary groups that were observed during baseline at 100 ppb ($p = 0.0360$, Fig. 42 b).

4.4.4.3 PbTx-2 Visual Stimulus

Exposure to PbTx-2 did not affect the ability of fish to respond to the presentation of the simulated predator (Fig. 33). No alterations in the frequency of interactions in PbTx-2 exposed groups were observed. No deviations in shoaling behaviors were observed from exposure to PbTx-2, however, increases in general activity were noted in the PbTx-2 exposures. Shoaling velocities in fish exposed to PbTx-2 displayed

significant increases when compared to baseline at 40 ppb, an opposite response to MS-222 exposure, which may describe an increase in activity due to exposure ($p = 0.0360$ and 0.0592 , Fig. 40 a). No significant changes in sedentary groups resulting from PbTx-2 exposure pre and post visual stimulus were found. Exposure to 55 ppb PbTx-2, however, significantly altered this response, with a significant decreases in solitary behavior in baseline stopping after exposure ($p = 0.0360$, Fig. 40 b).

4.4.5 Auditory/Vibratory Stimuli

The auditory/vibratory stimulus created a response in the groups of fish similar to that of the visual stimulus. However, differences in auditory/vibratory responses resulting from all toxin exposures were small when compared with visual stimuli, mainly due to small sample sizes and high variability in the observed behaviors. In contrast to the visual stimulus, exposure to MS-222, STX, or PbTx-2 did not significantly alter the number of groups of fish responding with a startle response. However, using the group software program to analyze group responses to auditory/vibratory stimuli after exposure to toxins resulted in several significant alterations.

4.4.5.1 MS-222 Auditory Stimulus

Exposure to MS-222 significantly reduced the percentage of fish per group that startled in response to the auditory/vibratory stimulus (40 and 60 ppm, $p = 0.0125$ and 0.0001 , Fig. 34 a). However, 24 hours later, the startle response returns to baseline levels

(Fig. 34 a). The amount of time fish spent motionless after the stimulus was additionally altered with exposures to MS-222 (60 ppm, $p = 0.0518$, Fig. 34 b). Groups of fish significantly decreased frequency of interactions in the 60ppb MS-222 exposure, which deviated from the baseline response ($p = 0.0360$, Fig. 43 a). Time spent shoaling significantly increased in MS-222 exposed fish responding to auditory/vibratory stimuli (40 ppm, $p = 0.0591$, Fig. 43 b), along with a significant decrease in time shoaling during recovery (60 ppm, $p = 0.0591$, Fig. 43 b). No alterations from MS-222 exposure were observed in shoaling velocity and no group behaviors.

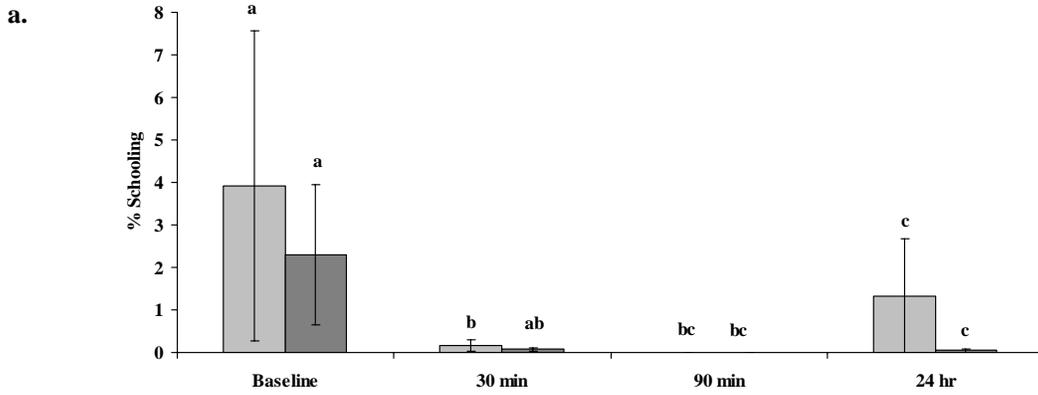
4.4.5.2 STX Auditory Stimulus

The percentage of fish per group that startled in response to the auditory/vibratory stimulus was significantly increased by STX exposure 24 hours later (150 ppb, $p = 0.0332$, Fig. 35 a). The amount of time fish spent motionless after the stimulus was altered with exposures to STX 24 hours later (100 ppb, $p = 0.0819$, Fig. 35 b). No significant alterations in interactions were observed in groups exposed to STX. Exposure to STX negates the decrease in shoaling time in groups of fish responding to the auditory/vibratory stimuli at 150 ppb (Fig. 44 a). No alterations from STX exposure were observed in shoaling velocity or group behaviors. Groups of fish after exposure to STX for 30 minutes displayed a significant decrease in sedentary groups when responding to the stimulus when compared with baseline (100 ppb, $p = 0.0592$, Fig. 45 a).

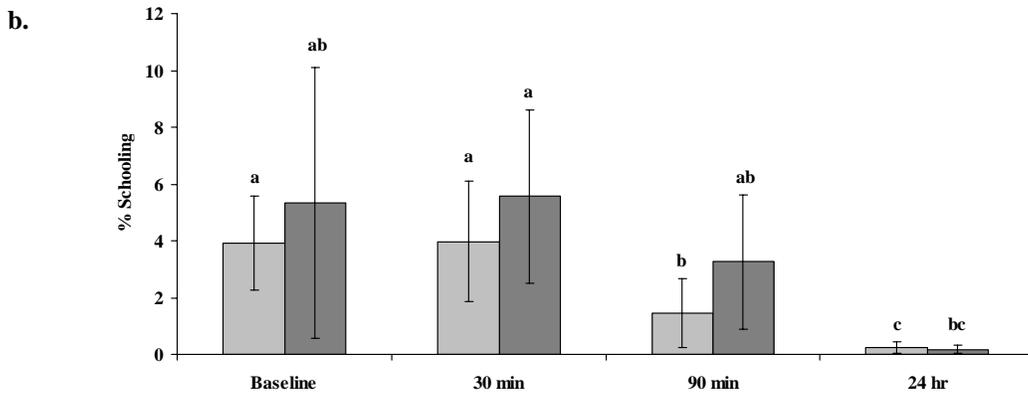
4.4.5.3 PbTx-2 Auditory Stimulus

The percentage of fish per group that startled in response to the auditory/vibratory stimulus was not altered by PbTx-2 exposure (Fig. 36 a). However, the amount of time fish spent motionless after the auditory stimulus was additionally altered with exposures to PbTx-2 (40 and 55 ppb, $p=0.0522$, Fig. 36 b). No significant alterations in interactions were observed in groups exposed to PbTx-2. Additionally, no significant alterations in shoaling percentage of groups responding to the auditory/vibratory stimuli were found (Fig. 44 b). No alterations from PbTx-2 exposure were observed in shoaling velocity or group behaviors. However, the significant increase in sedentary groups resulting from the stimulus is significantly reduced from PbTx-2 exposure (55 ppb, $p=0.0592$, Fig. 45 b).

MS-222 Percent Schooling



STX Percent Schooling



PbTx-2 Percent Schooling

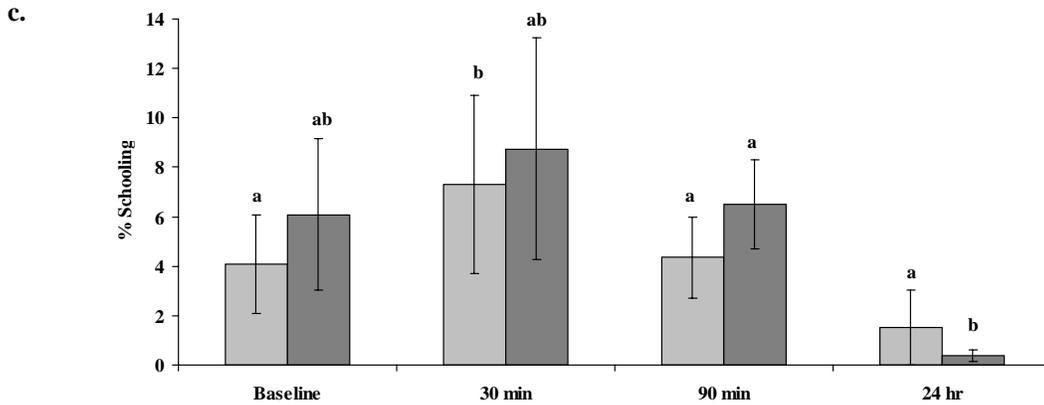
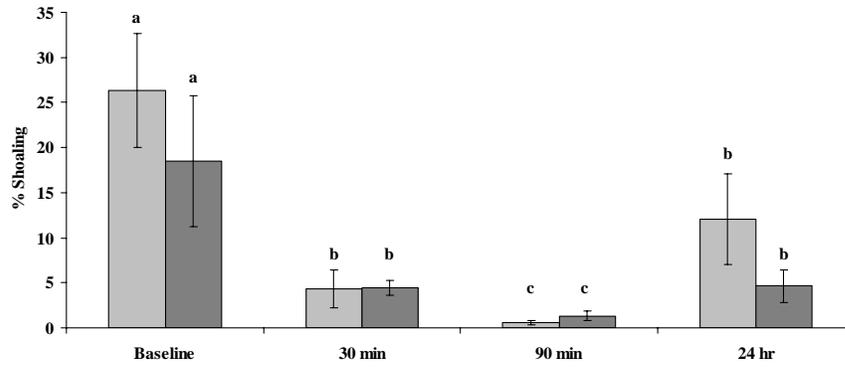


Figure 14 Percentage of time schooling between groups of mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in schooling through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

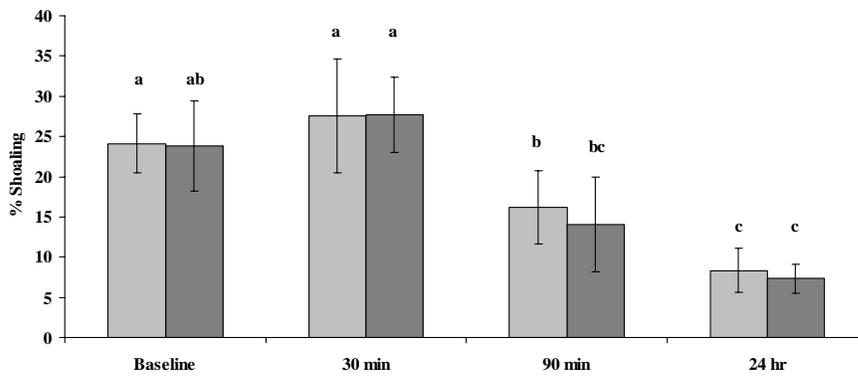
MS-222 Percent Shoaling

a.



STX Percent Shoaling

b.



PbTx-2 Percent Shoaling

c.

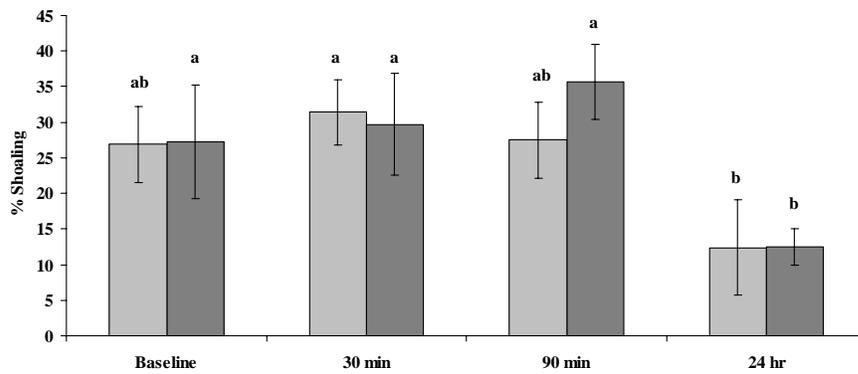


Figure 15 Percentage of time shoaling between groups of mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in shoaling through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

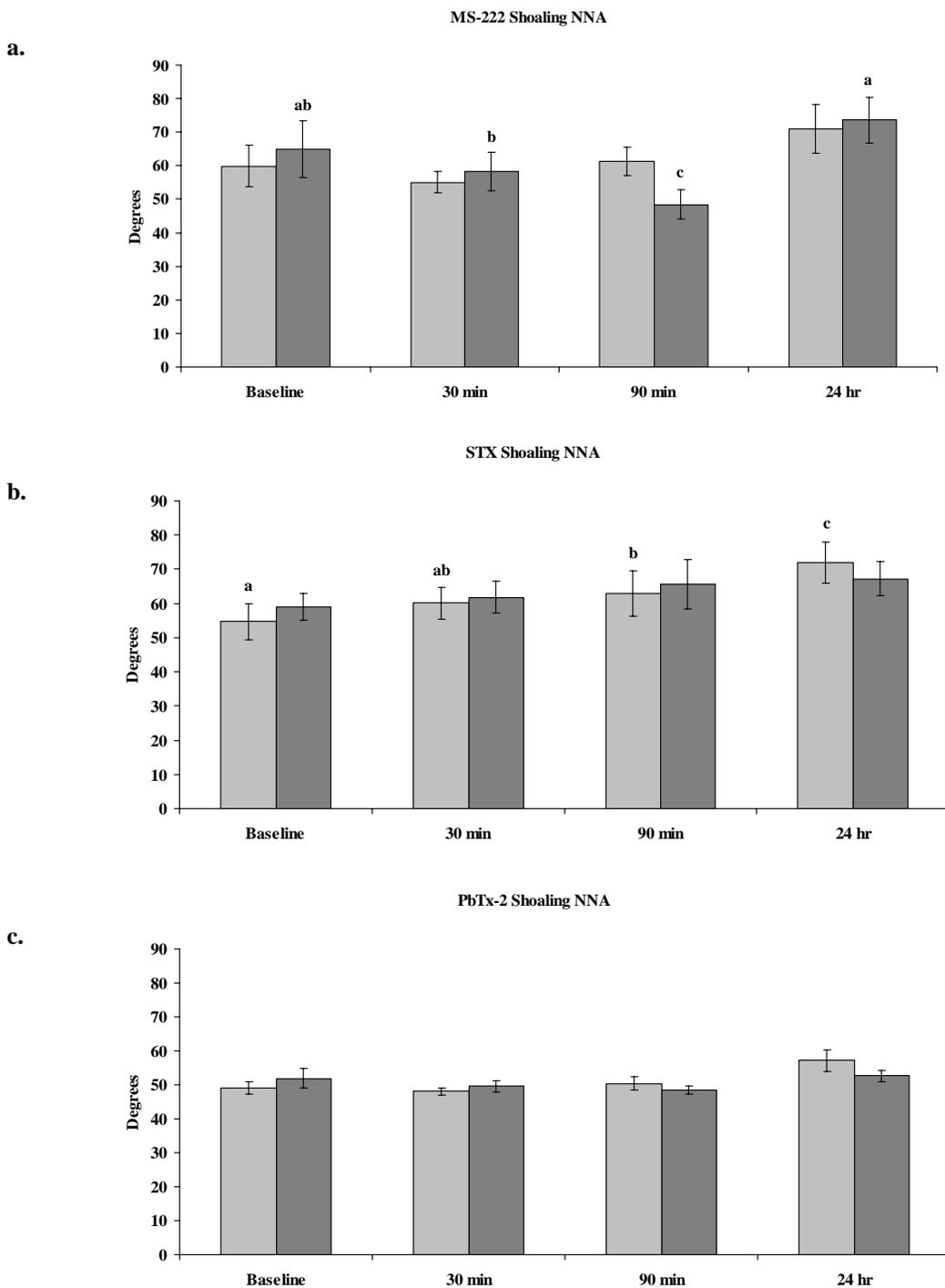


Figure 16 Nearest neighbor angle (NNA) between shoaling mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in shoaling NNA through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

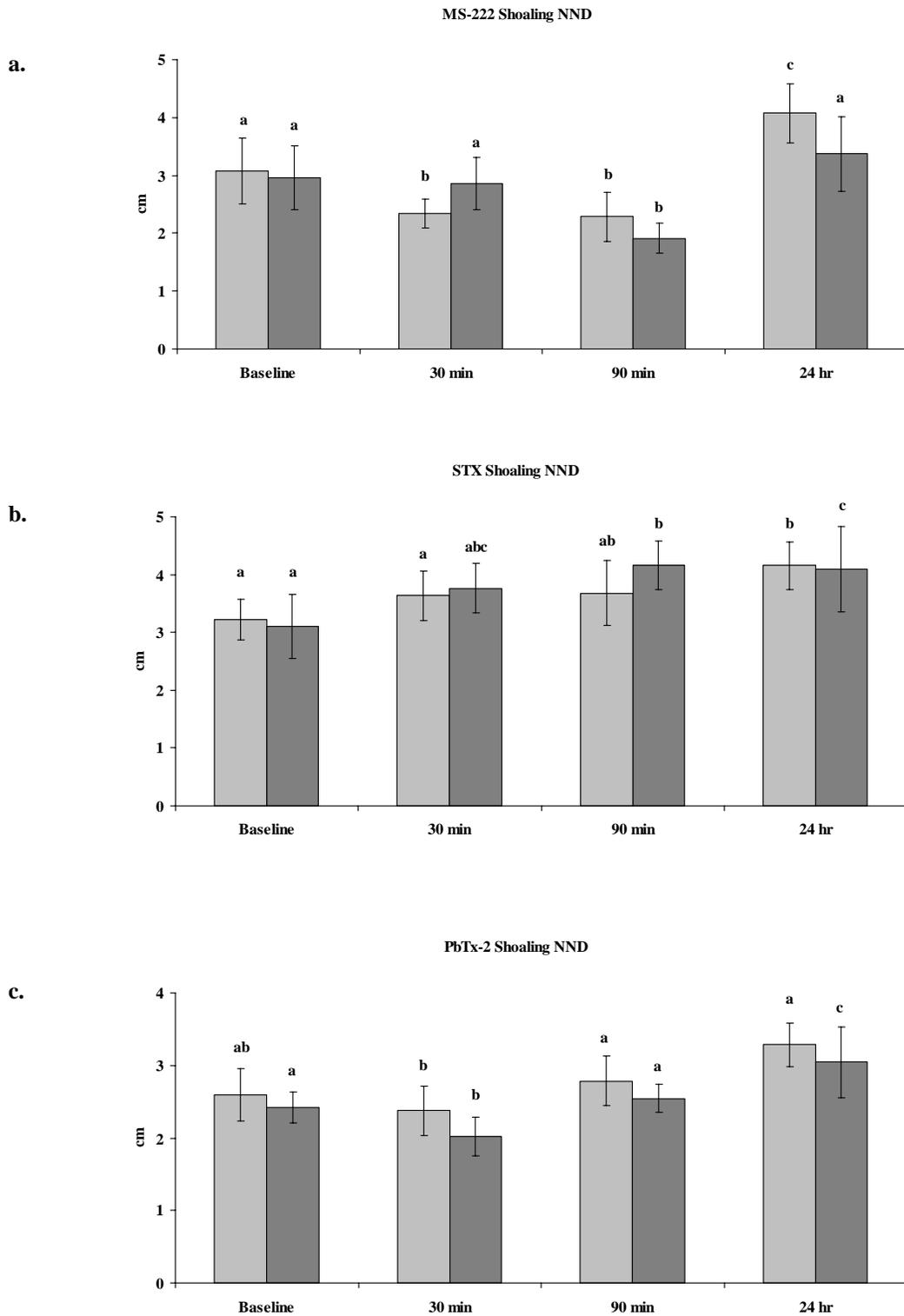


Figure 17 Nearest neighbor distance (NND) between shoaling mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in shoaling NND through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

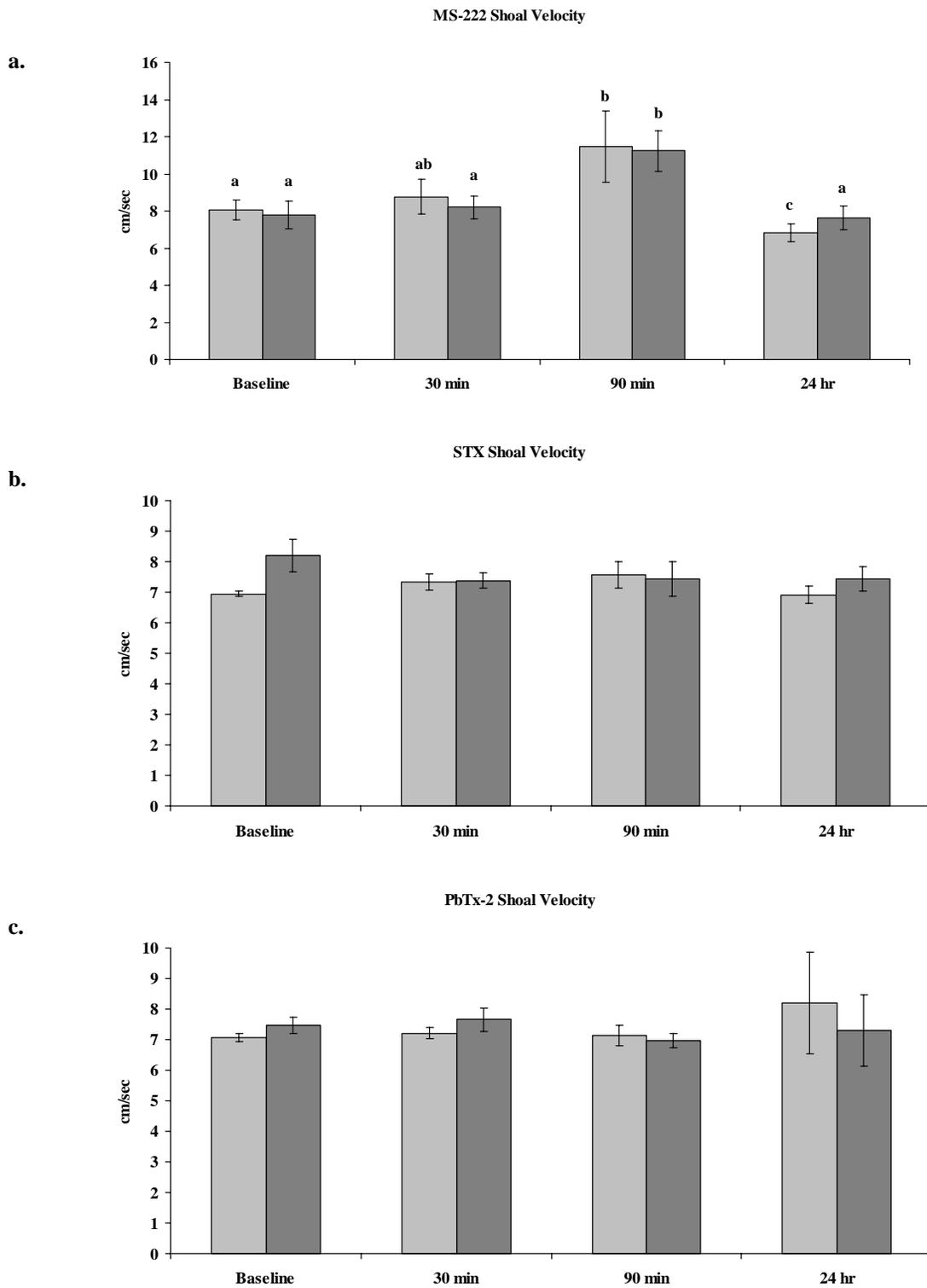


Figure 18 Shoaling velocity between groups of mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in shoaling velocity through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

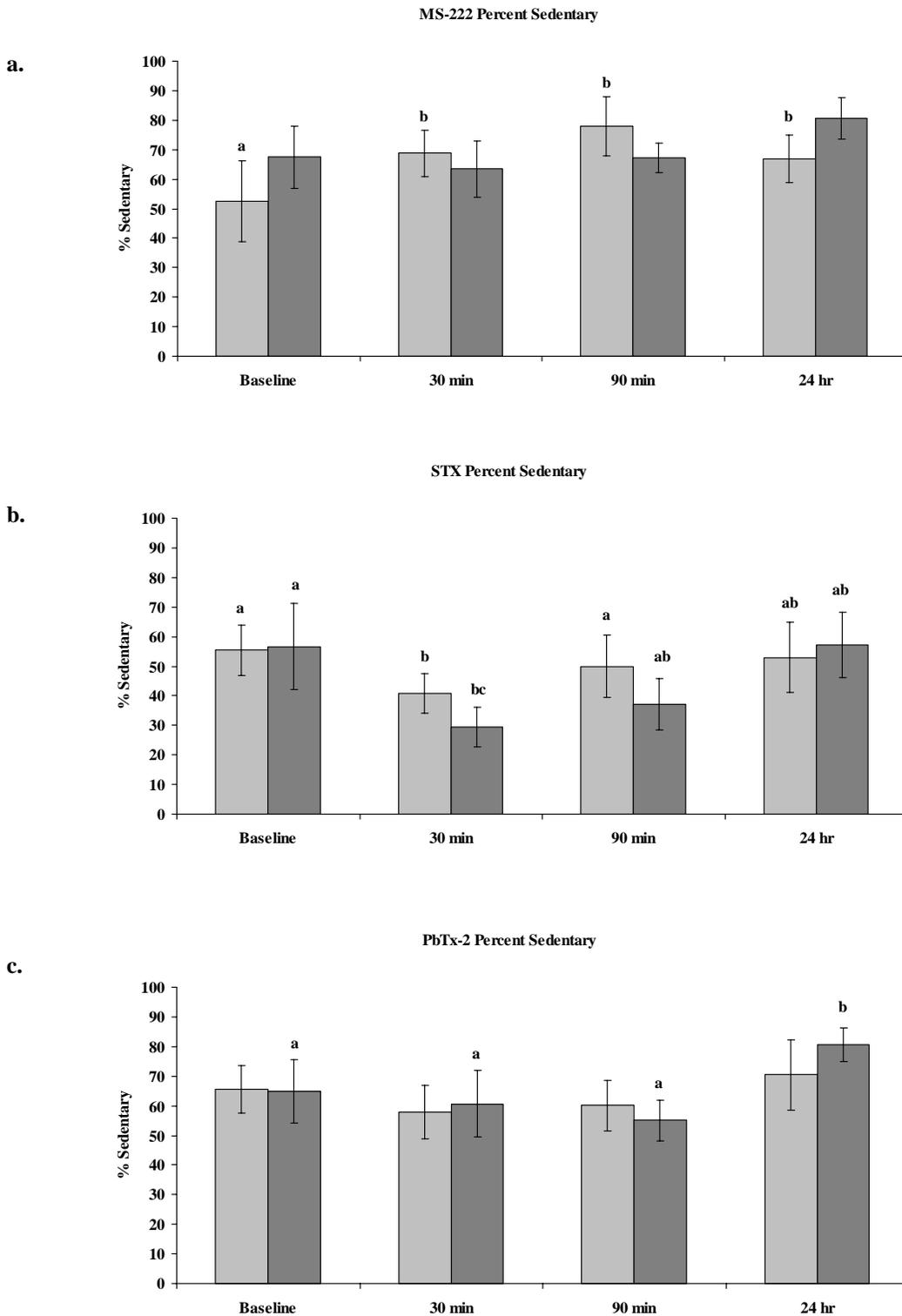


Figure 19 Percentage of time sedentary between groups of mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in aggregation through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

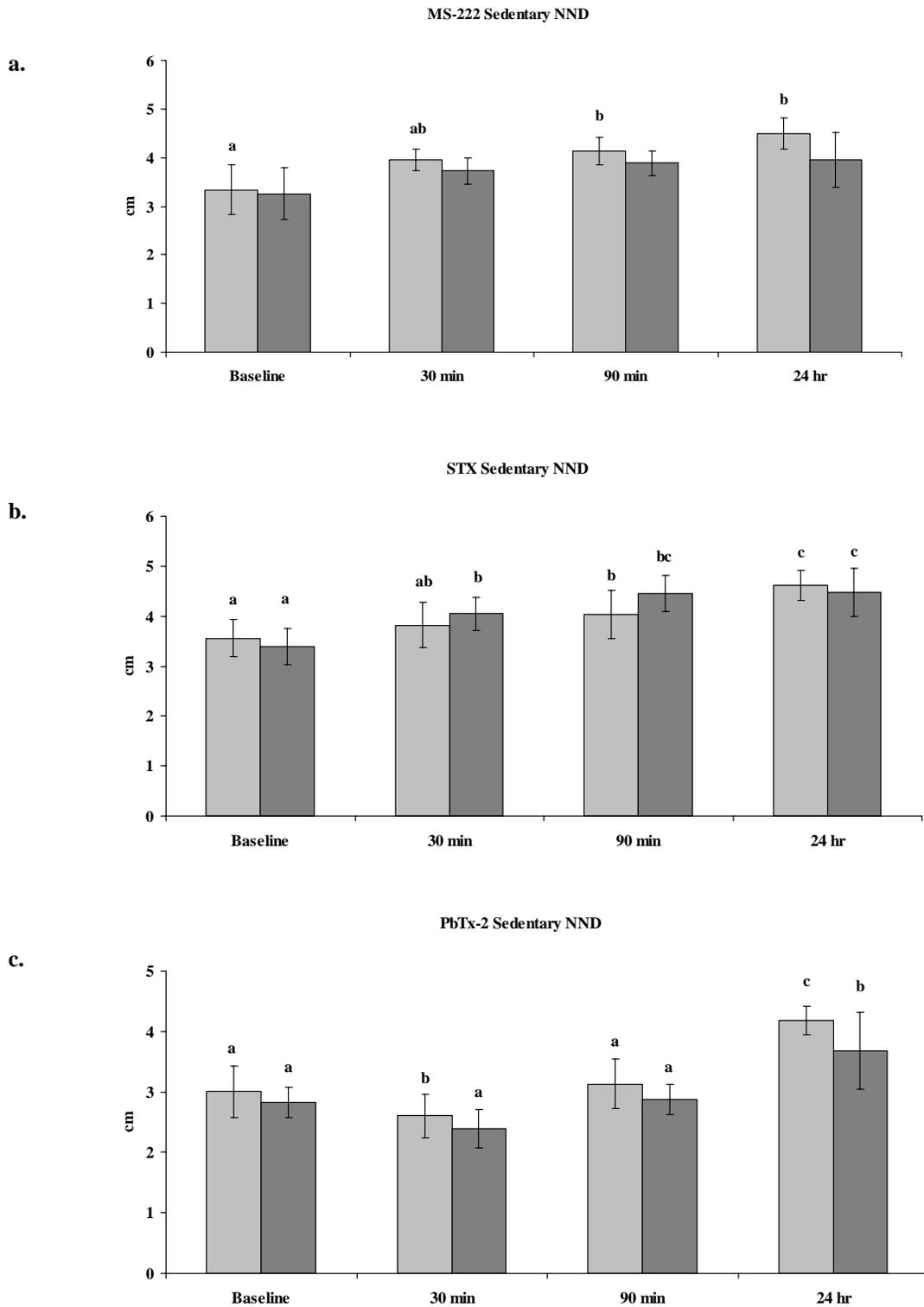


Figure 20 Nearest neighbor distance (NND) between sedentary mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in aggregation NND through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

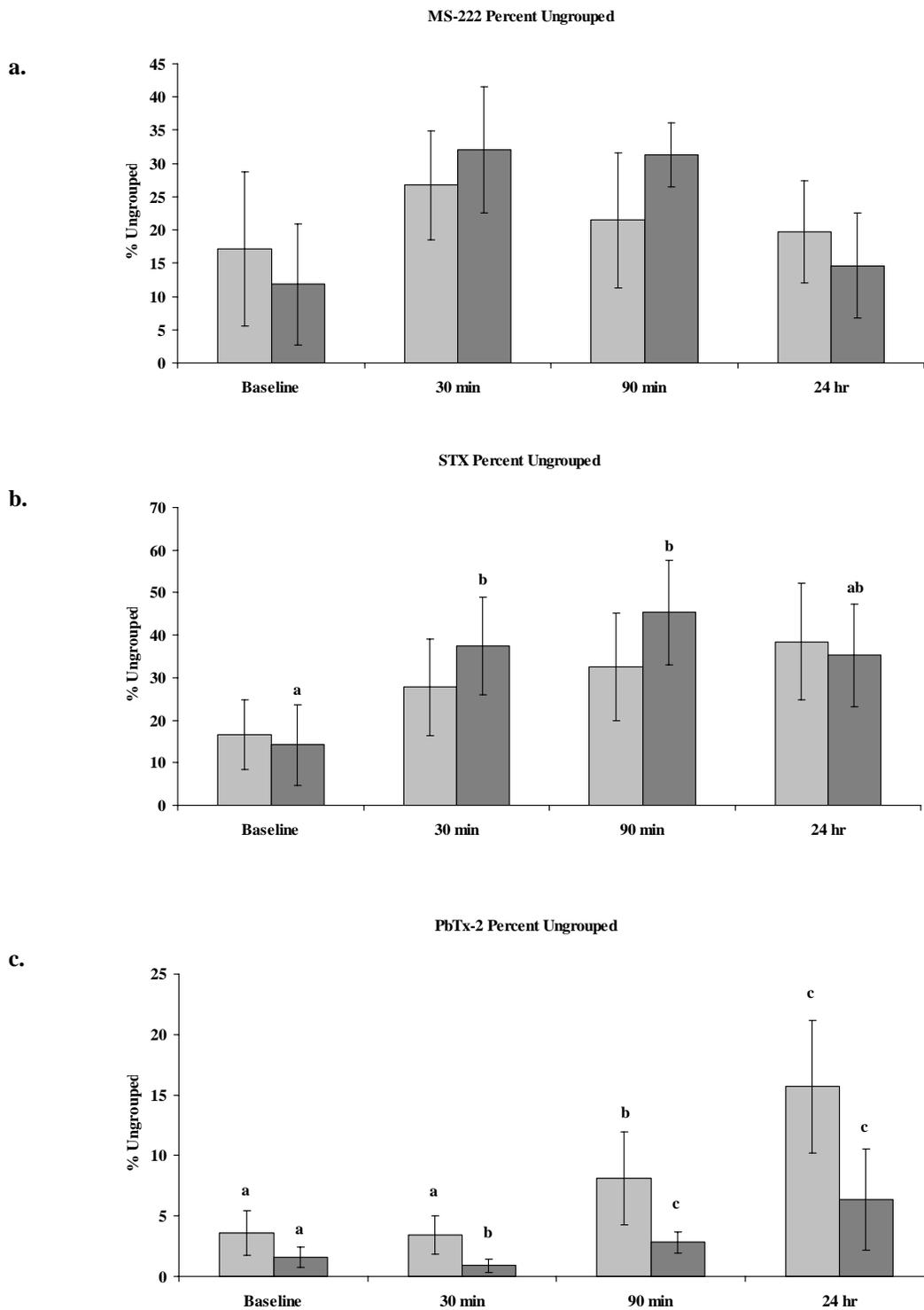


Figure 21 Percentage of time mummichog were ungrouped before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in percentage ungrouped through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

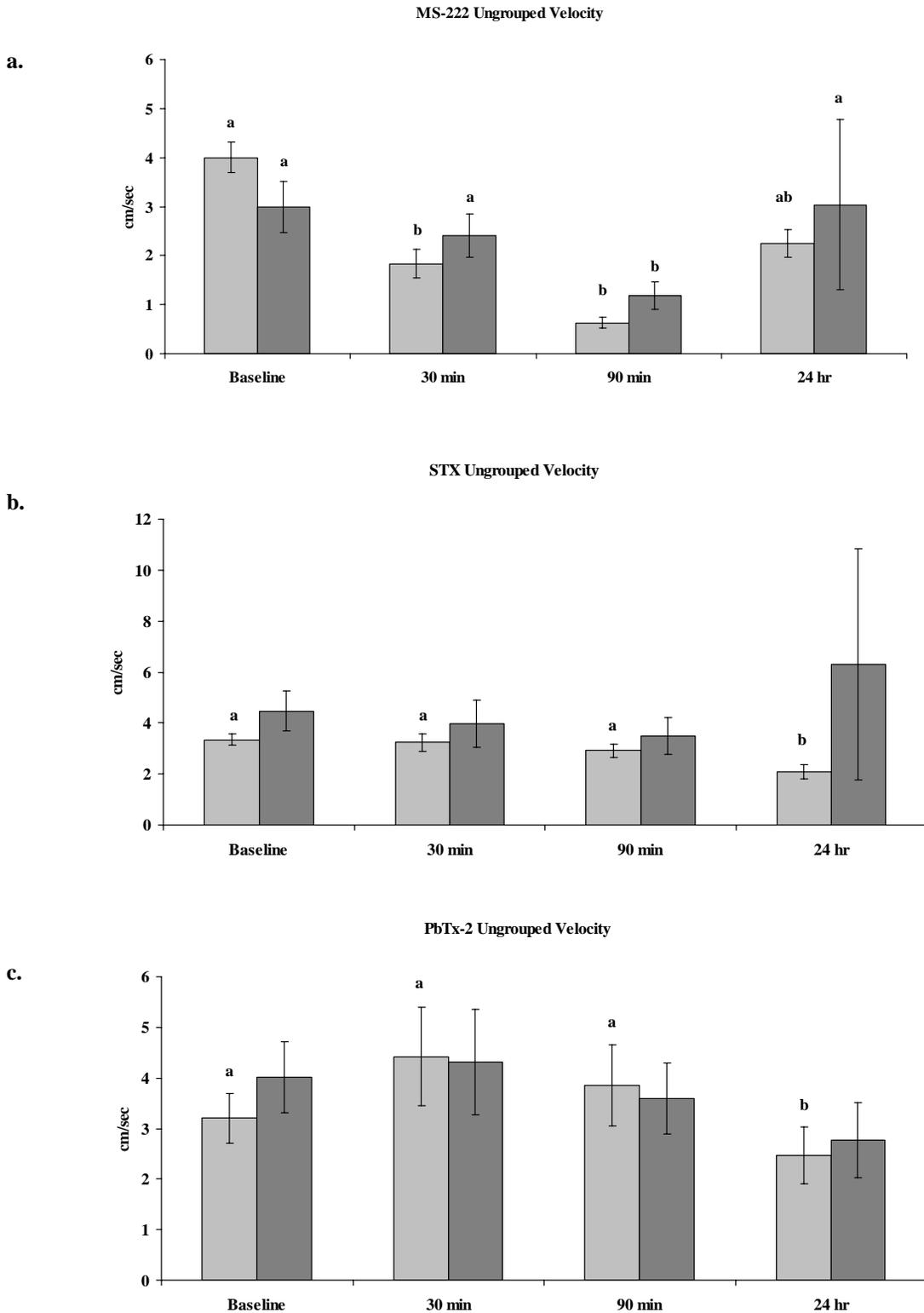
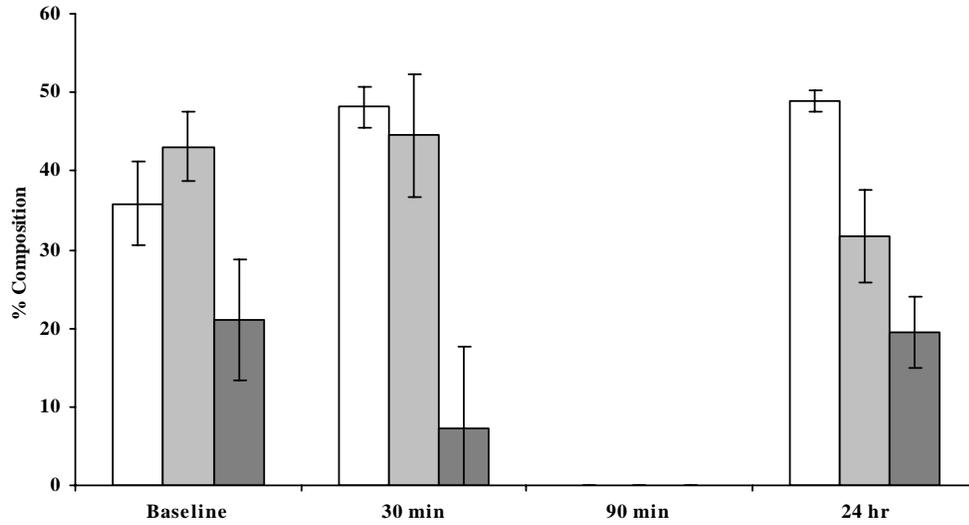


Figure 22 Velocity between ungrouped mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in velocity through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.

MS-222 40 ppm School Composition



b.

MS-222 60 ppm School Composition

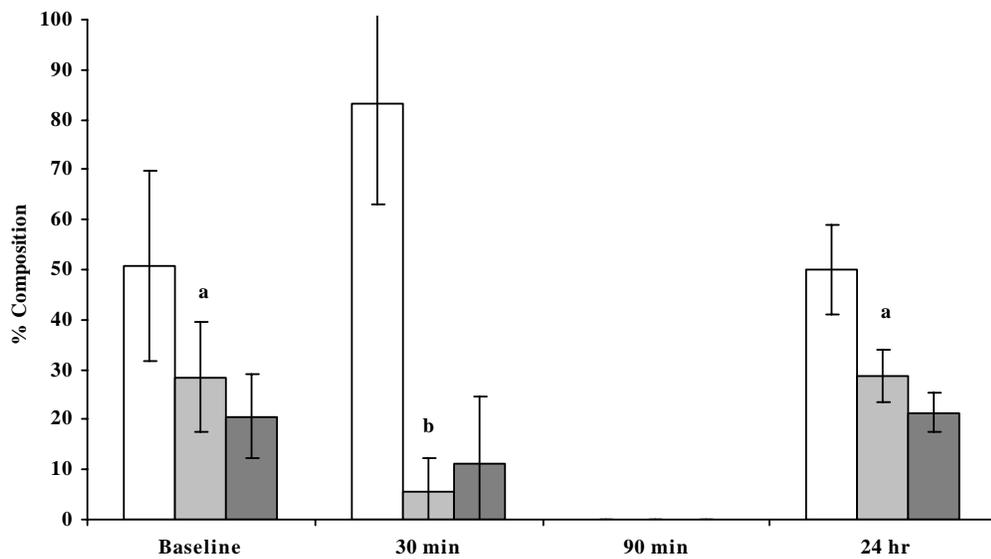


Figure 23 Schools of mummichog containing 3 (white bars), 4 (light. gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 40 (a) and 60 ppm (b) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

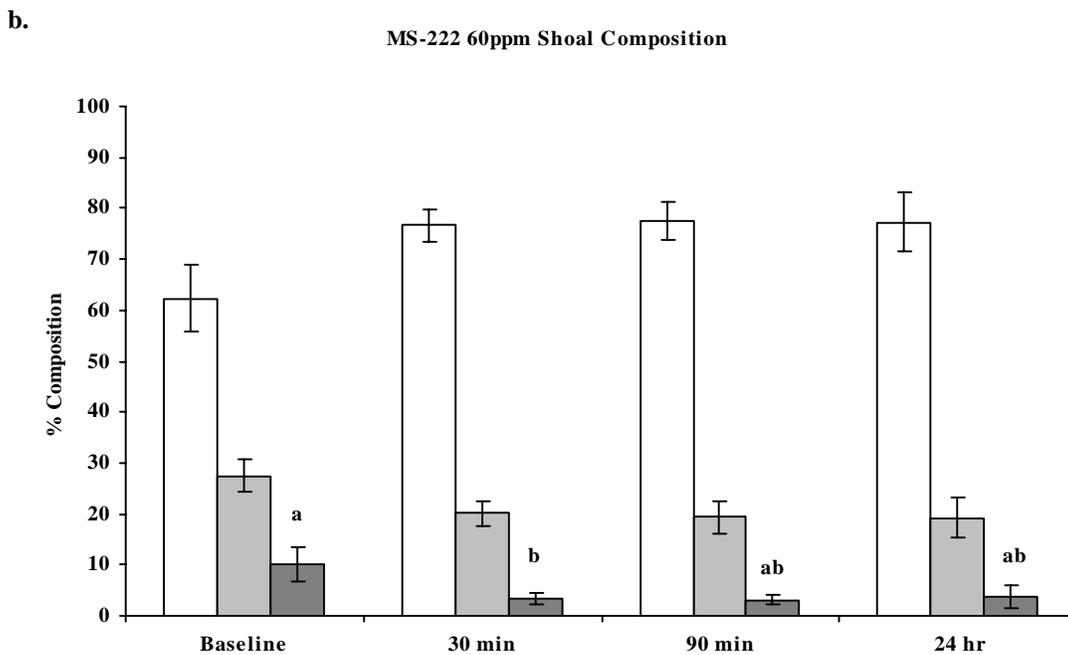
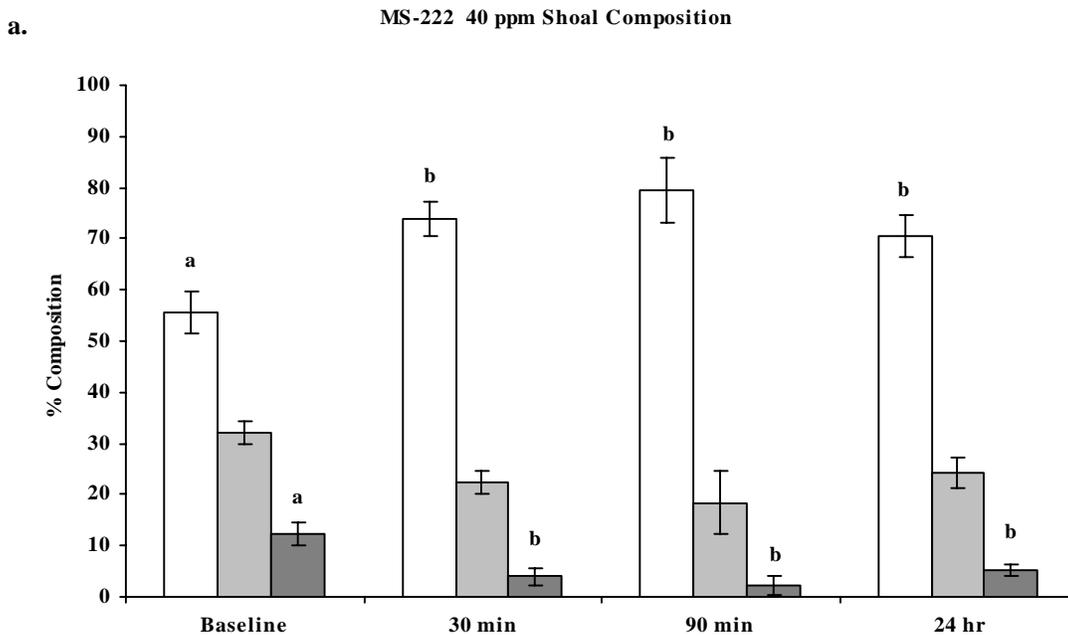
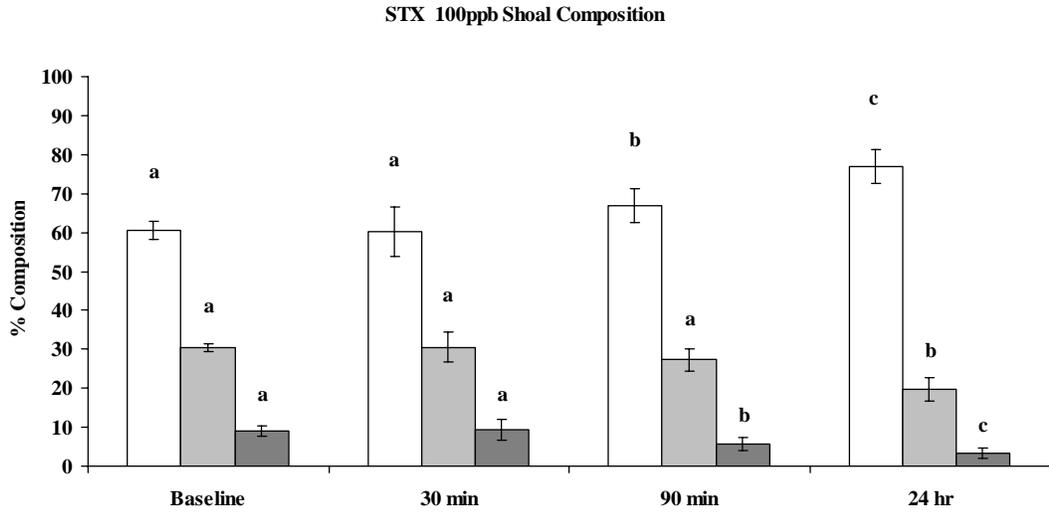


Figure 24 Shoals of mummichog containing 3 (white bars), 4 (light gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 40 (a) and 60 ppm (b) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.



b.

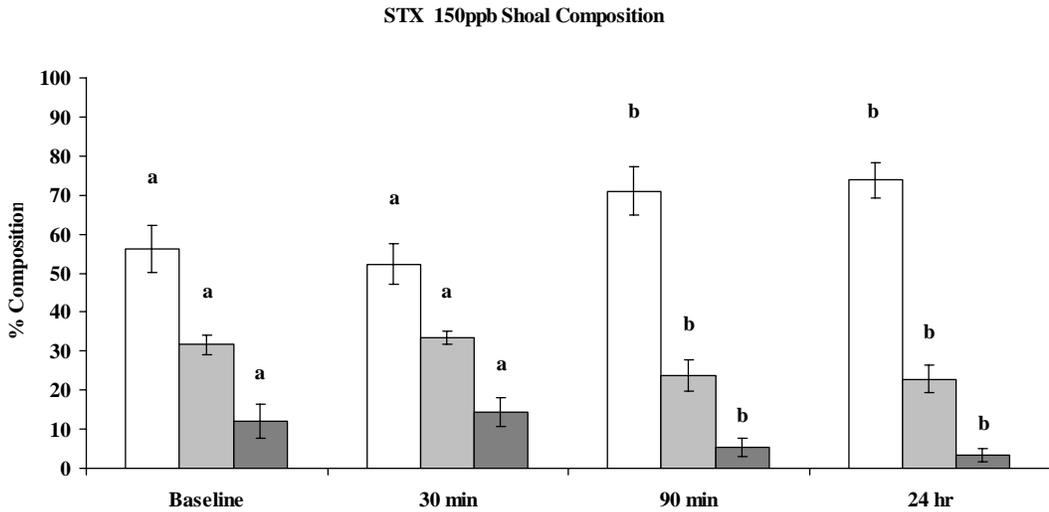


Figure 25 Shoals of mummichog containing 3 (white bars), 4 (light gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 100 (a) and 150 ppb (b) STX. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

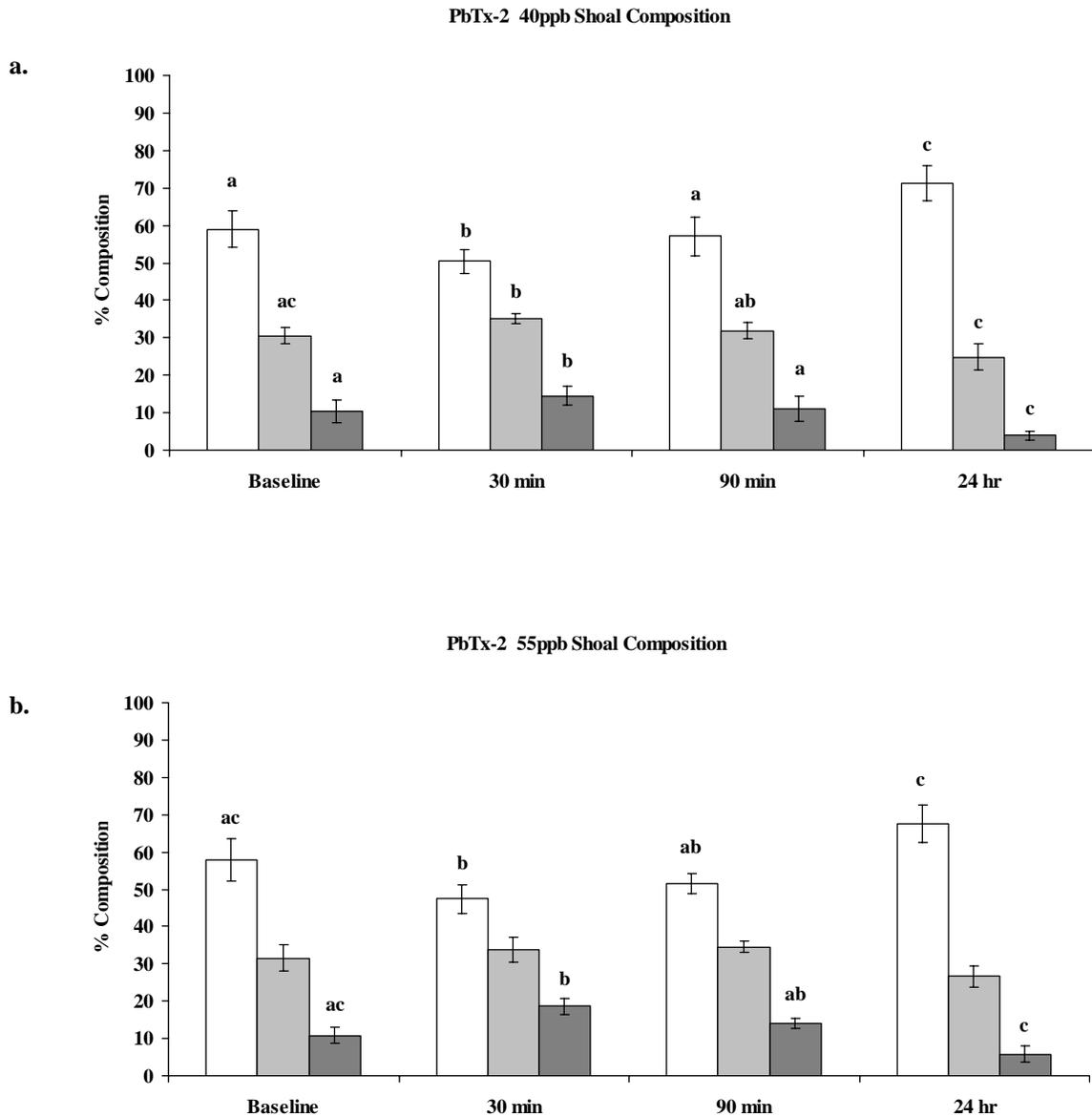
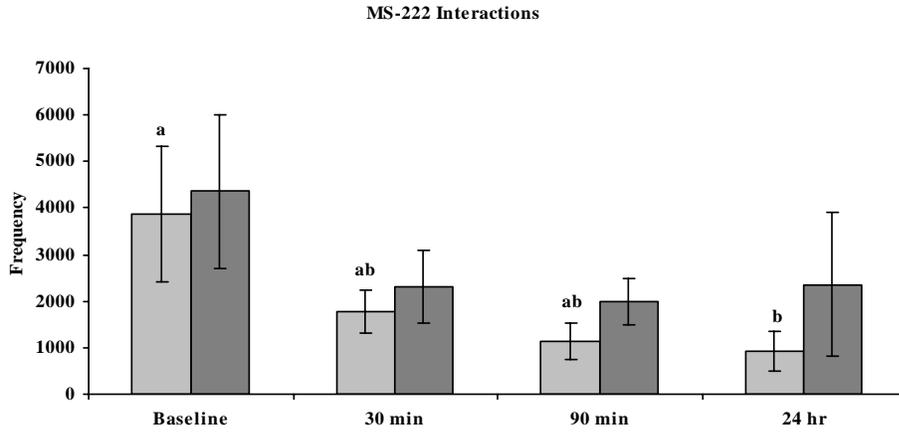
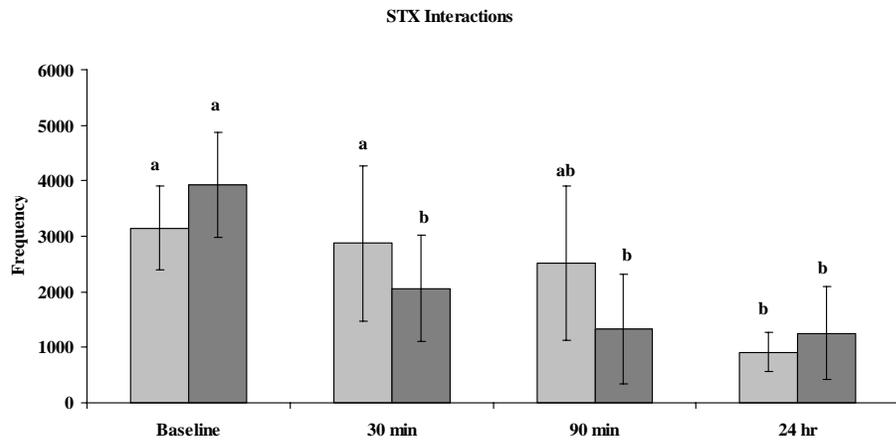


Figure 26 Shoals of mummichog containing 3 (white bars), 4 (light gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 40 (a) and 55 ppb (b) PbTx-2. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.



b.



c.

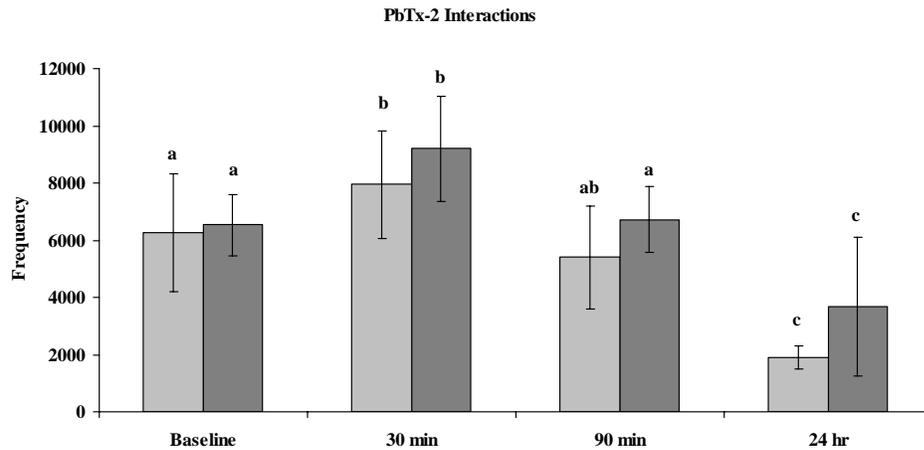


Figure 27 The number of interactions between groups of mummichog before, during, and 24 hours after exposure to MS-222 (a), STX (b), and PbTx-2 (c). Light gray bars represent the low concentration and dark gray bars represent the high concentration. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in interactions through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

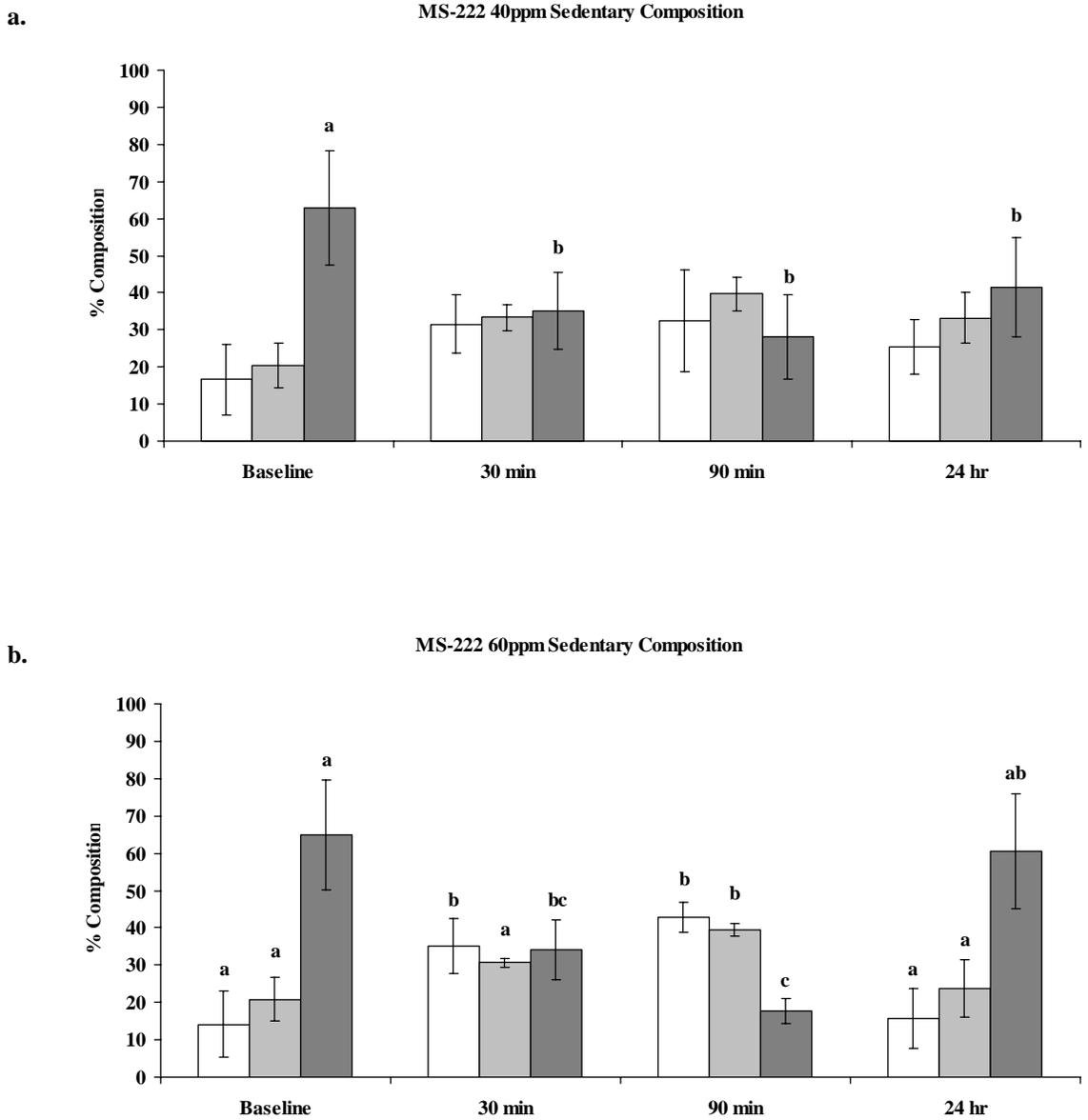


Figure 28 Sedentary groups of mummichog containing 3 (white bars), 4 (light gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 40 (a) and 60 ppm (b) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

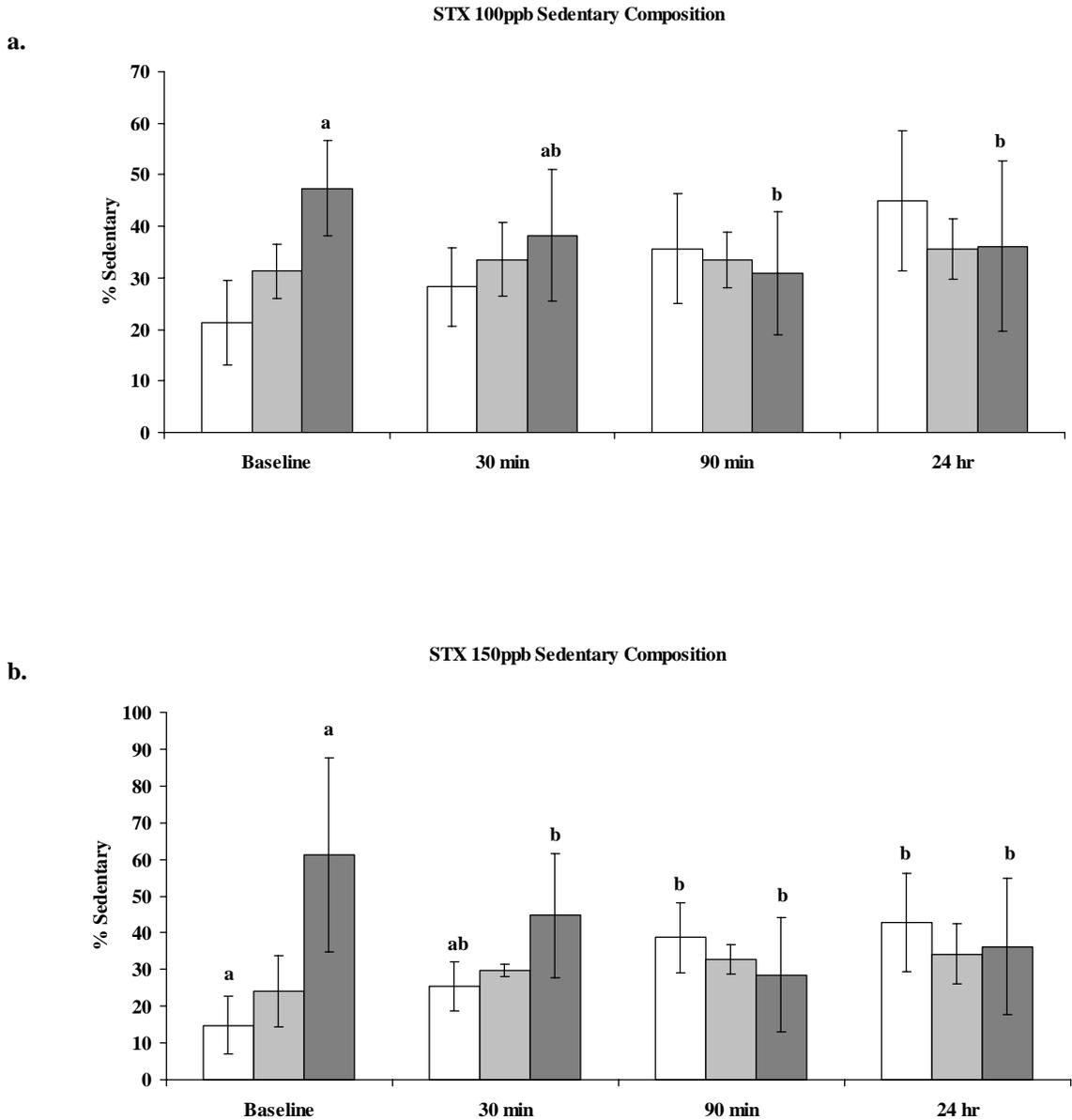


Figure 29 Sedentary groups of mummichog containing 3 (white bars), 4 (light gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 100 (a) and 150 ppb (b) STX. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

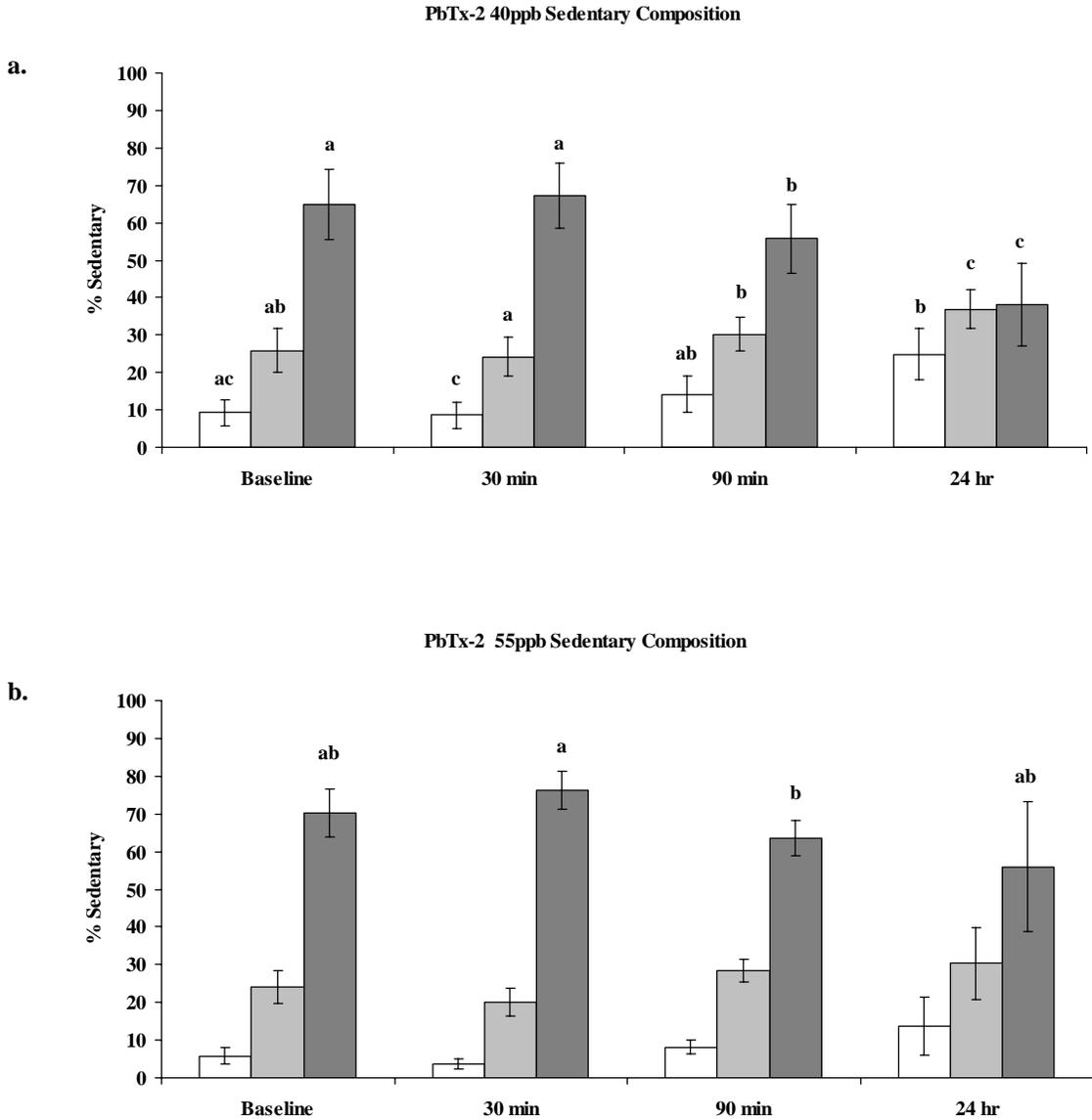


Figure 30 Sedentary groups of mummichog containing 3 (white bars), 4 (light gray bars), and 5 (dark gray bars) fish before, during, and after 24 hours exposure to 40 (a) and 55 ppb (b) PbTx-2. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in composition through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

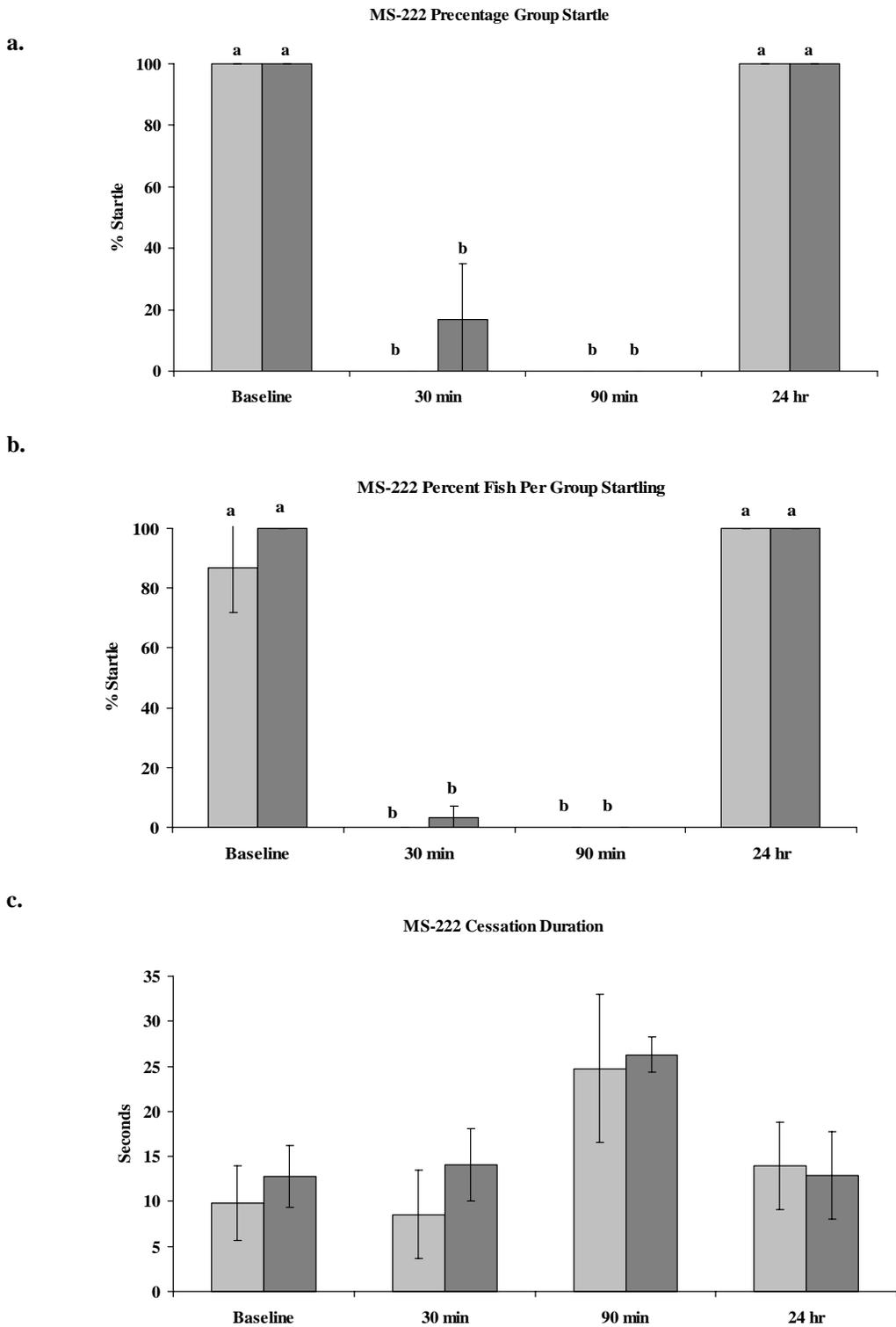


Figure 31 The percentage of groups (a) and of fish (b) exhibiting a startle response as well as the amount of time spent motionless (c) after the presentation of a visual predator with exposure to 40 ppm (light gray bars) and 60 ppm (dark gray bars) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in responses through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

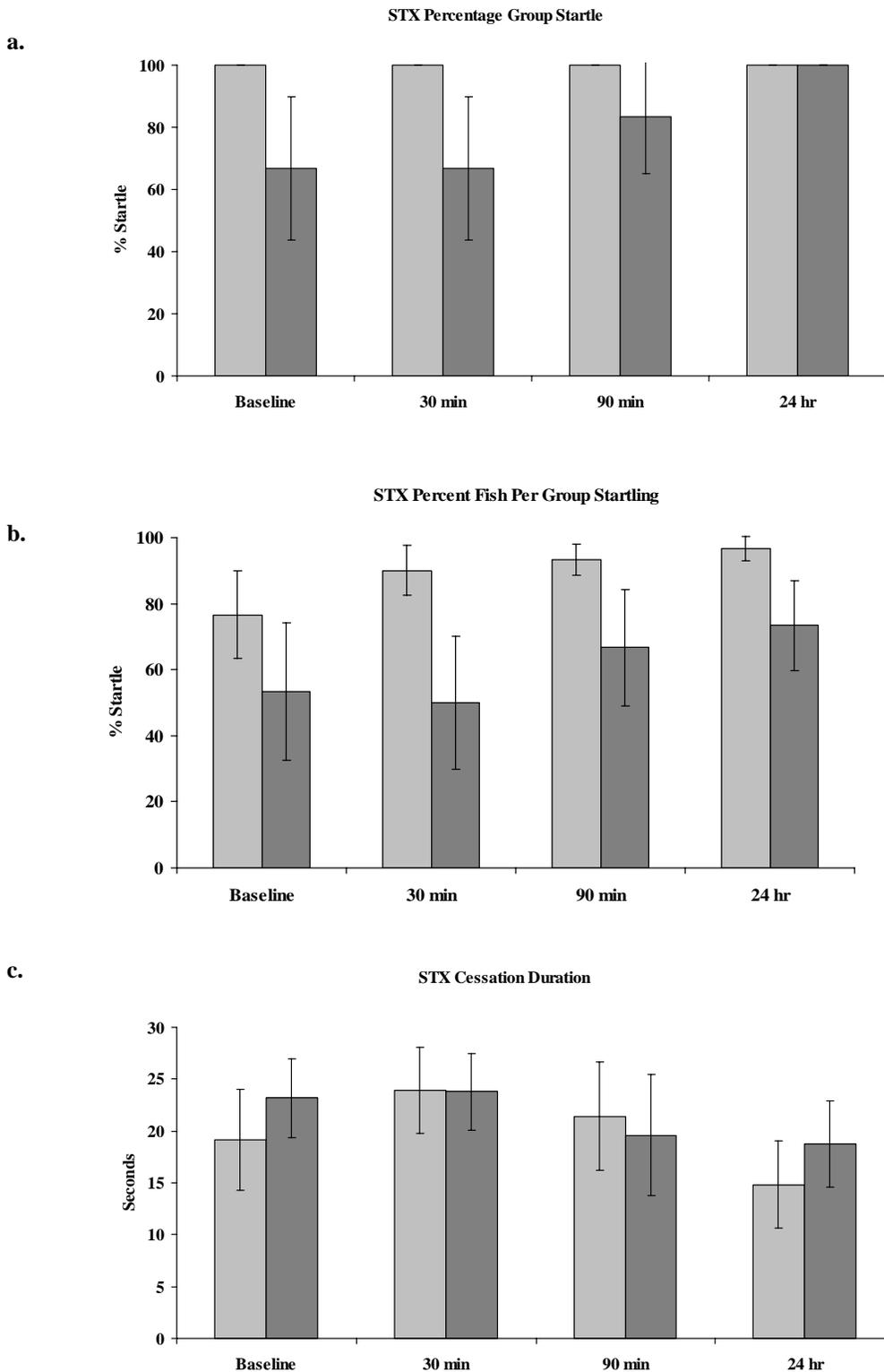


Figure 32 The percentage of groups (a) and of fish (b) exhibiting a startle response as well as the amount of time spent motionless (c) and after the presentation of a visual predator with exposure to 100 ppb (light gray bars) and 150 ppm (dark gray bars) STX. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in responses through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

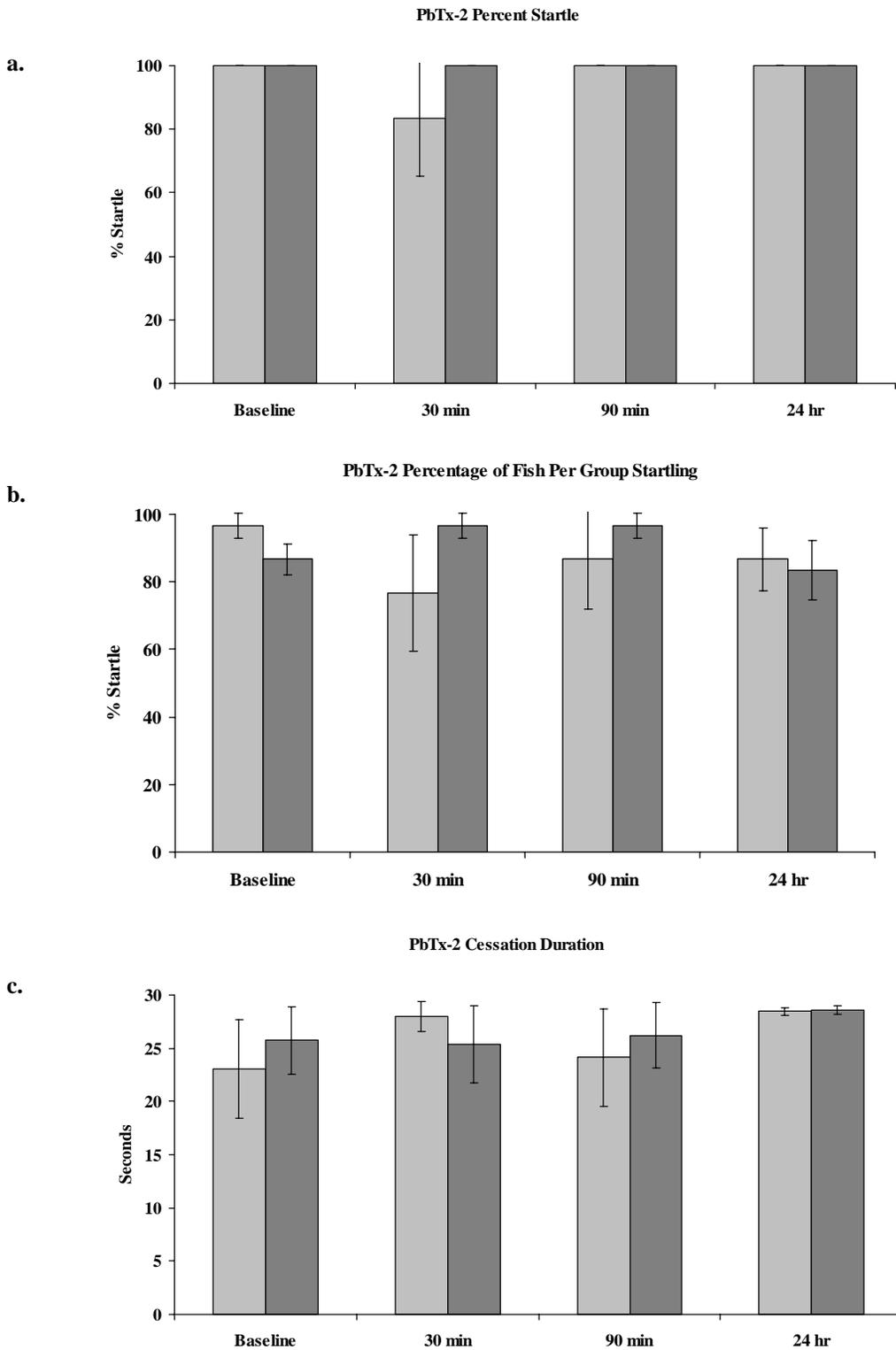


Figure 33 The percentage of groups (a) and of fish (b) exhibiting a startle response as well as the amount of time spent motionless (c) after the presentation of a visual predator with exposure to 40 (light gray bars) and 55 ppm (dark gray bars) PbTx-2. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in responses through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

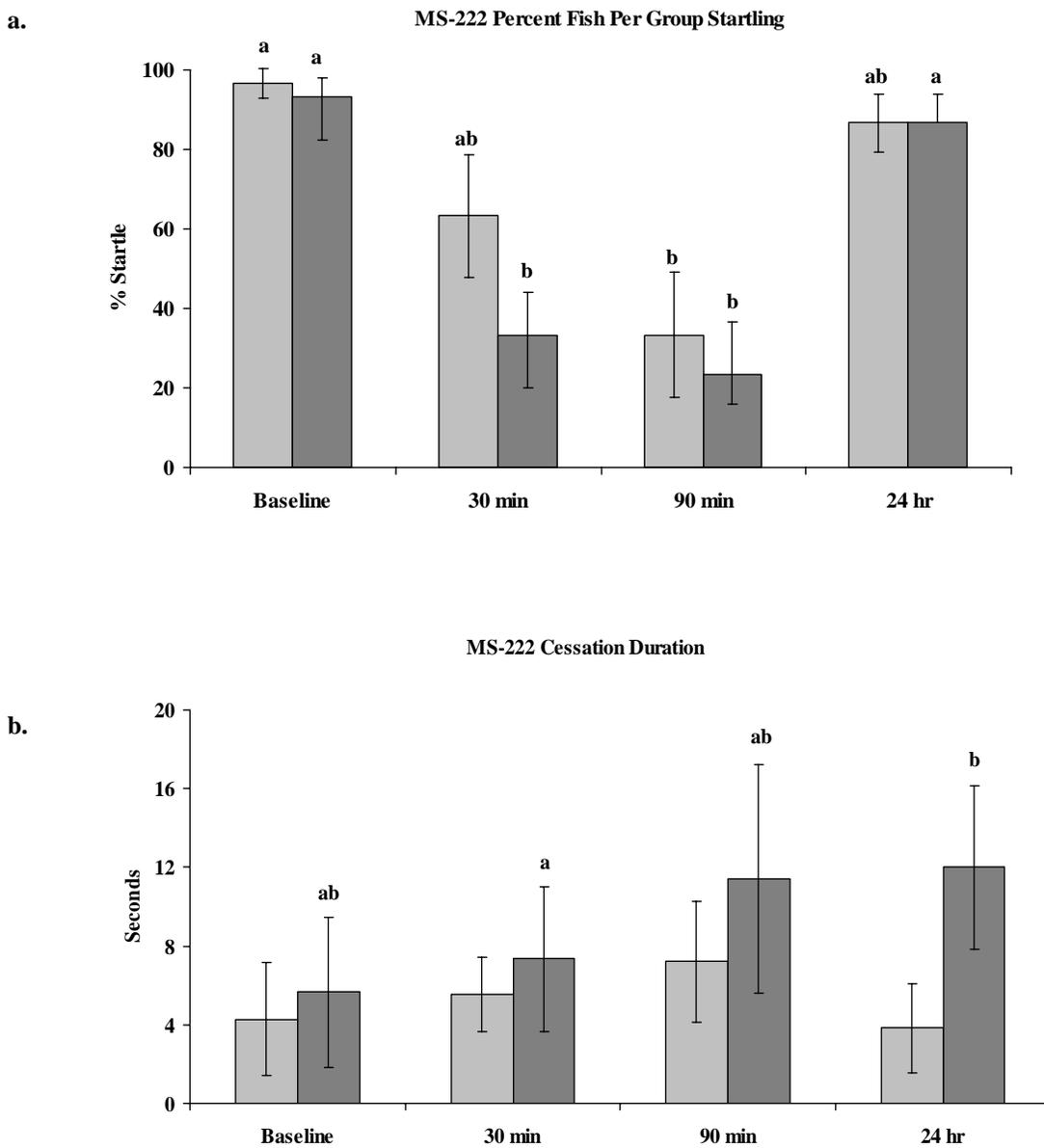
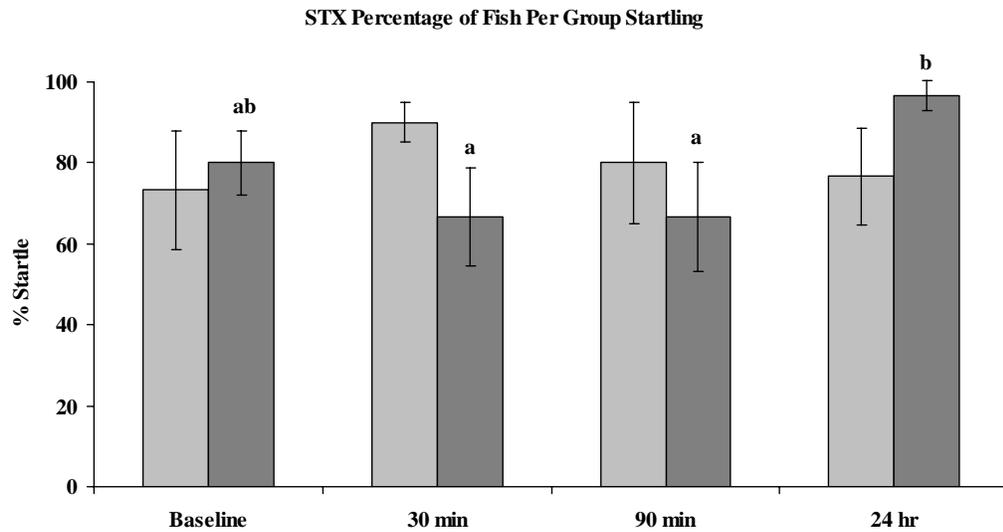


Figure 34 The percentage of groups (a) exhibiting a startle response as well as the amount of time spent motionless (b) after the presentation of an auditory/vibratory stimulus with exposure to 40 ppm (light gray bars) and 60 ppm (dark gray bars) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in responses through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.



b.

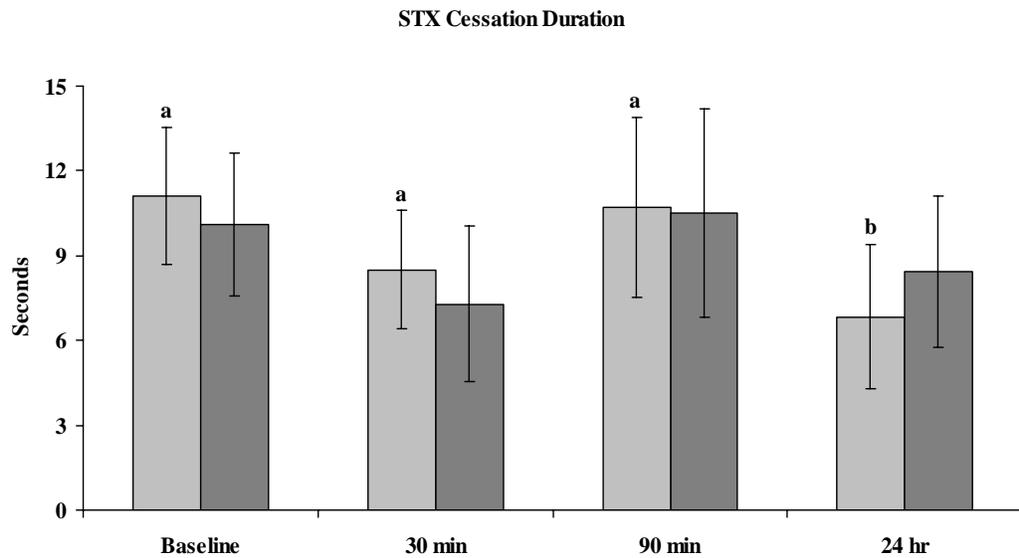
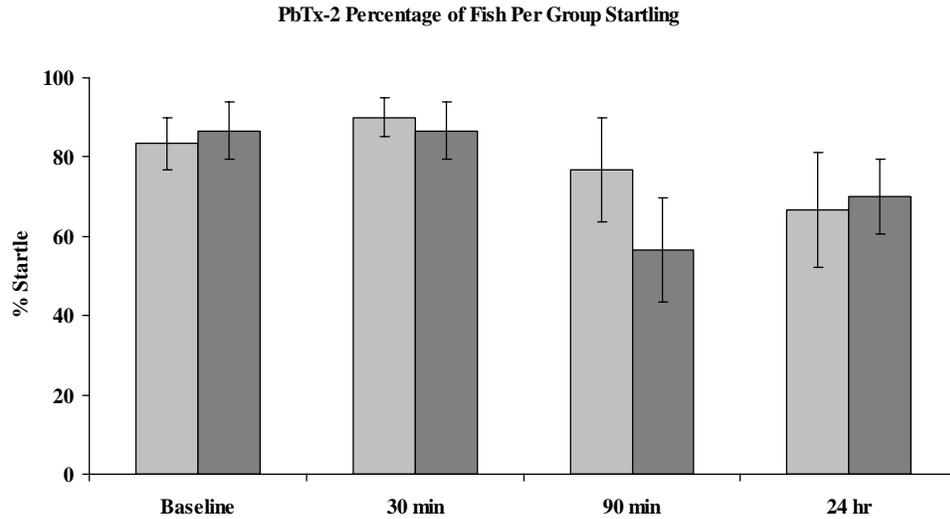


Figure 35 The percentage of fish (a) exhibiting a startle response as well as the amount of time spent motionless (b) after the presentation of an auditory/vibratory stimulus with exposure to 100 ppb (light gray bars) and 150 ppb (dark gray bars) STX. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in responses through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.



b.

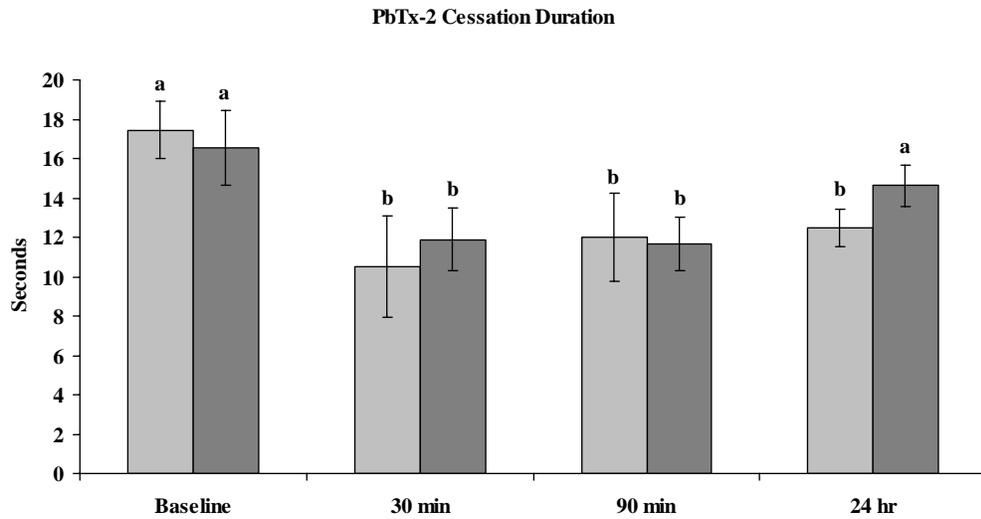


Figure 36 The number (a) of fish exhibiting a startle response as well as the amount of time spent motionless (b) after the presentation of an auditory/vibratory stimulus with exposure to 40 ppb (light gray bars) and 55 ppb (dark gray bars) PbTx-2. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A repeated measures ANOVA was used to analyze alterations in responses through time. Means with different letters are statistically different at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

MS-222 Interactions

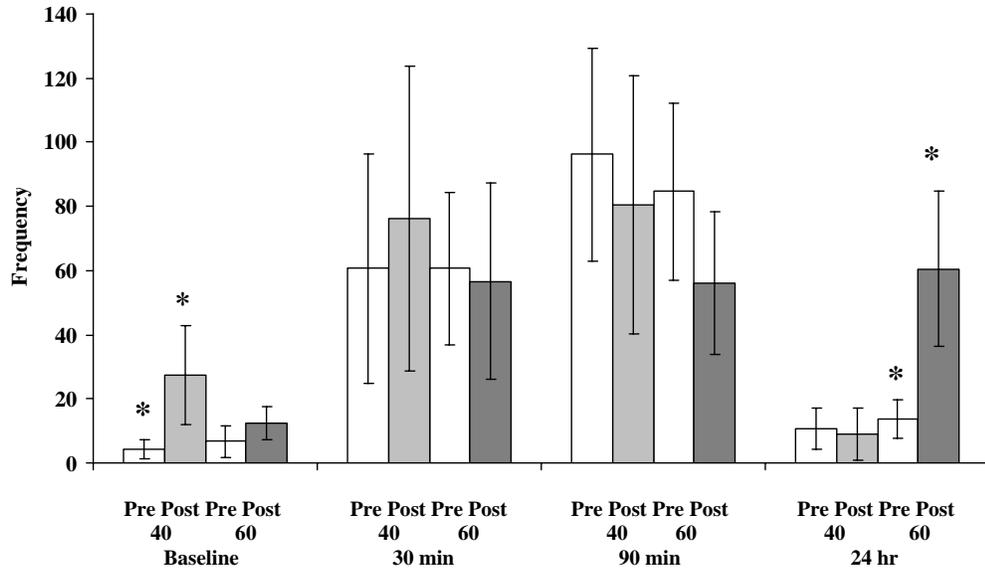


Figure 37 Mean frequency of interactions before (white bars) and after (gray bars) the presentation of a visual predator with exposure to 40 ppm (light gray bars) and 60 ppm (dark gray bars) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

MS-222 Shoaling Percentage

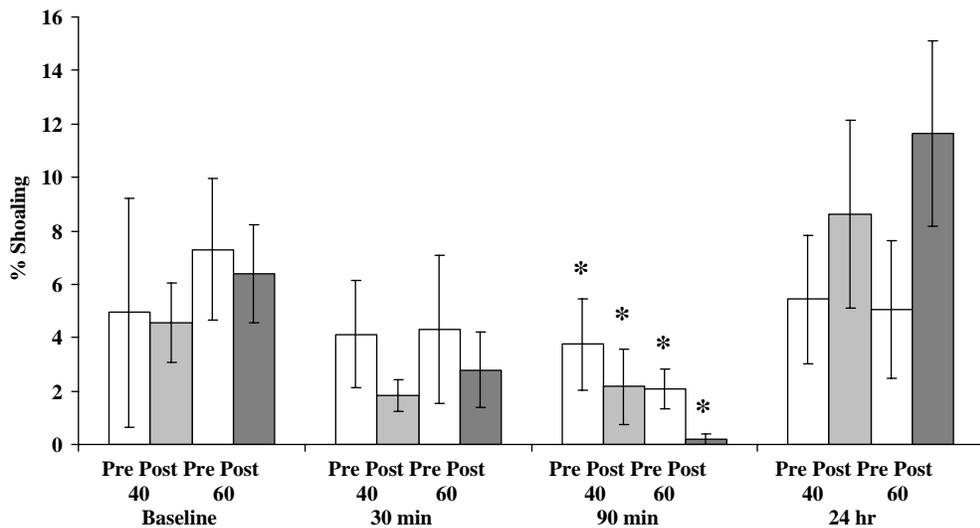
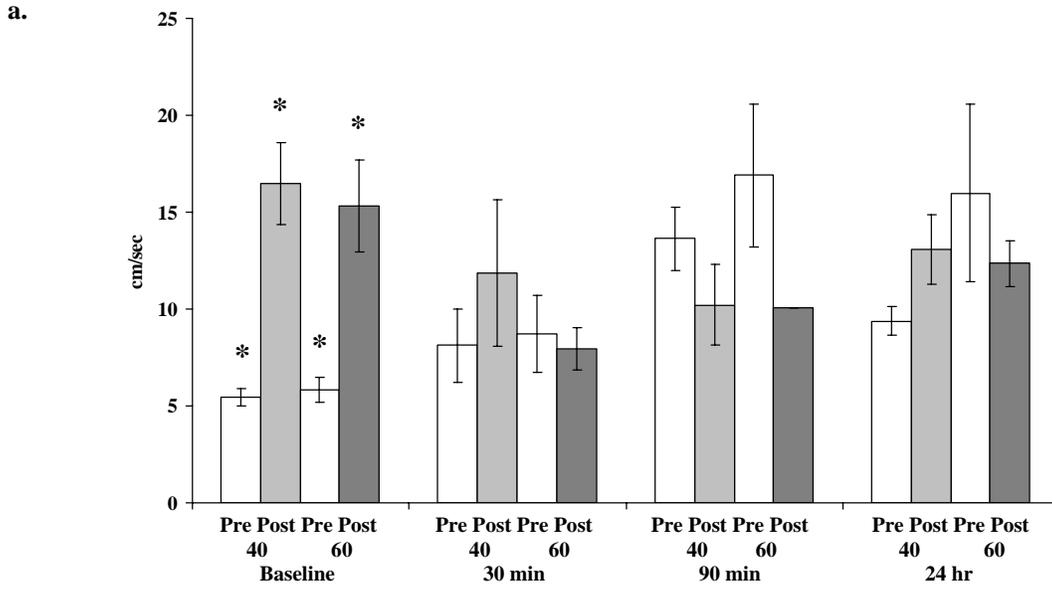


Figure 38 Mean time spent shoaling before (white bars) and after (gray bars) the presentation of a visual predator with exposure to 40 ppm (light gray bars) and 60 ppm (dark gray bars) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

MS-222 Shoaling Velocity



STX Shoaling Velocity

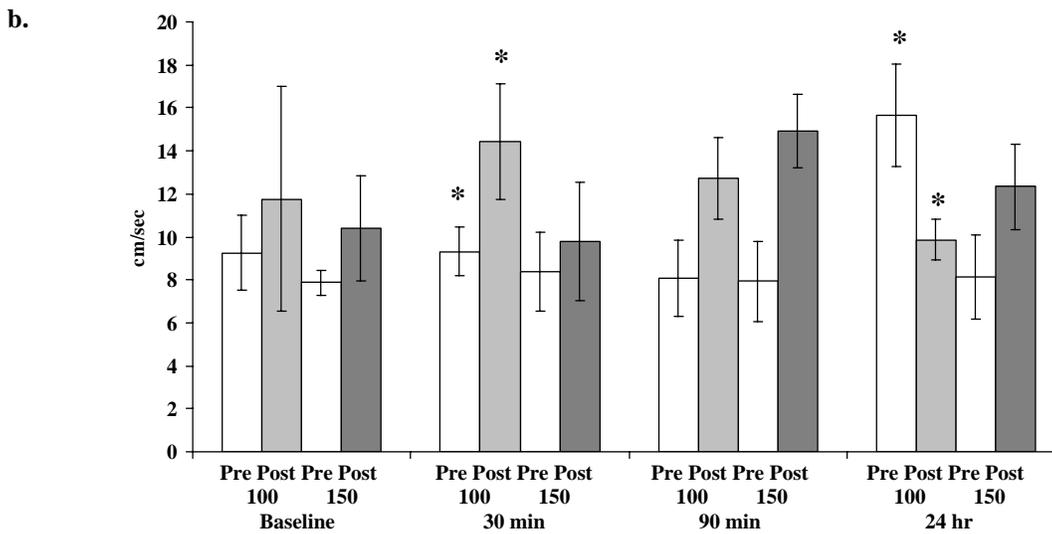


Figure 39 Mean shoaling velocity between groups of mummichog before, during, and 24 hours after exposure before (white bars) and after (gray bars) the presentation of a visual predator with exposure to MS-222 (a) and STX (b). A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

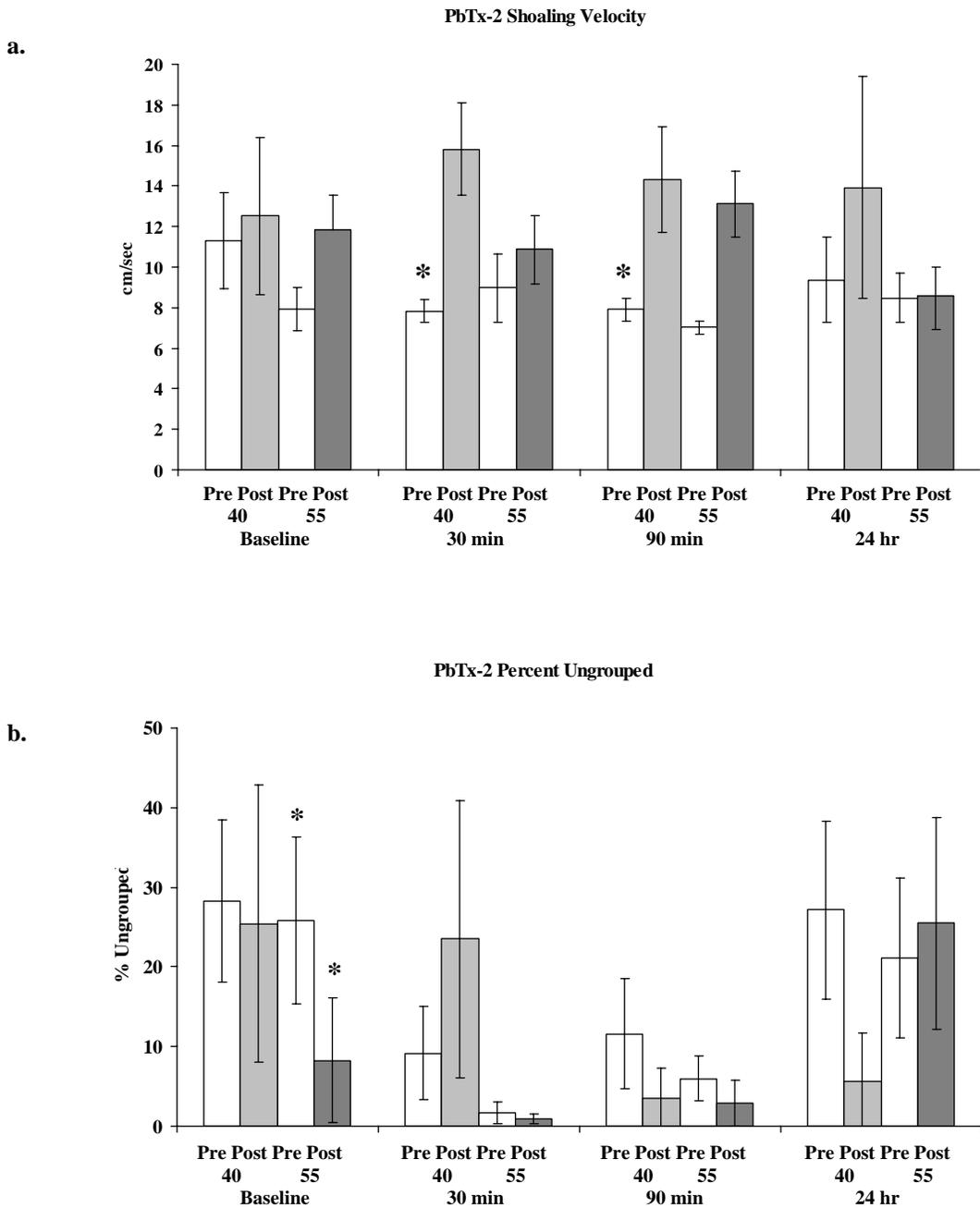


Figure 40 Mean shoaling velocity (a) and time spent ungrouped (b) before and after the presentation of a visual predator with exposure to 40 ppb (light gray bars) and 55 ppb (dark gray bars) PbTx-2. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

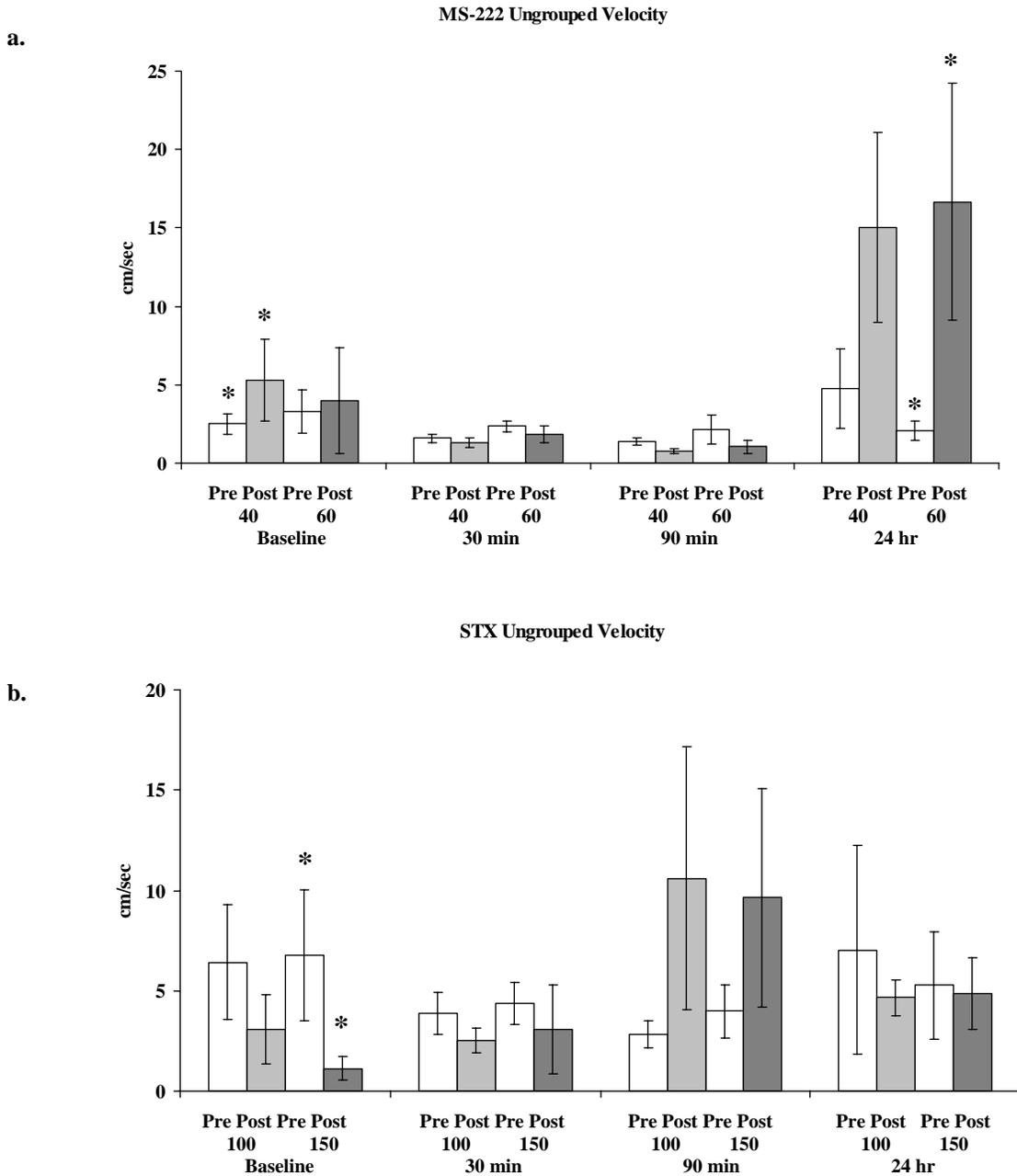


Figure 41 Mean ungrouped velocity between groups of mummichog before, during, and 24 hours after exposure before (white bars) and after (gray bars) the presentation of a visual predator with exposure to MS-222 (a.), and STX (b.). A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

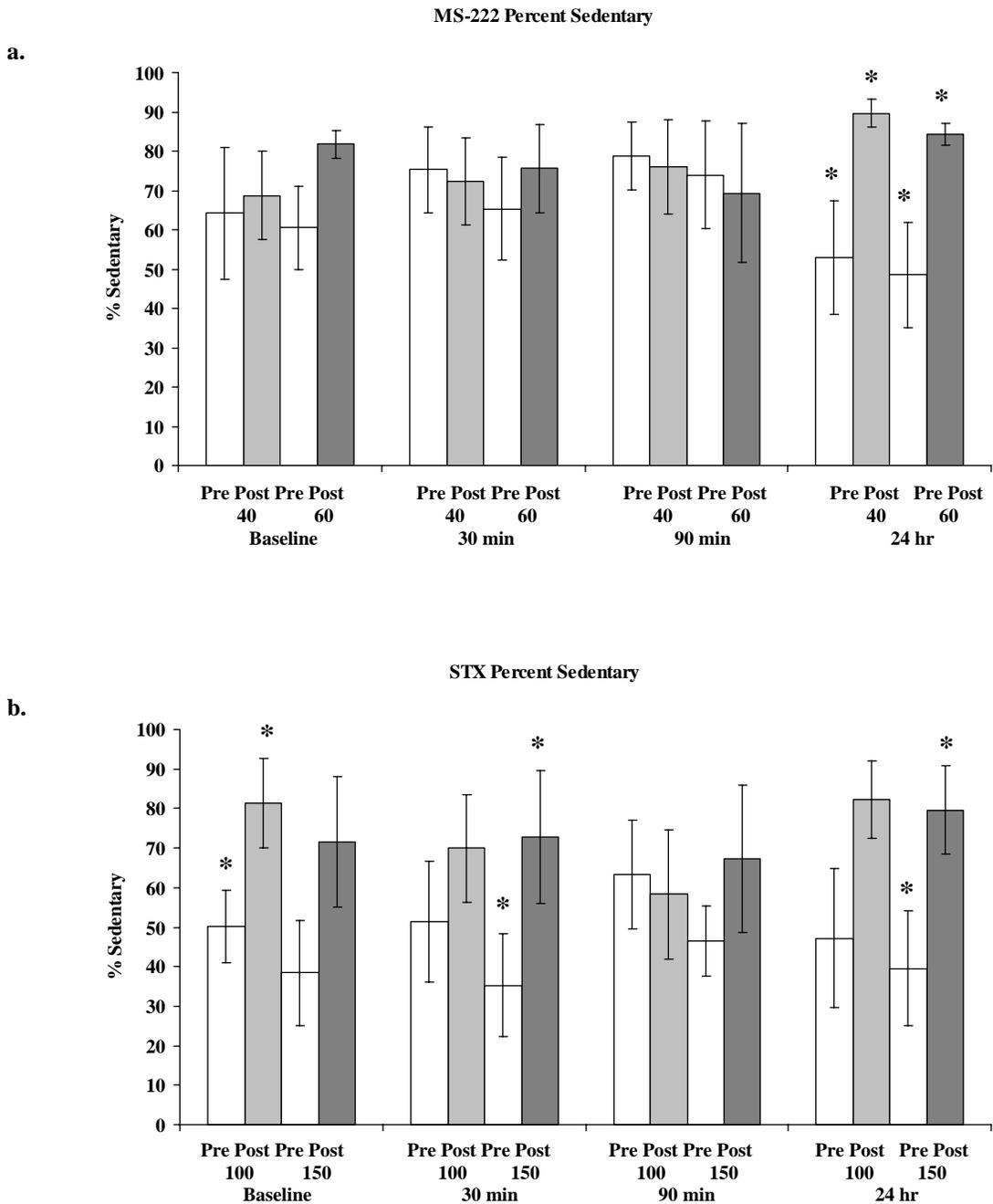
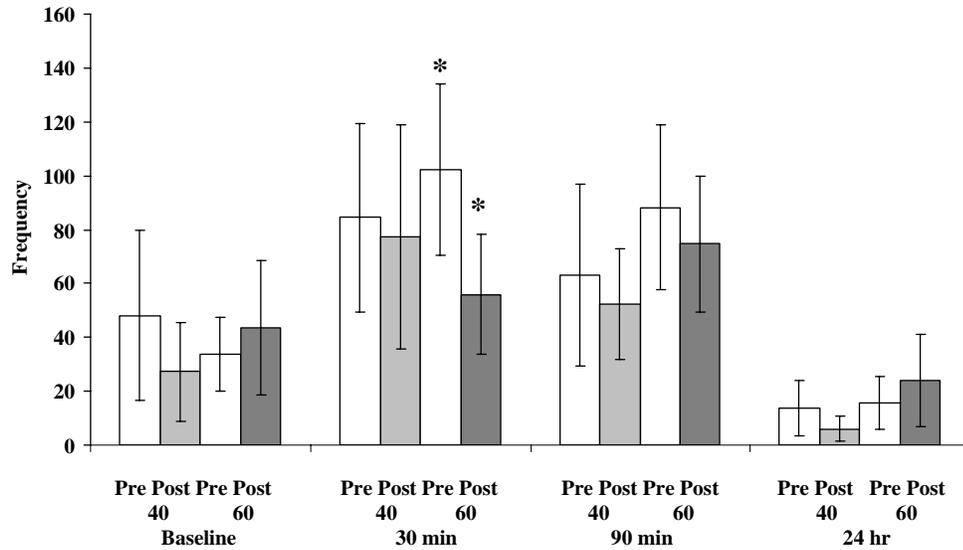


Figure 42 Mean time spent sedentary between groups of mummichog before, during, and 24 hours after exposure before (white bars) and after (gray bars) the presentation of a visual predator with exposure to MS-222 (a) and STX (b). A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.

MS-222 Interactions



b.

MS-222 Percent Shoaling

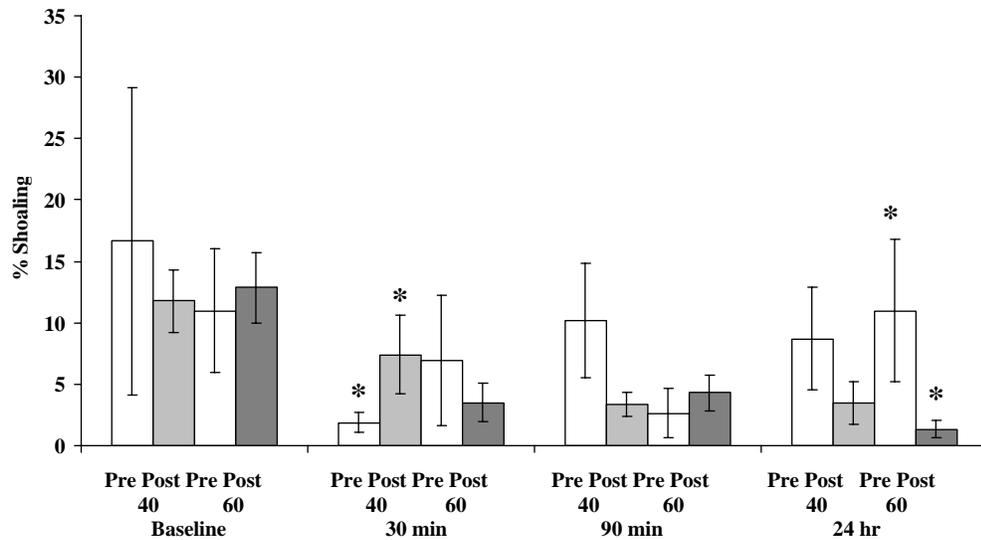


Figure 43 Mean frequency of interactions (a) and shoaling velocity (b) before (white bars) and after (gray bars) the presentation of a auditory/vibratory stimuli with exposure to 40 ppm (light gray bars) and 60 ppm (dark gray bars) MS-222. A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

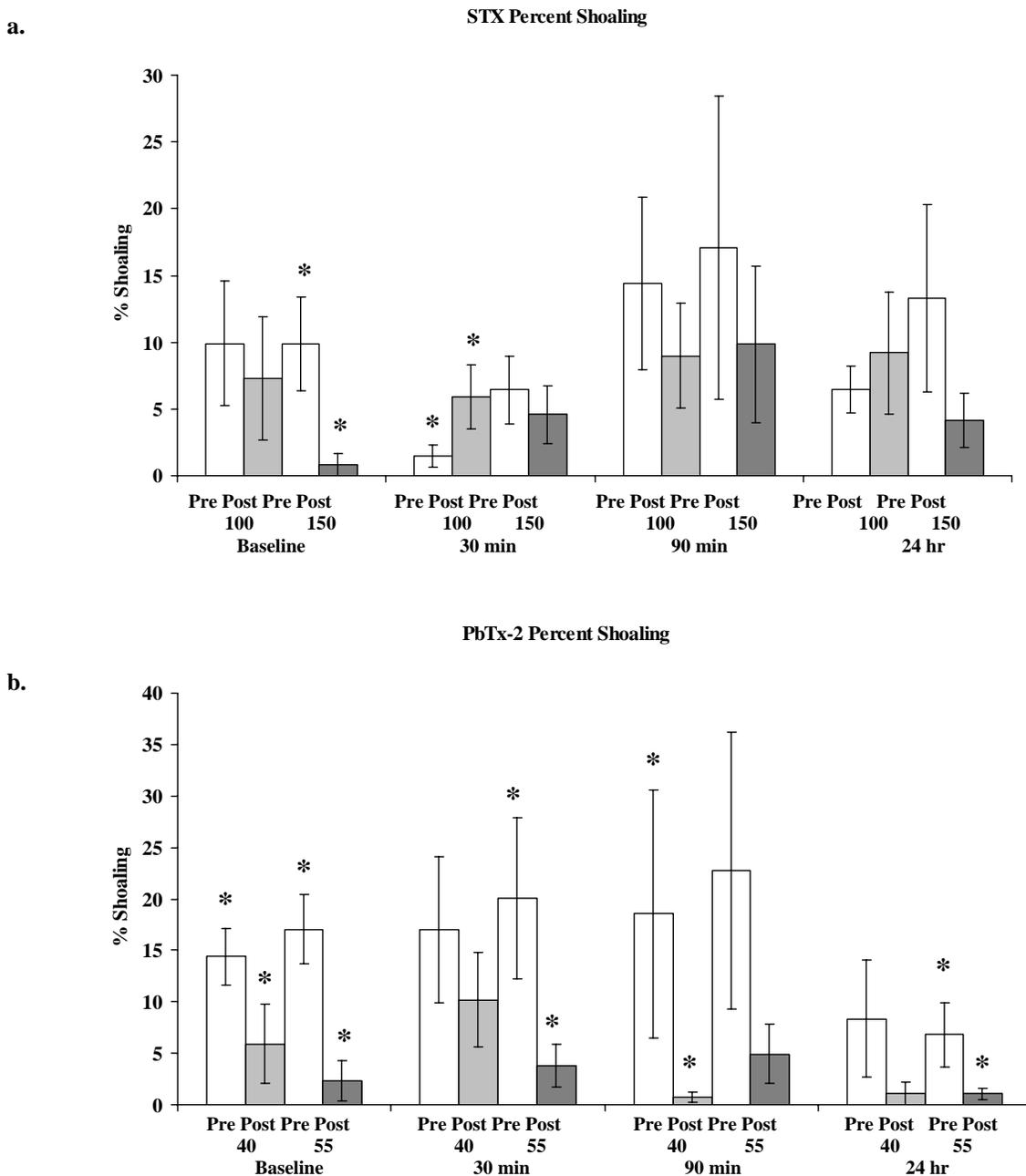
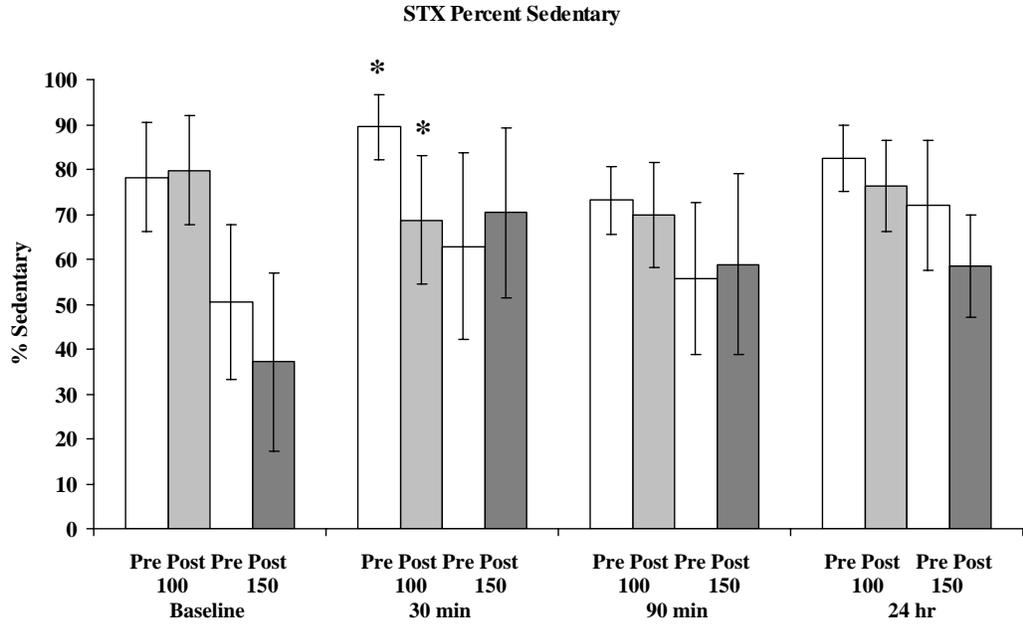


Figure 44 Mean shoaling percentage between groups of mummichog before, during, and 24 hours after exposure before and after the presentation of a visual predator with exposure to STX (a) and PbTx-2 (b). A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

a.



b.

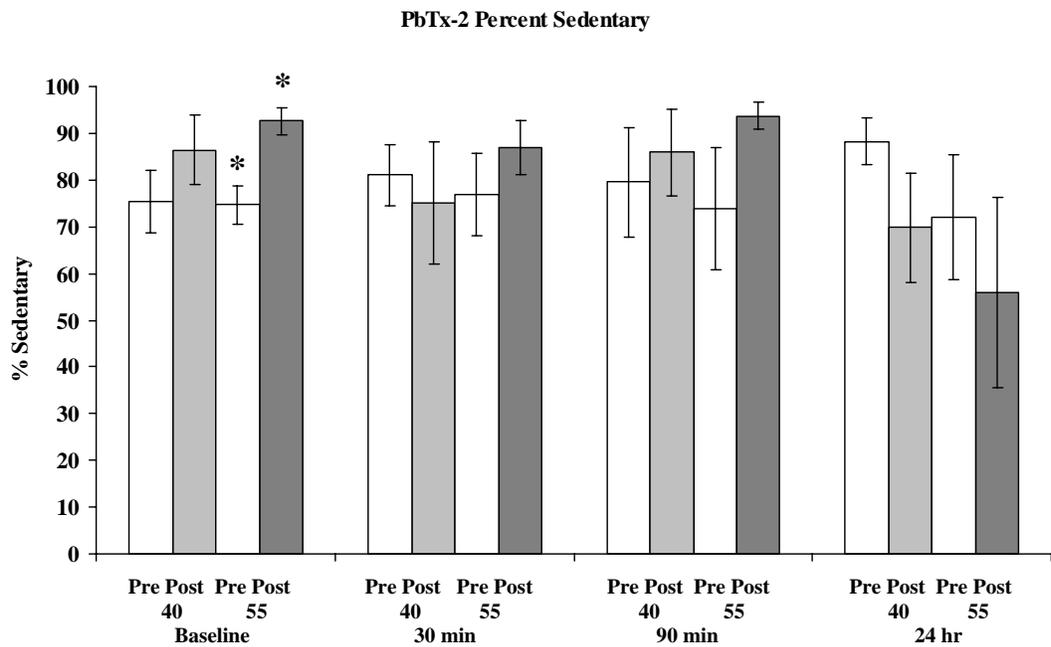


Figure 45 Percentage of time spent sedentary between groups of mummichog before, during, and 24 hours after exposure before (white bars) and after (gray bars) the presentation of a visual predator with exposure to STX (a), and PbTx-2 (b). A 30 minute baseline period along with 2 exposure periods, 30 minutes and 90 minutes post exposure, and 24 hours later were evaluated. A Wilcoxon signed-rank test was used to analyze alterations in interactions before and after the stimulus. Asterisks represent a difference between pre and post response at $\alpha \leq 0.10$. Error bars represent \pm one standard error (SE) of the mean.

4.5 Discussion

Behavior is a highly structured and predictable sequence of activities designed to ensure maximal fitness and survival. Therefore, behavioral endpoints serve as valuable tools to discern and evaluate effects of exposure to environmental stressors. Quantifiable behavioral changes in chemically-exposed fish provide novel information that cannot be gained from traditional toxicological methods; including short-term and sublethal exposure effects, mechanisms of effect, interaction with environmental variables, and the potential for mortality (Birtwell & Kruzynski, 1989; Giattina et al., 1981; Henry & Atchison, 1986; Kleerekoper et al., 1972; Little & Finger, 1990; Saglio & Trijasse, 1998). Ecologically relevant behaviors affected by sublethal concentrations include: altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Zhou & Weis, 1998; Little & Finger, 1990). Ultimately, changes in individual fitness and population dynamics resulting from alterations in behavior from sublethal stress exposure need investigation. This study was designed to investigate sublethal HAB exposure on fish behavior. However, unexpected mortality occurred in the PbTx-2 exposures 24 hours post exposure. The unexpected mortality may have been due to a combination of stressors; including the PbTx-2 exposure combined with acclimation to the exposure vessels and fasting for 48 hours.

In this study, HAB toxins and MS-222 induced alterations in schooling, shoaling, and aggregative behaviors in mummichog by forcing fish apart. Exposure to STX, MS-222 and PbTx-2 significantly decreased the frequency of interactions with no recovery up

to 24 hours later, demonstrating that fish are less cohesive over time. Additionally, MS-222 and STX displayed significant decreases in schooling and shoaling behaviors with increases in stagnant aggregations and solitary behaviors over time. Schools and shoals of mummichog were unable to recover to baseline values, describing that behavioral alteration can last upwards of 24 hours. In contrast, PbTx-2 exposed fish displayed no relevant changes during exposure, but significantly decreased schooling and shoaling behaviors 24 hours later, displaying a significant delay in response. Recovery data suggests that PbTx-2, MS-222, and STX may induce long-term alterations from a short exposure to sublethal concentrations. The analysis of aggregative swimming in mummichog over 3 days displayed no significant alterations in behaviors after a 24-hour acclimation period (Chapter 2). Therefore, alterations in aggregative swimming behaviors in this study, after the 24-hour acclimation, are due to HAB toxin exposure.

Mummichog exposed to HAB biotoxins alter their frequencies of interaction, schooling, and shoaling behaviors, which can have deleterious effects at the individual and population level of organization. It has been demonstrated that contaminant exposures can alter shoaling, schooling, and grouping behaviors in other species as well. Atlantic silversides (*Menidia menidia*) exposed to carbaryl displayed alterations in parallel orientation and increased distances between fish when compared to controls (Weis and Weis, 1974). Similarly, schooling declined following exposure of yearling common carp (*Cyprinus carpio*) to DDT and of fathead minnows (*Pimephales promelas*) to the herbicide 2,4-dinitrophenol (Holcombe et al., 1980). In contrast, Atlantic silversides (*Menidia menidia*) exposed to copper decreased distances between fish and increased activity, describing an increase in polarization after exposure (Koltes, 1985).

Swimming orientation and cohesion in schools of threespine sticklebacks (*Gasterosteus aculeatus*) were disturbed following exposures to the organotin bis(tributyltin)oxide and butyl benzyl phthalate (Wibe et al., 2001; Wibe et al., 2002). Golden shiners (*Notemigonus crysoleucas*) decreased shoal cohesion after exposure to methyl mercury (Webber & Haines, 2003). Increased water temperatures decreased polarization, increased interactions and disorientation, and retarded schooling development in larval sand smelt (Williams & Coutant, 2003). Goldfish (*Carassius auratus*) exposed to different herbicides altered the frequency of grouping behaviors (Saglio & Trijasse, 1998; Saglio et al., 2003). This is the first study, to our knowledge, to utilize and measure simultaneously, schooling and shoaling as endpoints of exposure to HAB toxins and MS-222 in fish.

Exposure to HAB toxins and MS-222 not only altered the frequency of schooling and shoaling behaviors, but the properties of these behaviors as well. When groups of fish were shoaling during exposure, which was rare, distance and angles of orientation decreased when exposed to MS222, increasing cohesion. When exposed to STX and PbTx-2 shoaling fish increased distance and angles of orientation, decreasing cohesion.

These exposure-related decreases in schooling and shoaling correspond with significant increases in solitary and aggregative behaviors. The percentage of time fish spent aggregated and not moving increased with exposure to MS-222, describing a reduction in activity and increase in congregation. In addition, velocity of solitary fish decreased from exposure to MS-222. Similarly, exposures to STX and PbTx-2 resulted in significant increases in percentage of time fish were not in a group combined with decreases in velocity of solitary fish. After exposure, groups of mummichog spent less

time together, slowed down, and increased distances between individuals. Mummichogs spent less time in groups and more time solitarily after exposure, describing affected individuals separating from the group and slowing down. These individual responses to exposure can alter school/shoal dynamics of the group over time.

HAB and MS-222 toxin exposure resulted in significant alterations in the dynamics of schools, shoals, and aggregations, with exposure decreasing group sizes. When exposed to PbTx-2, STX, and MS-222, schools, shoals, and aggregations containing 5 fish significantly decreased in occurrence, while groups containing 3 fish increased. The ability to quantify alterations in group size dynamics resulting from HAB exposure is novel and provides interesting insight into how HAB events may impact fish populations. These alterations in dynamics and shift from 5 fish to 3 fish groups may have serious consequences in nature. However, group dynamics in the PbTx-2 exposures is reversed during exposure only, with groups increasing from 3 to 5 fish instead of 5 to 3. This result is similar to other PbTx-2 endpoints, in which exposure responses are opposite to MS-222 and STX, with similar responses 24 hours later. Initial responses to PbTx-2 exposure are opposite to that of MS-222 and STX, which may have to do with the fact PbTx-2 is excitatory and STX and MS-222 are paralytic. This increase in group size observed in the PbTx-2 exposures describes an initial increase in polarity and cohesion in exposed fish.

In addition to schooling and shoaling behaviors, altered startle response and predator avoidance behaviors occurred from HAB and MS-222 exposure. Several behavioral responses were altered by exposure, resulting in an inconsistent response to threatening stimuli. Exposed mummichogs did not react to a model visual predator or

auditory stimuli after exposure to MS-222. These fish were able to recover 24 hours later, but during exposure periods, mummichog were unable to properly react to stimuli, which would compromise survival in the wild. In contrast, mummichog exposure to STX and PbTx-2 did not induce alterations in startle or predator avoidance behaviors. However, mummichog exposed to STX displayed alterations in reaction 24 hours after exposure, demonstrating possible long-term exposure consequences. Mummichog exposed to PbTx-2 increased shoaling velocity during and decreased aggregation after exposure. This may have resulted because PbTx-2 is an excitatory neurotoxin, which would increase conspicuousness, reducing the ability to aggregate, decreasing activity, and possibly increasing the chance of predation. Significant increases in percent aggregation in PbTx-2 exposure, similar to MS-222, were abolished by exposure and then reversed in recovery, describing fluctuations in long term effects. Exposure to environmental mercury resulted in alterations in foraging (prey strikes and captures) as well as predator avoidance in adult and larval mummichog (Smith & Weis, 1997; Zhou & Weis, 1998). Similarly, sticklebacks exposed to TBTO (bis tributyltin oxide) had a significantly reduced response to a heron model, which included a delay in the response with less fish startling (Wibe et al., 2001). Results of this study confirm, through the use of a behavioral quantification system, that exposures to neurotoxic agents alter schooling and shoaling behaviors as well the composition of these groups, describing alterations in behavioral responses from sublethal HAB exposures.

The cellular targets for both PbTx-2 and STX in fish are voltage sensitive sodium channels (VSSCs) located in excitable skeletal, muscle, and neural tissue. As such, VSSCs are the target of a wide variety of neurotoxins that can bind to a specific receptor

site on the channel, altering functionality. Through binding to receptor site one on VSSCs, STX blocks sodium conductance (Henderson et al., 1973). This action eliminates ion current, rendering channels impermeable, blocks electrical signaling, and leads to temporary paralysis of neuronal signaling. This is one possible explanation for the decreases in schooling and shoaling in mummichog and alterations in predator avoidance responses. Brevetoxin, in contrast, binds to site 5 on VSSCs, altering the channel gating mechanism and forcing the channel to remain open. This results in disrupting the flow of sodium ions by maintaining the channel in an open configuration and preventing repolarization of the membrane (Deshpande et al., 1993; Rodriguez et al., 1994). This disruption shifts action potentials negatively by two fold, elongating the action potential and depolarization (Trainer & Baden, 1999; Jeglitsch et al., 1998; Dechraoui et al., 1999). This results in continuous neuronal signaling, which may explain the initial increases in behavior observed in PbTx-2 exposed mummichogs.

Few studies have investigated the effects of HAB neurotoxins on fish behavior. Changes in fish behavior may decrease their ability to evade predation, thereby increasing transfer of toxins to higher trophic levels (Scholin et al., 2000; Lefebvre et al., 2001). In zebrafish, STX induced significant effects on physiology, growth, and survival of larvae including a rapid loss of sensory function and paralysis within 48 hours with possible long-term effects (Lefebvre et al., 2004). Additionally, larval Japanese anchovy, *Engraulis japonica*, fed STX exposed sea bream larvae, demonstrated high mortality and behavioral alterations (White et al., 1989). Similarly, behavioral alterations, including loss of equilibrium and irregular swimming were observed in Atlantic herring, *Clupea harengus harengus*, fed crude extracts of *A. tamarense* (White, 1977). STX exposure

was implicated in mortalities of chub mackerel, *Scomber japonicus*, in Argentina (Montoya et al., 1996). Dying fish were seen gasping, swimming upside down and on their sides at the surface. Gut contents of dead mackerel contained STX laden salps as well as *A. tamarensis* cells (Montoya et al., 1996). In the laboratory, goldfish IP injected with PbTx-3 exhibited corkscrew swimming, loss of balance, and reduced sensitivity of hearing that is recoverable (Lu et al., 1997). This demonstrates the possibility that sublethal HAB toxin exposure may alter sensory systems during blooms, causing the inability of fish to respond to stimuli. HAB toxin exposed mummichogs exhibited alterations in startle response and predatory avoidance behaviors, which may have been linked to exposure alterations in sensory systems. It has been demonstrated that exposure of mummichog to HAB toxins increases brain activity, which may be an indication of CNS alteration (Chapter 5).

Using group behaviors as endpoints in toxicology studies enables the use of complex behaviors in bioassay testing and providing conclusions that may extrapolate more accurately to what occurs in nature. Inappropriate response to environmental and physiological stimuli due to toxicant exposure can have severe implications for survival (Webber & Spieler, 1994). Additionally, fish are an excellent model for studying ecological environmental pollutant effects since many behaviors are easily observed and quantified in a laboratory setting (Scott & Sloman, 2004). Even if fish are not overtly harmed through exposure to a chemical, their ability to function in an ecological context may be altered (Scott & Sloman, 2004). Ecological relevance of such exposures may stem from altered vigilance, startle response, schooling, feeding, prey conspicuousness, migration, and diurnal rhythmic behaviors (Zhou & Weis, 1998; Little & Finger, 1990).

Thus, behavior is a selective response that is constantly adapting through direct interaction with physical, chemical, social, and physiological aspects of the environment.

The name mummichog is a Native American word that translates to “going in crowds”, but exposure to HAB neurotoxins leaves them more vulnerable to environmental flux and predation by separating them. Groups of mummichog exposed to HAB neurotoxins displayed altered schooling, shoaling, and aggregative behaviors, which ultimately could cause changes in fitness. Mummichogs in this experiment decreased schooling and shoaling behaviors when exposed to neurotoxins, which would increase their susceptibility to predators. Increased predation not only would negatively impact fish populations, but it also transfers more HAB toxins to higher level predators and humans. Alterations in schooling, shoaling, and predator avoidance behaviors described here could increase toxin transfer to higher level predators through increasing consumption rate of exposed fish (Lefebvre et al., 2001). In addition to a defense against predation, groups of fish may also have reproductive and competitive benefits by living in groups. These benefits may decrease when exposed to HAB events, thereby reducing reproductive and foraging success. This study describes, with the use of a behavioral analysis system, groups of mummichog altering group behaviors and startle response behaviors as a consequence of low-level HAB exposure. Quantified alterations in group swimming dynamics and predator avoidance as shown in this study demonstrate complex changes in behavioral dynamics in mummichog exposed to HAB stress agents.

Chapter 5: Altered c-Fos Protein Expression in the CNS of Mummichog, *Fundulus heteroclitus*, Related to Harmful Algal Bloom Stress Agents

5.1 Abstract:

The immediate early gene *c-fos*, and its protein product c-Fos, are known to be induced in neurons of mammals and fish as a result of neuronal stimulation. The purpose of this study was to qualitatively examine alterations in the mummichog, *Fundulus heteroclitus*, brain in relation to harmful algal bloom (HAB) stressor exposure. c-Fos expression in the central nervous system (CNS) of mummichog was visualized using immunocytochemistry. Brevetoxin (PbTx-2), domoic acid (DA), and saxitoxin (STX), neurotoxins released from HAB species, were selected for laboratory exposures. In addition, a simulated transport stress experiment was conducted to investigate effects of physical stress on c-Fos induction. In a series of experiments, groups of fish were exposed to one of the selected stress agents, and brain sections were examined for c-Fos staining and expression was quantified by brain region. Fish exposed to DA, STX, and transport stress displayed significant alterations in neuronal c-Fos labeling when compared to controls ($p \leq 0.05$). DA, an excitatory amino acid, and transport stress significantly increased c-Fos expression in the optic lobes. In contrast, STX, a sodium channel blocker, significantly decreased expression in the optic lobes. Alterations in swimming behaviors were observed during DA and STX exposures and may correspond

to alterations in regional neuronal activity. This is the first study to quantify c-Fos protein expression in fish exposed to chemical stressors. c-Fos expression may serve as a biomarker of exposure that can link HAB related alterations in fish swimming behavior from HAB exposure to changes in brain activity. General alterations in brain activity, as well as knowledge of specific regions within the brain activated during stress, can provide valuable insights into the neural control of fish behavior as well as sublethal effects of HAB stressors, chemical and physical, on neurons.

5.2 Introduction

Behavioral changes in organisms can result from complex alterations at the biochemical and physiological level of organization. Knowledge of underlying biochemical and physiological processes that control behavior is crucial to the understanding of behavioral responses to stimuli. In the central nervous system (CNS), the command center of behavior, organisms possess mechanisms within neurons that provide biochemical responses to extracellular stimulation. These mechanisms can induce cellular changes, either by adapting to stimuli or through repair and regeneration, which are essential for survival. In many instances, these adaptations and repairs require alterations in gene expression. Adaptation to environmental conditions is only possible due to the rapid flexibility of neuronal signal transduction and downstream changes in gene expression (Rybnikova et al., 2003).

Immediate early genes (IEGs), located within the nucleus of neurons, are induced as a result of neuronal stimulation. This stimulation involves electrical or second

messenger, and requires no synthesis of transcription factors (Herdegen & Leah, 1998). As a result, IEGs can be induced rapidly with activation observed within 15-30 minutes of stimulus application. IEG activation then codes for new proteins (e.g. cytokines, enzymes, HSP70, oxygenase) as well as transcription factors to activate other genes. IEG encodes transcription factors that lead to expression of late genes that are responsible for phenotypic changes in cells (Herrera & Robertson, 1996). *c-fos* is an IEG that codes for the protein transcription factor, c-Fos. c-Fos then forms heterodimers with other transcription factors, such as c-jun or junB, creating an AP-1 complex (Herdegen & Leah, 1998). AP-1 complex proteins then bind to regulatory sites on DNA and activate other genes for transcription, thereby regulating a cellular response (Herdegen & Leah, 1998; Hansson et al., 2003). Neuronal excitation leads to an increase in *c-fos* mRNA, which in turn leads to increased AP-1 complex binding and regulation of other IEG expression (Sonnenberg et al., 1989; Zhang et al., 2002). These newly synthesized proteins and cellular products resulting from the AP-1 complex binding then initiate, regulate, or modify neuronal responses to stimuli.

The response of *c-fos* to extracellular stimuli functions as a transcription factor network to couple extracellular stimuli and gene expression (Bakin & Curran, 1999). This increase in genetic activity leads to a prolonged change in neurons, which can occur through alterations in depolarization, neurochemistry, or synaptic transmission (Hunt et al., 1987). Induction of IEG expression in the brain occurs under a variety of conditions and is believed to link external stimuli and long-term phenotypic alterations (Thiriet et al., 2001). It has been demonstrated that the *c-fos* pathway can regulate neuronal excitability and survival (Zhang et al., 2002). As a result, c-Fos induction can be used as

a biomarker of neuronal stress and to assess regional brain activation when animals are exposed to different types of stressful stimuli (Rybnikova et al., 2003; Martinez et al., 2002).

Studies have been conducted in rats to investigate the role of *c-fos* in the regulation of downstream genes or in behavioral or physiological responses associated with *c-fos* activation (Hansson et al., 2003). Previous studies have shown that *c-fos* can be induced in rats through glutamate receptor agonists, ion channel flux, light, magnetism, dioxins, spinal cord injury, stress (trauma), and the mind altering drugs haloperidol and clozapine (Abraham & Brewer, 2001; Ceccatelli et al., 1989; Cheng et al., 2002; Morgan & Curran, 1986; Morris et al., 1998; Murphy & Feldon, 2001; Nemeč et al., 2001; Sonnenberg et al., 1989; Zhang et al., 2002). In addition, *c-fos* induced genetic changes can allow habituation to substances and increase long-term memory (Tischmeyer & Grimm, 1999; Kaufer et al., 1998; Herdegen & Leah, 1998).

Immunohistochemistry (IHC) and immunocytochemistry (ICC) have been used to detect c-Fos protein as a marker of neuronal activity and to map neuronal pathways activated by certain stimuli (Morgan & Curran, 1991; Sagar et al., 1988). IHC and ICC have been used to label physiologically activated neurons in the brain from a variety of stress exposures (Hoffman & Lyo, 2002; Sagar et al., 1988). In addition, IHC has been used to compare and differentiate activated and inactivated neurons in specific regions of the fish brain in relation to a variety of stressors (Bosch et al., 1995).

An advantage of c-Fos quantification using IHC and ICC over other methods, such as 2-deoxyglucose (2DG), is increased resolution and detection of regional and cellular activity. In rats, c-Fos expression and ICC labeling is localized to neuronal

nuclei, providing a high degree of specificity by only labeling active cell bodies (Sagar et al., 1988). Further c-Fos activation does not provide the same information as the 2DG method, they provide different aspects of neuronal activation. c-Fos is activated in areas in which plastic change is occurring with activation, rather than in areas of activation without change (Sadananda & Bischof, 2002). Thus, c-Fos activation provides better resolution than 2DG when investigating adaptive alterations in neurons resulting from stress exposure.

Currently, research involving expression of c-Fos is mostly conducted with rats in an attempt to better understand human brain function. However, fish are exposed to many of the same stressors (xenobiotics, neurotoxins, and environmental estrogens) in the environment, and c-Fos induction may serve as an important measure of exposure. The *c-fos* gene maintains a strong homology between species and is highly conserved among fish species (Bosch et al., 1995). In fish, the nature of the c-Fos protein has not yet been elucidated (Bosch et al., 1995). Previous studies using fish have described c-Fos expression in the brainstem following startle response behaviors using IHC and quantification of RT-Fos mRNA following exposure to kanic acid (Bosch et al., 1995; Bosch et al., 2001; Matsuoka et al., 1998). However, there are no studies to date that have investigated the use of c-Fos expression in fish exposed to ecologically relevant stressors. The goal of this work was to develop and test the utility of c-Fos expression, visualized using ICC, as a biomarker of exposure and effect in mummichog, *Fundulus heteroclitus*, to chemical, physical, and transport stress.

The purpose of this study was to qualitatively examine harmful algal toxin exposure on alterations in brain activity using mummichog. This was accomplished

through the examination of brains from stressed and control fish using anti c-Fos polyclonal antibody staining. The stressors included brevetoxin (PbTx-2), domoic acid (DA), saxitoxin (STX), and simulated transport stress. All aqueous exposures were static. The goal was to link alterations in swimming behavior from harmful algal bloom (HAB) exposure and other stressors to changes in brain activity. In addition to general changes in brain activity, knowledge of the specific regions within the brain activated during stress will provide valuable insight into the neural control of fish behavior.

5.3 Methods

5.3.1 Exposures

Fish were exposed in 4 liter glass beakers containing 3 liters of static artificial seawater (6 PSU, pH 8, 25°C) to brevetoxin, saxitoxin, domoic acid, or simulated transport stress. Brevetoxin (PbTx-2, Dr. Dan Baden, UNCW) exposures were aqueous (40 ppb nominal concentration) and contained an emulsifying vehicle, EL-620 (0.001%). Saxitoxin (STX, Dr. Sherwood Hall, FDA) exposures were also aqueous (75 and 150 ppb, nominal concentrations). Domoic acid (DA, Sigma-Aldrich) exposure was through IP injection (5mg /kg). Three sets of controls were conducted: an artificial seawater control, EL-620 control, and a sham IP injection for the DA exposed fish (phosphate buffered saline, PBS). Sample sizes were 4 fish per treatment in the PbTx-2 and DA exposures and 6 fish per treatment in the STX and simulated transport stress. To simulate

transport stress, fish were placed in a bucket and gently agitated for 60 minutes. Control fish were placed in the same bucket but not agitated.

Fish were exposed and observed for 60 minutes in all treatments prior to processing. Behavioral observations were conducted every 20 minutes during the exposures, and any alterations were recorded. After exposure, fish were anesthetized with buffered MS-222 and perfused with ice cold heparinized PBS. Fish were opened on the left flank exposing the heart, and a syringe was inserted into the posterior aspect of the ventricle and into the bulbus arteriosus. The atrium was then cut and perfusion was initiated. The perfusion concluded after the gills blanched, indicating the perfusate had reached the brain. Once perfused, the cranium of each fish was dissected out leaving the brain exposed. The brain and cranium of the fish were then preserved in 10% neutral buffered formalin containing 2.5% acrolein for three hours. After three hours, the brain was dissected out of the cranium and transferred into 30% sucrose, where it remained until embedding.

Fish brains were then embedded into egg gel molds and post fixed in 4% paraformaldehyde overnight. Sucrose was then added to make a 10 % solution and remained at this concentration until the brains sunk to the bottom of the container. Sucrose was then added 2 more times in 10% increments, until the brains were immersed in 30% sucrose. The brains were frozen with dry ice and sectioned on a freezing sliding microtome at 25 μ m. The tissue was collected into a cryoprotectant/anti-freeze solution in a 1-12 series (Watson et al., 1986). Sections were stored at -20°C until processed for immunocytochemistry.

5.3.2 c-Fos Immunocytochemistry

A 1-3 series of brain sections was selected and rinsed in potassium phosphate buffered saline (KPBS), and incubated in sodium borohydride for 20 minutes to remove any residual acrolein. In order to enhance the signal resolution and reduce background staining, an antigen retrieval procedure was followed using a sodium citrate buffer (pH 6.0). Sections were rinsed several times with KPBS and then rinsed twice and microwaved in the citrate buffer for 30 seconds (5:1 solution of 0.01 sodium citrate and 0.01 M citric acid). Sections were then immediately rinsed with KPBS and immersed into the polyclonal primary antibody, sheep anti-c-Fos, (Chemicon, Temecula, CA) at a concentration of 1:1,000 in KPBS containing 0.4% Triton-X, and incubated at room temperature for 60 minutes, and then at 4°C for 48 hours.

After incubation in the primary antibody for 48 hours, sections were rinsed in KPBS, immersed in the secondary antibody, biotinylated rabbit-anti sheep IgG, (Vector, Burlingame, CA) 1:600 dilution in KPBS and 0.4% Triton-X, and incubated for 60 minutes at room temperature. Sections were rinsed again in KPBS, immersed into avidin-biotin complex (Vector Stain ABC kit, 45µl of avidin and 45µl of biotin per 10 ml of KPBS with 0.4% Triton-X), and incubated at room temperature for 60 minutes. After 60 minutes, sections were rinsed in KPBS followed by rinses of 0.175 M sodium acetate. Sections were then stained with nickel-DAB chromogen (0.002g 3,3 diaminobenzidine, Sigma-Aldrich, 0.25g Nickel Sulfate, and 8.3µl of H₂O₂ 10 ml of sodium acetate) for 20 minutes or until the sections were stained by observing under a light microscope. Upon

completion of the stain, sections were rinsed in sodium acetate followed by KPBS and stored at 4°C until mounting.

Sections of each fish brain were then floated in saline and mounted in order, anterior to posterior, onto gelatin subbed microscope slides and allowed to dry overnight. Once dry, slides were immersed through a series of ethanol concentrations (50 and 75% for 5 minutes, 95% for 10 minutes, followed by 100% ethanol for 20 minutes) and histoclear for 20 minutes. Finally, slides were cover slipped using histomount (National Diagnostics, Atlanta, GA), cleaned, and observed for differences in c-Fos expression between treatments using standard light microscopy.

5.3.3 Data Collection and Analysis

Six regions in the fish brains were compared (Fig. 46): the anterior telencephalon (area ventralis telencephali pars ventralis (Vv) and dorsalis (Vd)), the posterior telencephalon (diencephalic ventricle (DiV) and anterior parvocellular preoptic nucleus (PPa)), two regions in the optic tectum (anterior and posterior periventricular grey zone, L1 & L2), the midbrain tegmentum (ventrolateral nucleus of the torus semicircularis (TSvl), nucleus lateralis valvulae (NLV)), and the rhombencephalon (medial longitudinal fascicle (MLF)) (Peter et al., 1975; Wulliman et al., 1996). Images from each brain region were captured at 10X and levels of brightness and contrast were adjusted using Adobe Photoshop 6.0[©]. Once the pictures were adjusted, they were imported into NIH Image[©] (v. 1.61) and tissue area (square microns) and stain area (cells stained black, cytoplasmic and/or nuclear, square microns) were calculated. The percentage of stain

area to total region area was then calculated and the percentages were compared between treatments. A one tailed, two-way ANOVA was used to analyze the effects of concentration (stress verses control) and region of the brain (figure 46) on c-Fos expression, with the *a priori* hypothesis that DA, PbTx-2, and transport stress fish would increase expression (PROC MIXED, SAS, vs. 8.1, Cary, N.C.). DA, PbTx-2, and transport data were log transformed to meet the assumptions of the ANOVA procedure prior to analysis. For the STX exposure, concentration was treated as a continuous variable for the analysis. For all experiments, a Tukey-Kramer *post-hoc* mean comparison test was used to evaluate differences ($\alpha \leq 0.05$) between control and exposed fish.

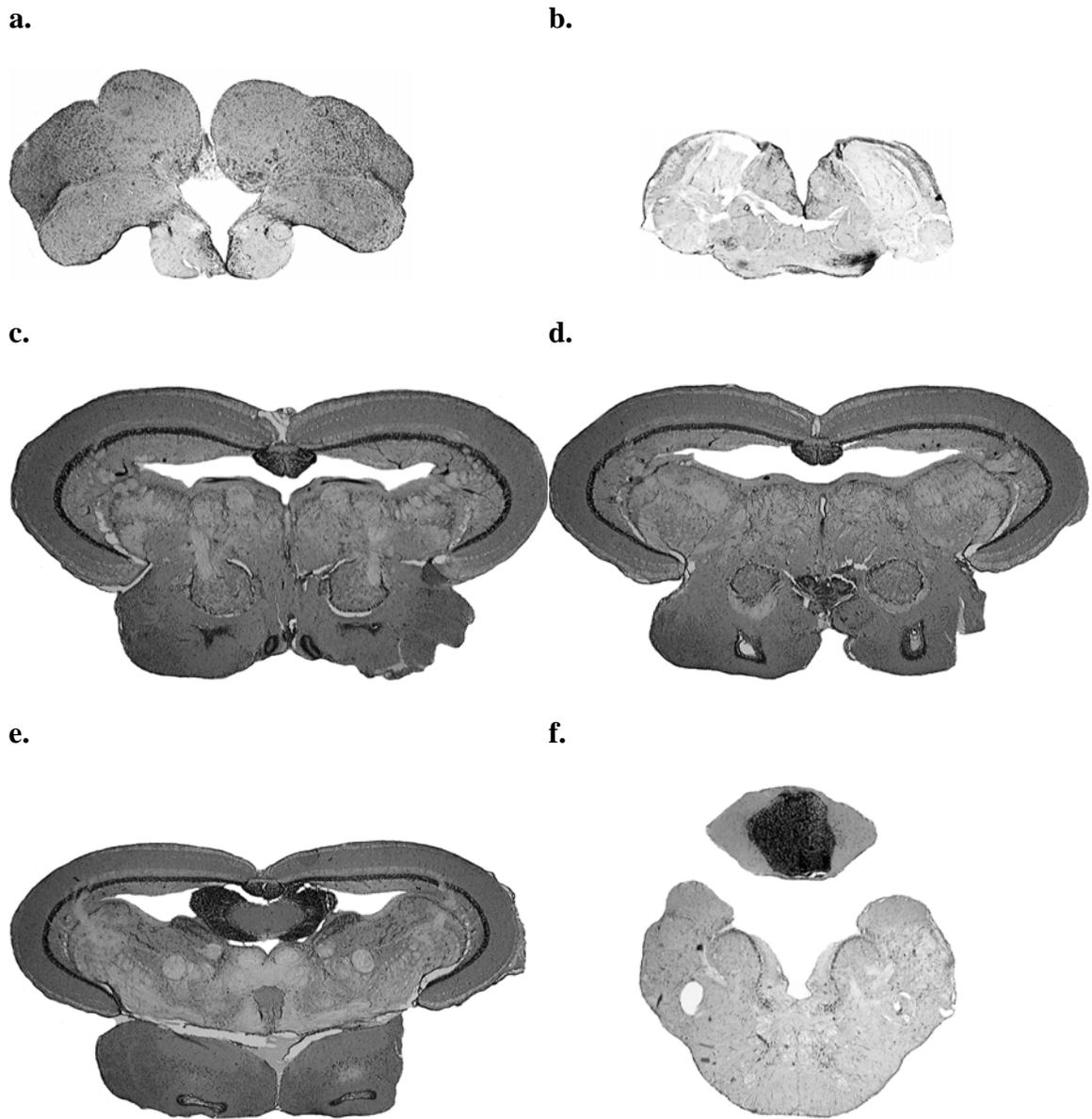


Figure 46 Regions (section level) of the mummichog brain investigated for alteration in c-Fos expression. Anterior telencephalon (a), posterior telencephalon (b), anterior optic lobe (c), posterior optic lobe (d), midbrain tegmentum (e), rhombencephalon (brain stem, f).

5.4 Results

Through the use of ICC, we were able to visualize consistent, reproducible c-Fos expression in the nucleus of mummichog neurons. Nuclear staining of c-Fos protein in mummichog neurons has not been previously demonstrated. Interestingly, Bosch et al. (1995; 2001), using IHC, illustrated cytoplasmic staining of c-Fos protein but found no evidence of nuclear staining. In this study, nuclear staining of c-Fos in the mummichog was found throughout the brain, but was quantitatively observed in the greatest amount in the optic lobes (Figs 51 - 54). This is the first report we know of describing neuronal nuclear staining of c-Fos protein in a teleost fish brain using ICC, which is reasonable considering c-Fos is a nuclear transcription protein. Similarly to Bosch et al. (1995), cytoplasmic staining was observed in the rhombencephalon and anterior brain stem of mummichog. Results demonstrated strong, punctate staining with exposure to chemical and physical transport stress. However, high variances were observed within all treatments with control fish expressing low levels of c-Fos protein in all regions investigated (Figs. 47 - 49). As a result of high variances between regions, no significant differences in c-Fos expression were found across the entire brain from exposure ($p > 0.05$). However statistically significant alterations in c-Fos expression were found within the optic lobe regions.

Regional differences were found between controls and simulated transport stressed DA, and STX, exposed fish, with only minor increases in PbTx-2. Significant alterations in c-Fos expression between control and exposed fish brains were observed in the anterior and posterior optic tecta (Figs 51 - 54). Stressor specific alterations in c-Fos

expression were observed, with simulated transport stress and DA significantly increasing, and STX significantly decreasing expression.

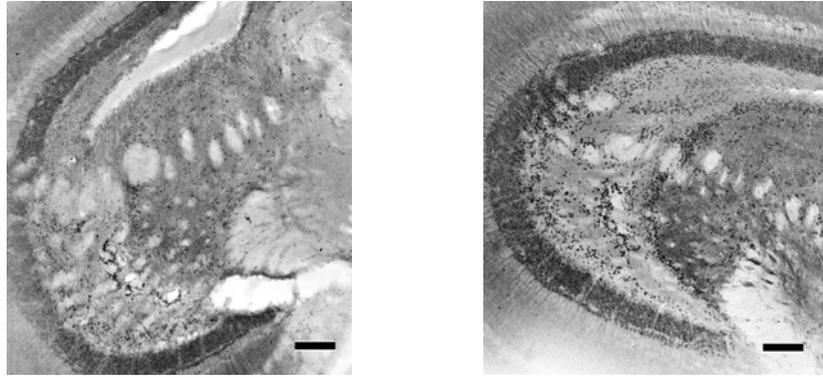


Figure 47 Representative optic tecta from control (left) and transport stressed (right) mummichog (10X magnification). Note the increase in c-Fos expression represented by black punctate nuclei in the stressed brain. Scale bar = 100 μ m.

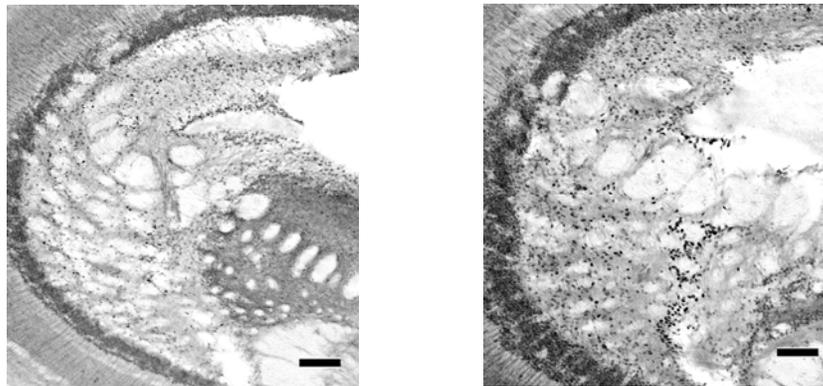


Figure 48 Representative optic tecta from sham control (left) and DA exposed (right) mummichog (10X magnification). Note the increase in c-Fos expression represented by black punctate nuclei in the exposed brain. Scale bar = 100 μ m.

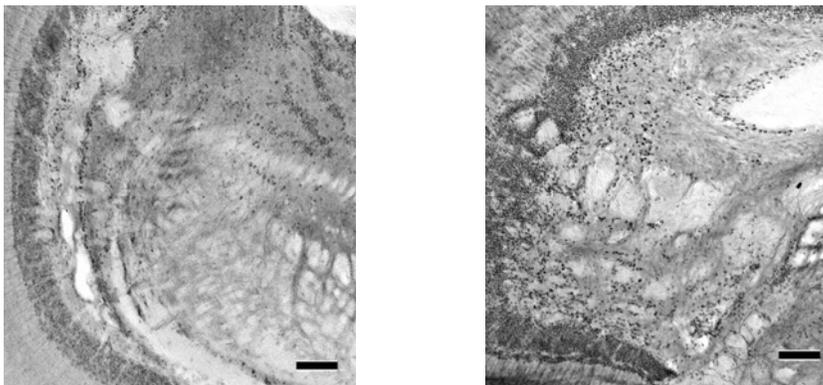


Figure 49 Representative optic tecta from control (left) and PbTx-2 exposed (right) mummichog (10X magnification). Note the minor increase in c-Fos expression represented by black punctate nuclei in the exposed brain. Scale bar = 100 μ m.

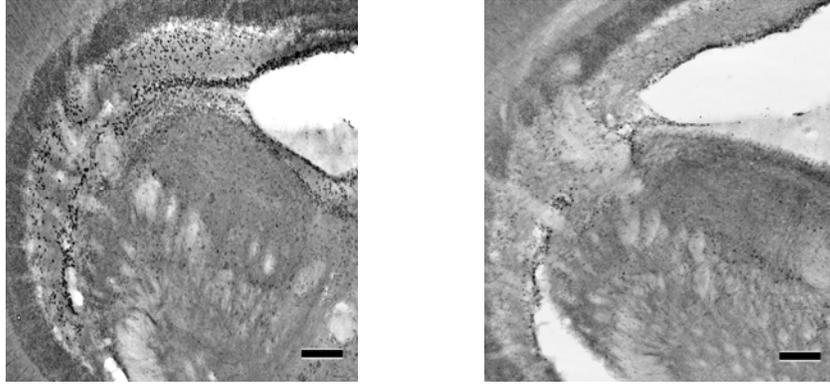


Figure 50 Representative optic tecta from control (left) and STX exposed (right) mummichog (10X magnification). Note the decrease in c-Fos expression represented by black punctate nuclei in the exposed brain. Scale bar = 100 μ m.

5.4.1 Simulated Transport Stress

Significant increases in c-Fos expression in simulated transport stressed fish were found in the anterior and posterior optic tecta ($p = 0.038$ and $p = 0.052$, respectively, Figs 47 and 51). The anterior and posterior telencephalon (area ventralis telencephali pars dorsalis, lateralis, and ventralis), and the dorsal midbrain tegmentum were not significantly different from controls, but did exhibit notably increased labeling ($p = 0.173$, 0.135 , and 0.335 , respectively Fig. 51). The rhombencephalon (brain stem) was not significantly different from controls ($p = 0.305$, Fig 51).

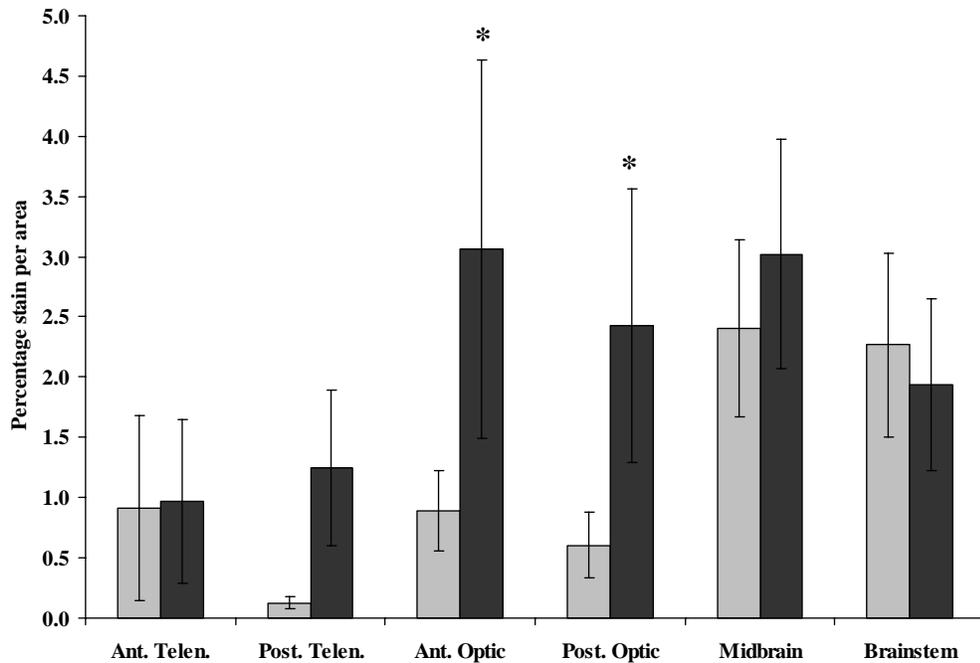


Figure 51 c-Fos expression in control (light gray) and transport stressed (dark gray) mummichog brains (N = 6). Ant. Telen. = anterior telencephalon, Post. Telen. = posterior telencephalon, Ant. Optic = anterior optic lobe, Post. Optic = posterior optic lobe, Midbrain = midbrain tegmentum, Brainstem = rhombencephalon. The y-axis is the percentage of c-Fos expression per area (sq. mm). Significant increases in c-Fos expression occurred in the Ant. and Post. Optic regions ($p = 0.05$, *).

5.4.2 DA Exposure

In addition to simulated transport stress, DA exposed fish displayed significant increases in c-Fos expression in the anterior optic lobe when compared to sham injected controls (Fig. 48). Behavioral alterations in DA exposed mummichogs were observed and recorded over the 60-minute exposure. Upon injection of DA, fish displayed hyperactivity followed by corkscrew swimming, listing, and twitching. DA exposed fish had a significant increase in c-Fos expression in the anterior optic tectum ($p = 0.055$, Fig. 52). The following brain areas were not significantly different in DA exposed fish when

compared to sham controls: anterior telencephalon (area ventralis telencephali pars dorsalis, lateralis, and ventralis), posterior telencephalon (area ventralis telencephali pars dorsalis, lateralis, and ventralis), posterior optic tectum, dorsal midbrain tegmentum, and the rhombencephalon (brain stem) ($p = 0.165, 0.435, 0.301, 0.245,$ and 0.435 respectively, Fig. 52). However, non-significant increased expression was observed in the anterior and posterior telencephalon and the midbrain tegmentum of DA exposed fish.

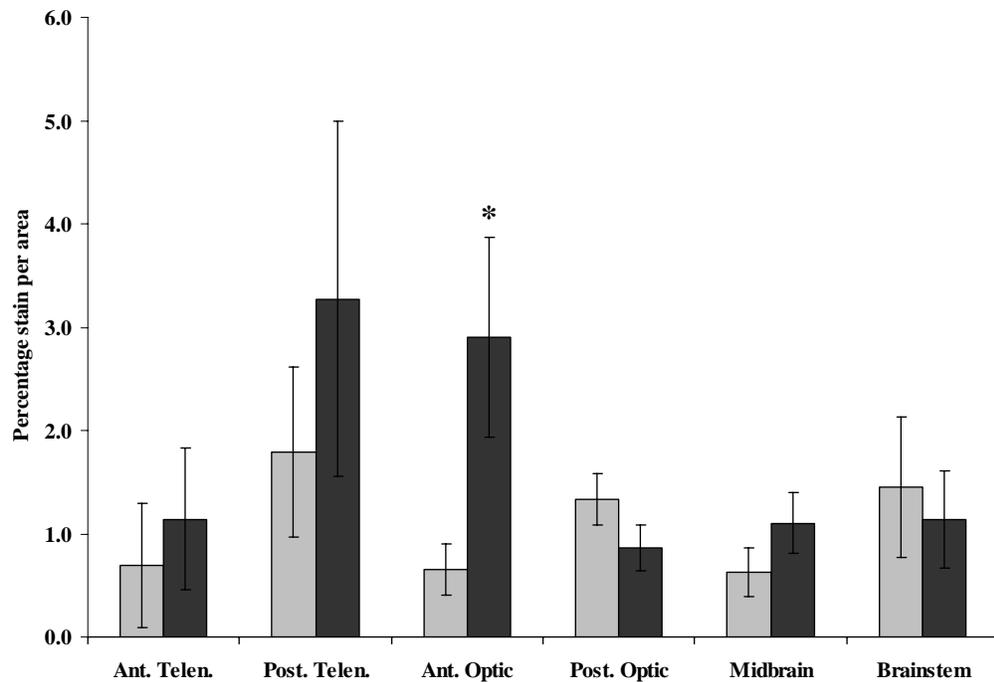


Figure 52 c-Fos expression in control (light gray) and domoic acid exposed (dark gray) mummichog brains ($N = 4$). Ant. Telen = anterior telencephalon, Post. Telen. = posterior telencephalon, Ant. Optic = anterior optic lobe, Post. Optic = posterior optic lobe, Midbrain = midbrain tegmentum, Brainstem = rhombencephalon. The y-axis is the percentage of c-Fos expression per area (sq. mm). Significant increases in c-Fos expression occurred in the Ant. Optic region ($p = 0.05, *$).

5.4.3 PbTx-2 Exposure

Non-significant increases in c-Fos labeling of PbTx-2 exposed fish did occur when compared to vehicle control (EL-620) in the anterior telencephalon, posterior optic tectum, midbrain tegmentum, and rhombencephalon (brain stem) (Figs 49 and 53). Emulphor EL-620, the vehicle control, is an emulsifying agent used to reduce sorption of the PbTx-2 to surfaces. Results demonstrate the need for appropriate controls when conducting c-Fos assays, as c-Fos increase from generalized stress may mask increases from the experimental stress exposure.

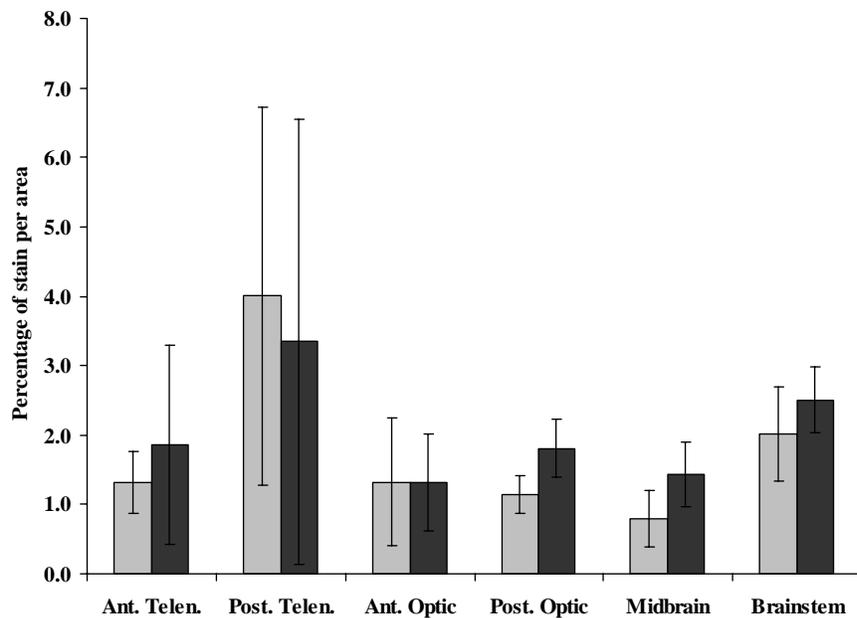


Figure 53 c-Fos expression in control (light gray) and PbTx-2 exposed (dark gray) mummichog brains (N = 4). Ant. Telen = anterior telencephalon, Post. Telen. = posterior telencephalon, Ant. Optic = anterior optic lobe, Post. Optic = posterior optic lobe, Midbrain = midbrain tegmentum, Brainstem = rhombencephalon. The y-axis is the percentage of c-Fos expression per area (sq. mm).

5.4.4 STX Exposure

In contrast to the PbTx-2, fish exposed to STX displayed significant decreases in c-Fos expression compared with controls (Figs 50 and 54). Decreased c-Fos expression occurred in the posterior telencephalon and rhombencephalon, with significant decreases in the optic lobes (0.0069, and 0.0005 respectively, Fig. 54). Behavioral alterations in STX exposed mummichogs were observed and recorded over the 60-minute exposure, including mild paralysis, floating at the surface and lying on the bottom, along with a decrease in general activity. Inhibition of c-Fos labeling in STX exposed fish is not surprising considering that the mechanism of action is neuronal signaling blockage of Na⁺ channels, opposite to that of PbTx-2, which maintains the channel in the open configuration, allowing for constant signaling.

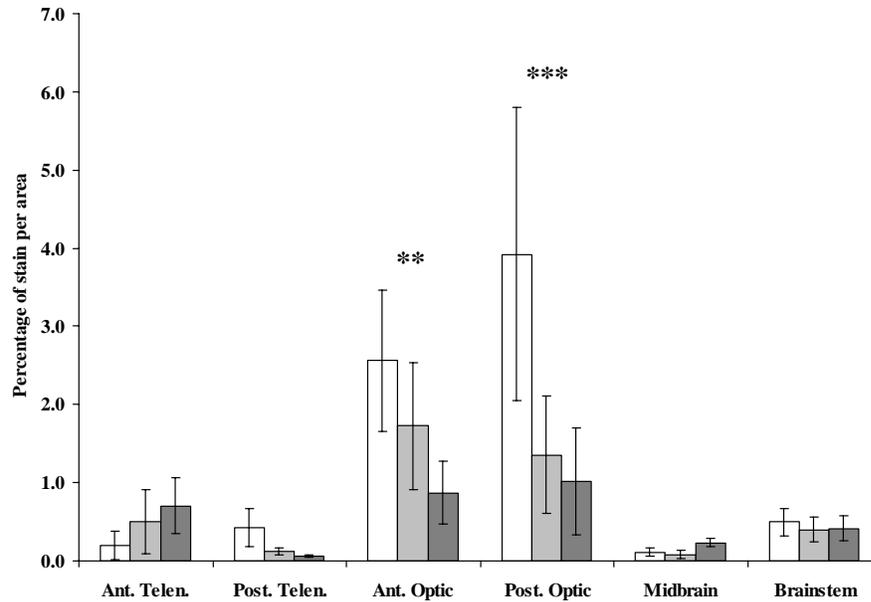


Figure 54 c-Fos expression in control (0 ppb, white bars), low (75 ppb, light gray) and high (150 ppb, dark gray) STX exposed mummichog brains (N = 6). Ant. Telen. = anterior telencephalon, Post. Telen. = posterior telencephalon, Ant. Optic = anterior optic lobe, Post. Optic = posterior optic lobe, Midbrain = midbrain tegmentum, Brainstem = rhombencephalon. The y-axis equals percentage of c-Fos expression per area (sq. mm). Dose dependant significant decreases in c-Fos expression occurred in the Ant. Optic and Post. Optic regions (p = 0.01, ** and 0.0001, *** respectively).

5.5 Discussion

c-Fos induction, measured through ICC and IHC staining, has been used as a biomarker of neuronal stress and stimulation from stress exposure (Hoffman & Lyo, 2002; Hansson et al., 2003; Zhang et al., 2002). In addition, *c-fos* induced genetic changes can allow habituation to substances and increase long-term memory (Kaufer et al., 1998; Herdegen & Leah, 1998). *c-fos* can be induced in 15 minutes in neurons *in vitro* resulting from extracellular concentration increases of potassium creating an influx in calcium (Morgan & Curran, 1986). Induction of *c-fos* decreased seizures and

increased neuronal survival in mice through down regulating NMDA receptors in the presence of increased calcium concentrations (Zhang et al., 2002).

This study successfully developed and reproduced an ICC method to detect c-Fos labeling in fish exposed to physical stress and neurotoxins using the mummichog model. Neuronal nuclear staining was visualized in the telen- (area ventralis telencephali), mesen- (optic tectum), and metencephalon (torus semicircularis), combined with cytoplasmic staining in the myelencephalon and rhombencephalon. The cytoplasmic staining observed in the rhombencephalon is consistent with previous studies of c-Fos expression in fish (Bosch et al., 1995; Bosch et al., 2001). These studies reported that differential expression of c-Fos was observed between brain regions, with the largest differences in staining between control and stressed fish occurring in the periventricular gray zone of the optic lobe. Stressor induced regional c-Fos alterations were found in mummichog in this current study, with physical stress and neurotoxin exposure altering expression in the optic lobes. In addition, we observed neuronal nuclear staining in the fore- and mid-brain in the mummichog in response to stress, which has not been previously described in fish.

These alterations in c-Fos expression were observed in mummichogs exposed to simulated transport stress (increase), domoic acid (increase), and saxitoxin (decrease) after sixty minutes. In addition, increases in c-Fos expression were noted in PbTx-2 exposures, however, no significant differences compared with vehicle controls were found. Additional, non-significant, increases in expression were noted in the posterior telencephalon (area ventralis telencephali), and midbrain tegmentum (torus ventrolateral nucleus of the torus semicircularis).

Increased c-Fos expression has been previously measured in rainbow trout, *Oncorhynchus mykiss*, exposed to vibratory stress. c-Fos protein and *c-fos* mRNA expression have been demonstrated to increase in rainbow trout resulting from vibratory stress and kanic acid exposures (Matsuoka et al., 1998; Bosch et al., 1995; Bosch et al., 2001). In rainbow trout exposed to vibratory stress, increased expression of c-Fos protein was detected in the preoptic nucleus, mesencephalon (medial longitudinal fasciculus), oculomotor nerve, torus semicircularis, and nucleus ruber (Bosch et al., 2001). Bosch et al. (2001) also located staining throughout the brain resulting from startle response stress, including areas not previously known to participate in the startle response, but significant differences were located in the rhombencephalon.

We found the largest difference between stressed and control mummichog in the optic tectum, a region where Bosch et al. (1995; 2001) found no staining in rainbow trout. In rainbow trout, c-Fos staining was located only in the cytoplasm and surrounding glial cells, with cells reacting with varying intensities (Bosch et al., 1995). Mummichog exposed in this study additionally displayed variance in stain intensity, but staining was nuclear in all regions investigated except for the brain stem. c-Fos expression occurred throughout the control mummichog as well, but in most cases, expression was less than in stressed fish. In rainbow trout, c-Fos expression was located in both control and treated brains, with significant increases in startled fish and controls expressing far less than stressed fish (Bosch et al., 1995; Bosch et al., 2001). Clearly, additional work is warranted to elucidate the patterns of c-Fos expression in fish. Results in mummichog from this study demonstrated c-Fos increases in the optic tectum, and suggest that there

may be variation in stress response and location of neuronal activity between different fish species.

The use of c-Fos induction as a measure of neuronal activity in fish is a more specific neuronal marker than 2DG, allowing for measures of activity at the neuronal level. Matsuoka et al. (1998) illustrated that mRNA quantification and IHC of *c-fos* provided better resolution than 2DG. Choich et al. (2004) demonstrated significantly increased 2DG labeling following PbTx-2 exposure in bluegill, but this method did not provide data showing regional specificity. This study, in contrast, was able to provide regional brain activity information, which in conjunction with 2DG experiments, can elucidate regional specificity in addition to activity.

The simulated transport stress experiment in this study constantly agitated the fish for 60 minutes, comprising a combination of stimuli, including visual, lateral line, and auditory cues. This overall stress is confirmed by the significant increase in c-Fos expression in the anterior and posterior optic tecta, as well as increases in all other regions investigated. Future work should investigate neuronal activation of c-Fos to simply visual or lateral line stimuli, in an attempt to better locate regional neuronal activation for each stimulus.

Domoic acid exposed mummichog in this study displayed significant increases in c-Fos expression in the anterior optic lobe. Additional non-significant increases in c-Fos expression over controls were observed in the telencephalon and optic lobe. In addition, behavioral alterations were observed in DA-exposed fish, including maintaining orientation and loss of equilibrium in the water column. Domoic acid is produced by *Pseudo-nitzschia* spp. blooms, and binds to AMPA/kinate/NMDA receptors and voltage

sensitive calcium channels (VSCCs). However, DA exposure is not the causative agent of fish kills in the wild (Lefebvre et al., 2002b). DA exposure increases intracellular calcium concentrations, creating alterations in neuronal signaling and survival (Berman et al., 2002). Exposure to DA can induce brain lesions, seizures, memory loss, and neuronal death in mice (Todd, 1993). In the wild, DA can bio-accumulate in fish, exposing predatory fish, marine mammals, birds, and humans to DA (Scholin et al., 2000; Lefebvre, 2001; Lefebvre et al., 2002a).

In wild caught anchovies, *Engraulis mordax*, tissue uptake of DA is minimal, with low concentrations in the brain (Lefebvre, 2001). However, anchovies exposed to DA through IP injection in the laboratory display alterations in swimming behavior, with an EC50 of 3.2 mg/kg, and an EC100 of 5mg/kg DA. Our study confirms alterations, at the neuronal level in mummichog, resulting from DA exposure in the laboratory. As a result, the use of c-Fos expression may provide location of neuronal activity when other standard measures of DA uptake fail to detect physiological alterations. c-Fos labeling may be used to investigate the effects of DA on neural activity in fish and may serve as an additional endpoint to DA exposure other than mortality and alterations in swimming. Results from this study support the notion of Lefebvre et al. (2002b) that anchovies exposed to DA in the wild may exhibit alterations in neural function, leading to alterations in swimming behaviors, and compromising predator avoidance. This may result in an increased predation rate, transferring DA to higher trophic levels such as seals, sea lions, and pelicans.

In contrast to the DA exposure, no significant differences between brevetoxin (PbTx-2) exposed and vehicle controls were found. However, non-significant increases

were observed in PbTx-2 exposed fish in the anterior telencephalon, posterior optic tectum, and midbrain tegmentum. This finding was surprising considering the alteration in neuronal signaling should be similar between DA and PbTx-2. PbTx-2 is a neurotoxin released by the harmful dinoflagellate species, *Karenia*. PbTx-2 has a specific binding affinity for voltage gated sodium channels (VSSC) in excitable membranes (Deshpande et al., 1993). PbTx-2 binding holds sodium channels open, which increases the influx of sodium ions, releases of excitatory amino acids, and activates NMDA receptors through increases of Ca^{2+} influx through the voltage gated calcium channel (VGCC) (Berman & Murray, 1999). Examples of ion channel modulators altering c-Fos expression include veratridine, carbamate, and pyrethroid pesticides (Imamura et al., 2002; Friedman et al., 1996; Morgan & Curran, 1986). This cascade of events, leading to Ca^{2+} influx and neuronal depolarization is similar to DA exposure, albeit through VSSCs instead of NMDA receptors.

There are several possible explanations for the difference in c-Fos expression observed in the DA and PbTx-2 exposures. c-Fos expression is a response to general stressors, and the increase resulting from PbTx-2 may have been masked by expression to other stressors. Increases in c-Fos expression resulting from EL-620 exposure suggest that the vehicle control may have increased c-Fos expression, leading to a lack of significant PbTx-2 response. This conclusion supports the notion of a clear and appropriate control group for comparison (Bosch et al., 1995). In addition, different exposure methods for the two neurotoxins were employed, DA were administered by IP injection (dose), while PbTx was added to the water (aqueous concentration), which may explain differences in c-Fos expression. Finally, effects of brevetoxin observed from this

experiment are based on relatively low, environmental concentrations. Higher, environmentally relevant brevetoxin concentrations may elicit greater c-Fos expression. Future studies are required, at higher concentrations, to investigate any significant effect of brevetoxin on c-Fos expression.

In STX-exposed fish, we found significant depression in optic lobe c-Fos expression compared to controls. This finding was expected since STX blocks VSSCs, resulting in a reduction in sodium influx and signal transmittance. STX mediates toxicity by obstructing the ion pore of the VSSC, thereby stopping flow of sodium into excitable cells, and resulting in nerve dysfunction (Anderson, 1997). STX is produced by approximately 11 *Alexandrium* species, a genus with a global distribution and a threat to human health, wildlife, and local economies (Landsberg, 2002; Velez et al., 2001). In addition, STX is produced from other dinoflagellate genera (*Gymnodinium* and *Pyrodinium*) as well as cyanobacteria (Landsberg, 2002). In fish, exposure to pure STX as well as algal cells caused edema, hyperplasia, and necrosis of the secondary lamellae, which was not proportional to concentration (Chen & Chou, 2001). STX could potentially affect neurons that control swimming and other motor functions, which may lead to a possible decrease in predator avoidance and prey capture behaviors.

Tetrodotoxin (TTX), a neurotoxin with the same mechanism of action as STX, has been demonstrated to inhibit c-Fos expression in the rat cortex, visual systems, and striatum (Hausmann et al., 2001; LaHoste et al., 2000; Lu et al., 2001). Additionally, scorpion venom neurotoxin (BmK) partially blocks VSSC channels and significantly inhibits c-Fos expression in rat spinal cords (Zhang et al., 2003). STX depression of c-Fos in fish further validates our experiments by demonstrating that fish neurons respond

similarly to mammalian neurons when exposed to similar stressors. In zebrafish, *Danio rerio*, STX induced significant effects on physiology, growth, and survival of larvae including a rapid loss of sensory function (Lefebvre et al., 2004). This study further demonstrates that exposure to sublethal concentrations of STX in adult fish can induce alterations in neuronal signaling and activity, possibly leading to individual and population level alterations.

Regional alterations of c-Fos expression in the brain of mummichog can have higher-level effects on behavior. Regions of the mummichog brain in which we observed increased c-Fos labeling, such as the optic lobe, are areas known to regulate locomotion and schooling behaviors (Smith, 1982). Teleost fish such as mummichog, have well-developed optic tecta, and cadmium exposures in fathead minnow, *Pimephales promelas*, resulted in alterations in the ability to school and maintain position in the column, thereby reducing predator avoidance (Sullivan et al., 1978). Locomotor behaviors are controlled by the diencephalon, mesencephalon, medulla, and ventrolateral peduncular neuropil (VLPN). The VLPN is located at the border of telencephalon and diencephalon and is the main integrative center for skeletal muscle, where sensory information is processed and translated to an appropriate motor response (Smith, 1982). In addition, the tegmentum motoricum in the metencephalon is an additional region of motor control. Increased c-Fos expression when coupled with observed alterations in swimming behaviors during exposures, may describe a link between regional brain activity and behavior.

Schooling behaviors are controlled by the telencephalon, diencephalon, mesencephalon (optic tectum), and medulla (Smith, 1982), regions in which increased c-

Fos expression was observed in mummichog. The optic tectum comprises a large area and is complex, with afferent and efferent fibers interconnecting almost all areas of the brain. Resultant processing of this input produces motor responses required to maintain proper position in the water column as well as position within the school. Our study confirms that alterations in behavior observed in the mummichog may be related to neuronal stress in the telencephalon, optic lobes, and mesencephalon. These resulting alterations may provide neuronal regions of behavioral control in fish, including general locomotor and schooling behaviors.

To our knowledge, this is the first study to investigate alterations in c-Fos expression resulting from HAB stress exposure in an environmentally relevant fish species. Mummichog is an ecologically important estuarine species with a cosmopolitan distribution on the East coast of the U.S., providing an important link into the effect of HAB events on estuarine trophic structure. High variability in expression may have resulted from other generalized stressors within the experiment increasing c-Fos expression, masking any real increase from HAB exposure. However, statistically significant alterations in c-Fos expression were observed in DA, STX, and transport stress exposed fish. The utility of c-Fos expression as a biomarker of HAB exposure in fish has potential for physical and vibratory stressors as well as chemical exposures. c-Fos expression, measured through ICC, can identify neural stress resulting from HAB exposure, prior to mortality and may link alterations in swimming behaviors with brain activity. These resulting physiological alterations may compromise the survival of populations of mummichog, and other estuarine fish species in the wild with short-term exposure to high concentrations of HABs having a deleterious effect. Alterations in c-

Fos expression provide an additional measure of HAB stressor exposure other than mortality in fish.

Chapter 6: Altered c-Fos Expression as a Measure of Neuronal Stress in Mummichog, *Fundulus heteroclitus*, Exposed to *Pfiesteria shumwayae* and *Chaetoceros concavicornis*

6.1 Abstract

In an attempt to better understand sublethal effects of physical stressors on fish, mummichog, *Fundulus heteroclitus*, were exposed to varying, environmentally-relevant densities of *Pfiesteria shumwayae* in the laboratory. In addition, we exposed fish to varying densities of *Chaetoceros concavicornis*, a harmful algal bloom (HAB) diatom species known to cause physical stress to gill lamellae and mortality in fish. This study qualitatively examined *P. shumwayae* (CCMP 2089) and *C. concavicornis* (CCMP 169) exposure on alterations in mummichog brain activity using c-Fos expression as a marker of exposure. c-Fos expression, quantified through immunocytochemistry (ICC), has been used as a biomarker of neuronal stress and stimulation from stress exposure in fish. Brains of exposed fish were removed, sectioned and stained, and neurons expressing c-Fos were quantified. *P. shumwayae* and *C. concavicornis* exposed fish brains showed significantly increased neuronal c-Fos labeling compared to control fish brains. A significant dose response relationship was observed, with increased labeling in fish exposed to higher cell densities for both HAB species tested. Fish exposed to both HAB species had increased labeling in the telencephalon, optic lobes, midbrain, and portions of the medulla. The most notable and statistically significant increases in expression were

observed in the telencephalon of *Pfiesteria*-exposed fish, and in the telencephalon and optic lobes of *Chaetoceros*-exposed fish. In addition, alterations in swimming behaviors were observed during the *P. shumwayae* exposures, which may be associated with the increased regional neuronal activity. Alterations in c-Fos expression as a biomarker of *P. shumwayae* exposure may link quantifiable changes in fish swimming behavior to changes in brain activity. Alterations in brain activity, as observed in this study, may lend insight into behavioral changes of fish exposed to *P. shumwayae* and *C. concavicornis* observed in the field and during laboratory assays.

6.2 Introduction

Pfiesteria shumwayae (CCMP 2089) and *Chaetoceros concavicornis* (CCMP 169) are harmful algal bloom (HAB) species that play a role in fish kills along the United States coastline. *P. shumwayae*, a member of the toxic *Pfiesteria* complex, are considered responsible for, and associated with, ulcerative lesions in fish along the mid-Atlantic of the United States (Burkholder et al., 1995; Burkholder & Glasgow, 1997; Glasgow et al., 1995; Glasgow et al., 2001a; Lovko et al., 2003). Fish kills in North Carolina and Maryland estuaries co-occurring with the presence of *Pfiesteria* complex species have brought HABs to the forefront of public attention in these coastal communities (Burkholder et al., 1992; Law, 2001; Magnien, 2001; Noga et al., 1996). In addition, *Pfiesteria* species have been discovered in other estuaries on the East coast including New York and South Carolina, as well as the Gulf Coast and in Europe (Ruble et al., 1999; Lewitus et al., 2002; Jakobsen et al., 2002; Glasgow et al., 2001a). In the

Chesapeake Bay region, *Pfiesteria* and *Pfiesteria*-like organisms were implicated in fish kills and human health issues in the Pocomoke, Kings Creek, and Chicamacomico river systems of the Chesapeake Bay in the Summer and Fall of 1997 (Magnien, 2001). As a result of the widespread distribution of the HAB species and public interest, research into the mechanisms of fish mortality and human illness continue.

It is unclear whether all strains of *Pfiesteria* complex organisms are toxic to fish, but knowledge regarding physical attraction and subsequent attachment to fish through the use of a peduncular organ, resulting in epidermal damage, is clearly recognized. *P. shumwayae* directly attaches to fish skin, gill, olfactory organs, and oral mucosa, causing extensive tissue damage and mortality in laboratory exposures (Berry et al., 2002; Vogelbein et al., 2001; Vogelbein et al., 2002). Physical damage to fish, resulting from exposure to *P. shumwayae*, leads to degradation of the mucal coat, necrosis, and sloughing of epidermal cells, leading to a complete loss of epidermal and respiratory epithelium (Vogelbein et al., 2001; Lovko et al., 2003). Similarly, Burkholder et al. (2001a) noted sloughing of epithelial tissue resulting from physical attack in a strain of *P. shumwayae* from the Neuse River, N.C. Strains of *P. piscicida* and *P. shumwayae* from the Neuse estuary displayed a strong positive attraction to fish excreta and mucus as well as digestion of cellular components (Cancellieri et al., 2001).

Physical exposure to *P. piscicida* and *P. shumwayae* concurrently are known to damage fish gill lamellae, and cause multi focal epidermal erosion and other skin lesions (Burkholder & Glasgow, 1997; Glasgow et al., 2001a; Noga et al., 1996). Direct physical contact between fish and *P. shumwayae* (CCMP 2089 and CAAE 101272) is the most consistent mechanism mediating fish mortality in laboratory exposures (Gordon & Dyer,

2005). Fish mortality resulting from *Pfiesteria* complex exposure is well-documented in the laboratory, however, less is known regarding fish kills in the field due to the complex assemblage of organisms present. Fish exposed to sublethal concentrations in the laboratory and then allowed to recover developed bacterial and fungal infections (Noga et al., 1996). Exposure to fungal, bacterial, and *Pfiesteria* complex organisms, combined with immune system changes may play synergistic roles in fish mortality in the wild (Dykstra & Kane, 2000; Law, 2001; Vogelbein et al., 2001).

Behavioral alterations have been observed and documented in fish from the wild and the laboratory. Menhaden were observed swimming “erratically,” writhing near the surface, and exhibiting “distress” in the Maryland *Pfiesteria* bloom of 1997 (Magnien, 2001). During the 1991 blooms in North Carolina, fish ‘walks’ were observed, including descriptions of fish writhing at the surface and actively trying to avoid bloom conditions (Burkholder et al., 1995). In the laboratory, exposure to *Pfiesteria* complex organisms causes a loss of equilibrium, disorientation, lethargy combined with periods of hyperactivity, general depression, decreased respiration, “wavering,” “fin twitching,” and settling to the bottom of the aquaria (Burkholder et al., 1995; Berry et al., 2002; Gordon et al., 2002; Lewitus et al., 1995; Noga et al., 1996). These documented alterations are observational only, at concentrations lethal to fish, with the mechanism(s) of action driving these behaviors unknown.

Similarly to *Pfiesteria* spp., *Chaetoceros* spp., have been linked to fish kills on the West coast of the U.S. through direct physical contact. *Chaetoceros* is a harmful diatom genus, commonly occurring in many temperate coastal waters, which can cause finfish mortalities at concentrations as low as 5 cells/ml in salmonid species (Bell et al., 1974;

Yang & Albright, 1992). *Chaetoceros* form long chains of bullet shaped cells that have hollow spines (setae). These spines are studded with even smaller spines (spinules) along their length (Yang & Albright, 1992). Upon contact, these silicon spines break off and penetrate the secondary lamellae of fish gill membranes, causing hyperplasia, hypertrophy, and partial or complete fusion of secondary lamellae. In addition, blooms as low as 5 cells/ml can cause gill edema, collapse of pillar cells, and detachment of epithelial cells in fish (Yang & Albright, 1992).

Fish mortality has been attributed to microbial infections due to damaged gill tissue, gill hemorrhage, or suffocation from excess mucus production (Yang & Albright, 1994; Bell, 1961). In addition, physiological parameters, including decreases in partial pressure of oxygen, ventilation volume, and oxygen consumption have been noted, with increases in respiration frequency, haematocrit, lactate, and glucose values. There is a general trend of increased mucus followed by hyperplasia, then hypertrophy in fish exposed to sublethal concentrations of *Chaetoceros* (Yang & Albright, 1992). The limitation on gill function leads to hypoxic conditions in the fish.

Mortality in salmonids exposed to blooms of *Chaetoceros* is caused by a combination of stressors, and not the algal bloom alone. *Chaetoceros* blooms exacerbate mortality due to other diseases such as vibriosis and bacterial renal disease. Exposure to 4-52 *Chaetoceros* cells/ml, suppresses neutrophils, lymphocytes, and thrombocytes of the immune system of salmon, with 15 cells/ml killing juvenile salmon (Yang & Albright, 1994). In addition, aquaculture facilities noted various effects of *Chaetoceros* blooms on penned salmon ranging from vibriosis and bacterial kidney disease (BKD) to high mortality (Albright et al., 1993). In aquaculture situations, vibriosis was the major cause

of death and no mortality occurred with exposure to other bloom species. This depression of the immune system is further compromised by the hypoxic conditions caused by the gill damage, with infected fish beings more susceptible to *Chaetoceros* blooms than healthy fish.

This present study investigated the effect of sublethal concentrations of *P. shumwayae* (CCMP 2089) and *C. concavicornis* (CCMP 169) on the physiological controls of behavior in fish, specifically, neuronal activity. *Pfiesteria* research in the past has used fish as “sentinels” of toxic bloom events, specifically using fish mortality and time to mortality as endpoints of interest to measure *Pfiesteria* strain toxicity in laboratory studies (Burkholder et al., 2001c; Gordon et al., 2002; Lovko et al., 2003; Vogelbein et al., 2001). In contrast, this study examines underlying neuronal control as an endpoint of exposure, since behavioral alterations are controlled by the central nervous system (CNS) and have been documented in the field and laboratory. Sublethal HAB exposure may lead to alterations in fish behavior, including compromised foraging behavior, prey capture, reproduction, and predator avoidance. These behavioral alterations, whether acute or chronic, may induce population and community changes in estuarine communities.

c-fos, an immediate early gene (IEG), and its protein product c-Fos, are induced in neurons as a result of neuronal stimulation. Through the use of c-Fos expression, neural stress can be used as a biomarker of stress in fish (Chapter 5). In mammals, c-Fos induction, visualized through immunocytochemistry (ICC) or immunohistochemistry (IHC), has been used as a specific biomarker of neuronal activation, stress, and stimulation (Hoffman & Lyo, 2002). ICC has been effectively used to map patterns of

functional activation of c-Fos to a variety of stimuli, including traumatic stimulation and physiological agents (Cheng et al., 2002). The result of the *c-fos* induction and c-Fos expression is a regulation of cell survival and excitability. c-Fos mediates excitotoxicity and neuronal survival, decreases seizures, neuronal excitability, and cell death.

Mediation occurs through direct gene regulation, which alters target gene expression, mediating neuronal excitability, and survival post stimulation (Zhang et al., 2002). As a first step toward the goal of understanding behavioral alterations resulting from *Pfiesteria* exposure, this study exposed an ecologically relevant estuarine fish species to *P. shumwayae* and *C. concavicornis*, and investigated exposure-related neuronal stress using alterations in c-Fos expression. These data will help to link exposure with behavioral change (Chapter 4) and neuronal stress in fish.

The purpose of this study is to qualitatively examine sublethal *P. shumwayae* and *C. concavicornis* exposures on alterations in regional brain activity using mummichog and c-Fos immunocytochemistry. This will be accomplished through the examination of exposed and control brains with anti c-Fos polyclonal antibody staining. The goal is to link alterations in swimming behavior from *Pfiesteria* exposure to alterations in brain activity. In addition to general changes in brain activity, knowledge of the specific regions within the brain activated during stress can provide valuable insights into the neural control of fish behavior, and may lend insight to behavioral alterations witnessed in the field and laboratory.

6.3 Methods

6.3.1 Test Species

Mummichog is an essential species to the success and productivity of estuarine marsh environments. These small cyprinidontid teleosts regulate the trophic structure of marsh systems through control of invertebrate populations (crustaceans and annelids), as well as by serving as prey for larger predators such as blue crabs, striped bass, and shore birds. These eurythermal and euryhaline fish live in a variety of salt, estuarine, and brackish marshes, maintaining a cosmopolitan distribution along the East coast of the United States. Due to their ubiquitous range, wide tolerances, and ecological importance, mummichog have become an important species of marine scientific research (Atz, 1986). Mummichog are year-round residents in most estuarine systems with a small home range, providing an excellent model to study as sentinels for toxicity (Eisler, 1986; Weis et al., 2001). Mummichog used in this experiment were collected from reference sites, treated for ectoparasites, and laboratory-acclimated for 4 weeks prior to experimentation. Temperature, pH, and salinity of the experimental arenas were maintained at the same values as holding tanks (Tables 6 and 7).

6.3.2 Exposures

6.3.2.1 *Pfiesteria* Exposure

P. shumwayae cultures, maintained at the Virginia Institute of Marine Science, were used to expose adult mummichog in 9.5 L tanks without aeration. Mummichog were exposed (static) for 120 minutes to 5 cell densities: 0, 1000, 2000, 26,000, and 70,000 c/ml. Cell densities selected for exposures fall within environmentally relevant concentrations as well as those of other laboratory studies (Burkholder et al., 1995; Burkholder et al., 2001a; Lewitus et al., 1995; Vogelbein et al., 2001; Vogelbein et al., 2002). Cell densities were determined similar to the methods of Lovko et al., (2003). Samples were fixed in Lugols iodine (0.5-1.0%), mixed, and then centrifuged at 3000 rpm for 10 minutes at room temperature. Samples were then gently decanted, resuspended, and counted in a hemacytometer (Neubauer). Cells were added to the water in the respective exposure aquaria, and then fish were added to the aquaria, 8 per treatment, 40 total. Water quality parameters were measured and adequately maintained throughout the exposures based on vessel loading (Table 6). Behavioral observations were conducted every 20 minutes during the exposure, and any alterations were recorded. In addition, 3 fish per treatment were sacrificed after the exposure for general gill and skin histology.

Table 6 Water quality parameters of the *Pfiesteria shumwayae* exposures

Treatment	Temperature (°C)	pH	Unionized Ammonia (ppm)	Nitrite (ppm)
Control	22	7.8	0.011	0.4
1000 c/ml	22	7.8	0.006	0.2
2000 c/ml	22	7.4	0.045	4.0
26000 c/ml	22	7.8	0.056	2.0
70000 c/ml	22	7.9	0.140	4.0

6.3.2.2 *Chaetoceros* Exposure

C. concavicornis cultures were obtained from Robert Andersen (CCMP, Bigelow Laboratory, Boothbay Harbor, ME) and maintained at the University of Maryland Aquatic Pathobiology Center. Mummichogs (N=6 per treatment) were exposed (static) to 3 cell densities, 0, 800, and 5000 c/ml in 1.5L vessels without aeration for 120 minutes. Cell densities were determined through the use of a hemacytometer. Behavioral observations were conducted every 20 minutes during the exposure, and any alterations were recorded. Water quality parameters were measured and adequately maintained throughout the exposures based on vessel loading (Table 7). In addition, 3 fish per treatment were sacrificed after the exposure for general gill and skin histology.

Table 7 Water quality parameters of the *Chaetoceros concavicornis* exposures

Treatment	Temperature (°C)	pH	Unionized Ammonia (ppm)
0 c/ml	24.5	8.31	0.038
800 c/ml	24.5	8.38	0.038
5000 c/ml	24.5	8.44	0.069

6.3.3 Tissue Collection and Preparation

Fish were exposed to either *P. shumwayae* or *C. concavicornis* for a total of 120 minutes and sacrificed with an overdose of buffered MS-222. Fish were then perfused using a method described in chapter 5 (5.3.2). Once perfused, the dorsal aspect of the cranium of each fish was dissected out leaving the brain exposed. The brain and cranium of the fish were then preserved in 10% neutral buffered formalin containing 2.5% acrolein for three hours. After three hours, the brain was dissected out of the cranium and transferred into 30% sucrose, where it remained until embedding. Samples of skin and gills were collected for histopathology (Kane, 1996). Three gill arches were carefully dissected out from the left side; a piece of skin including muscle was obtained from the left flank. Tissues were preserved in 10% neutral buffered formalin and processed for routine histology (Profet et al., 1992). Tissue sections on glass slides were observed for pathology using light microscopy. Gill and skin are sensitive organs known to be altered by exposure to *C. concavicornis* and *P. shumwayae*, respectively.

Preserved fish brains were then transported to the University of Maryland Medical School, embedded into egg gel molds, and placed into 4% paraformaldehyde overnight. Sucrose was then added to make a 10% solution: brains remained in this concentration until the brains sank to the bottom of the container. Sucrose was then added 2 more times in 10% increments, until the brains were immersed in 30% sucrose. The brains were frozen with dry ice and sectioned on a freezing sliding microtome at 25 μ m. Tissue slices were collected in a 1-12 series and stored in a cryoprotectant/anti-

freeze solution (Watson et al., 1986). Sections were stored at -20°C until processed for immunocytochemistry.

6.3.4 c-Fos Processing

A 1-3 series of brain sections were selected and rinsed in potassium phosphate buffered saline (KPBS), and incubated in sodium borohydride for 20 minutes to remove any residual acrolein. In order to enhance the signal resolution and reduce background staining, an antigen retrieval procedure was followed using a sodium citrate buffer (pH 6.0). Sections were rinsed several times with KPBS and then rinsed twice and microwaved in the citrate buffer for 30 seconds (5:1 solution of 0.01 sodium citrate and 0.01 M citric acid). Sections were then immediately rinsed with KPBS and immersed into the polyclonal primary antibody sheep anti-c-Fos, (Chemicon, Temecula, CA) at a concentration of 1:1,000 in KPBS containing 0.4% Triton-X, and incubated at room temperature for 60 minutes and then at 4°C for 48 hours.

After incubation in the primary antibody for 48 hours, sections were rinsed in KPBS, immersed in the secondary antibody (biotinylated rabbit-anti sheep IgG, Vector, Burlingame, CA, 1:600 dilution in KPBS and 0.4% Triton-X), and incubated for 60 minutes at room temperature. Sections were then rinsed again in KPBS, immersed into avidin-biotin complex (Vector Stain ABC kit, 45 μl of avidin and 45 μl of biotin per 10 ml of KPBS with 0.4% Triton-X), and incubated at room temperature for 60 minutes. After 60 minutes, sections were rinsed in KPBS followed by 2 rinses of 0.175 M sodium acetate. Sections were then stained with nickel-DAB chromogen (0.002g 3,3

diaminobenzidine, Sigma-Aldrich, 0.25g Nickel Sulfate, and 8.3 μ l of H₂O₂ in 10 ml of sodium acetate) for 20 minutes or until the sections were stained as observed under a light microscope. Upon completion of the staining process, sections were rinsed in sodium acetate followed by KPBS and stored at 4°C until mounting.

Sections of each fish brain were then floated in saline and mounted in order, anterior to posterior, onto gelatin-subbed microscope slides and allowed to dry overnight. Once dry, slides were immersed through a series of ethanol concentrations (50 and 75% for 5 minutes, 95% for 10 minutes, followed by 100% ethanol for 20 minutes) and histoclear for 20 minutes. Finally, slides were cover-slipped using histomount (National Diagnostics, Atlanta, GA), cleaned, and observed using standard light microscopy for differences in c-Fos expression between treatments.

6.3.5 Data Analysis

Six regions in the fish brains were compared: the anterior telencephalon (area ventralis telencephali pars ventralis (Vv) and dorsalis (Vd)), the posterior telencephalon (diencephalic ventricle (DiV) and anterior parvocellular preoptic nucleus (PPa)), two regions in the optic tectum (anterior and posterior periventricular grey zone, L1 & L2), the midbrain tegmentum (ventrolateral nucleus of the torus semicircularis (TSvl) and nucleus lateralis valvulae (NLV)), and the rhombencephalon (medial longitudinal fascicle (MLF)) (Peter et al., 1975; Wulliman et al., 1996), (Fig. 46, Chapter 5). Images were captured at 10X and adjusted levels of brightness and contrast using Adobe Photoshop 6.0[©]. Once the pictures were adjusted, they were imported into NIH Image[©] (v. 1.61)

where tissue area (square microns) and stain area (cells stained black, cytoplasmic and/or nuclear, square microns) were calculated. The percentage of stain area to total region area were then calculated and compared between treatments. A two-way ANOVA was then used to analyze the effect of concentration and region (Fig 46) of the brain on c-Fos expression (PROC MIXED, repeated, SAS, vs. 8.1, Cary, N.C.). *Pfiesteria* exposure data were log transformed to meet the assumptions of the ANOVA procedure prior to analysis. Concentration was treated as a continuous variable for the analysis and a Tukey-Kramer *post-hoc* mean comparison test was used to evaluate differences ($\alpha \leq 0.05$) between control and exposed fish.

6.4 Results

Results demonstrated strong, punctate staining of neurons expressing c-Fos activity with exposure to *P. shumwayae* and *C. concavicornis*. However, high variances were observed within treatments as well as with control fish expressing c-Fos in all regions of the brain investigated (Figs 55 and 56). Differential expression in c-Fos expression was observed between brain regions, with the largest differences in staining between control and stressed fish occurring in the telencephalon and optic lobe. These findings are similar to other studies utilizing c-Fos expression in fish (Bosch et al., 1995; Bosch et al., 2001) (Chapter 5, Figs. 47-50).

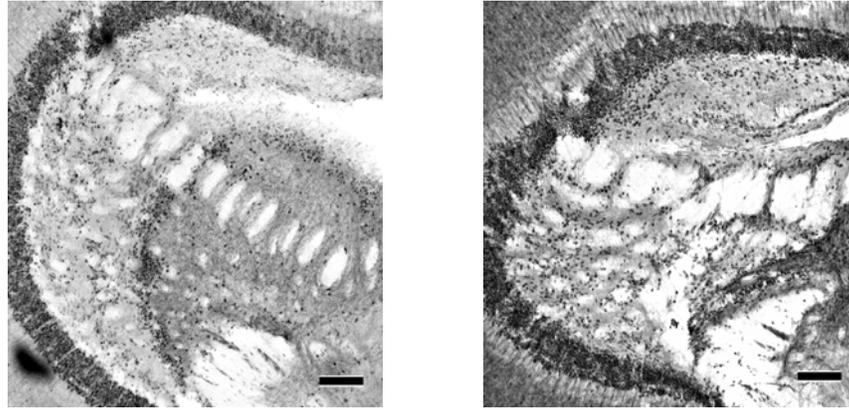


Figure 55 Representative optic tecta from control (left) and *Pfiesteria* exposed (2000 cells/ml, right) mummichog (10X magnification). Note the increase in c-Fos expression represented by black punctate nuclei in the exposed brain.

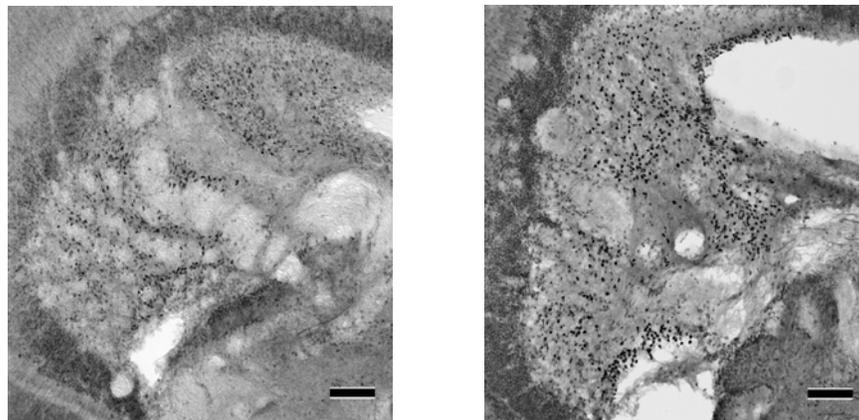


Figure 56 Representative optic tecta from control (left) and *Chaetoceros* exposed (5000 cells/ml, right) mummichog. Note the increase in c-Fos expression represented by black punctate nuclei in the exposed brain. (10X magnification)

6.4.1 *Pfiesteria* Exposure

c-Fos expression significantly increased as *P. shumwayae* densities increased in the anterior and posterior telencephalon ($p = 0.0001$ and 0.0002 respectively, Fig. 57). c-Fos expression in optic lobe region asymptotically increased as *P. shumwayae* densities increased, with a notable increase in the anterior optic lobe ($p = 0.0869$, Fig. 57). Non significant increases in labeling occurred in the midbrain tegmentum and rhombencephalon (brain stem) ($p > 0.05$, Fig. 57). All brain regions investigated displayed greater labeling from exposed fish than control fish, when exposed to increasing concentrations of *P. shumwayae*, (Fig. 57). Behavioral alterations were observed, with fish “wavering” in the water column and “twitching fins” (Berry et al., 2002). Additionally, no abnormalities were observed in the gill or skin samples from the exposed fish when compared with controls.

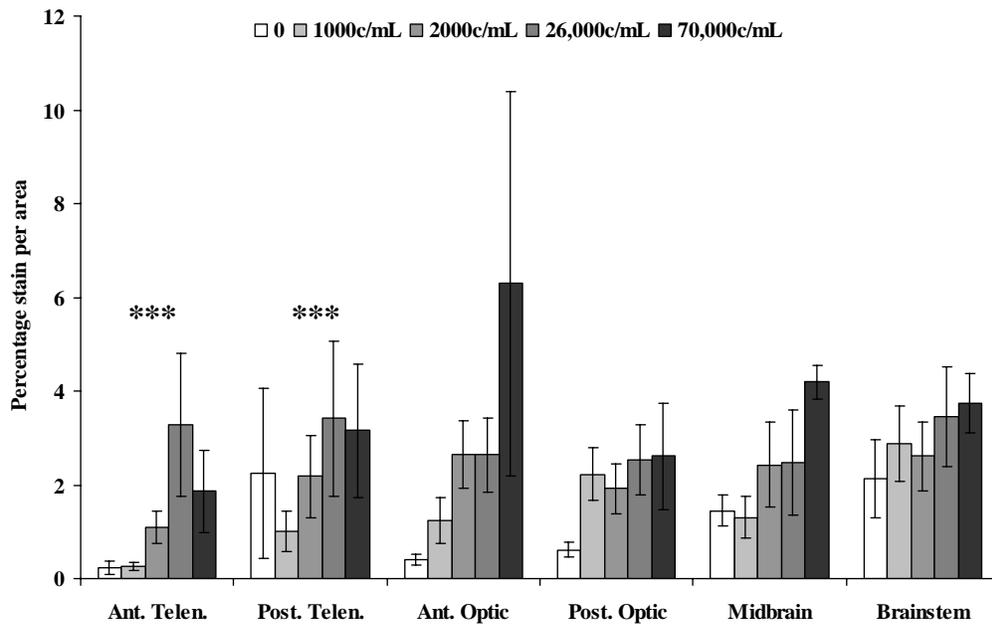


Figure 57 c-Fos expression at varying densities (0, 1000, 2000, 26,000, and 70,000 cells/ml) of *Pfiesteria shumwayae* in mummichog brains (N = 8). Ant. Telen. = anterior telencephalon, Post. Telen. = posterior telencephalon, Ant. Optic = anterior optic lobe, Post. Optic = posterior optic lobe, Midbrain = midbrain tegmentum, Brainstem = rhombencephalon. The y-axis equals percentage of c-Fos expression per area (sq. mm). Significant increases in c-Fos expression occurred in the Ant. Post. Telen. regions (p = 0.0001, *** and 0.0002, *** respectively).

6.4.2 *Chaetoceros* Exposure

Similarly to *Pfiesteria*, exposure to *C. concavicornis* resulted in an increased c-Fos expression when compared with controls. Increases were found with exposure to both densities of *Chaetoceros* in all regions, with significant increases in the anterior telencephalon, anterior optic lobe, and posterior optic lobe (p = 0.002, 0.0172, and 0.0542 respectively, Fig. 58). In addition, there was a dose response relationship, with increasing densities of *Chaetoceros* increasing c-Fos expression. The resultant data for exposures to both algal species were similar with increases in the same regions. In

contrast to the *Pfiesteria* exposures, no behavioral alterations were observed in *Chaetoceros* exposed fish. Similarly, no abnormalities were observed in the gill or skin samples from the *Chaetoceros* exposed fish when compared with controls.

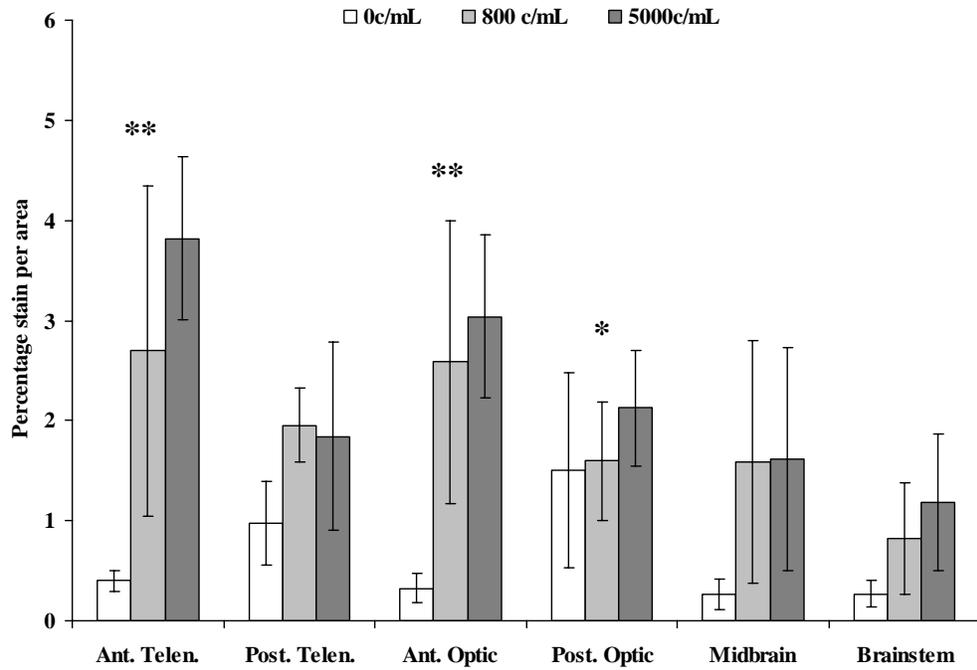


Figure 58 c-Fos expression at varying densities (0, 800, and 5000 cells/ml) of *Chaetoceros concavicornis* in mummichog brains (N = 6). Ant. Telen. = anterior telencephalon, Post. Telen. = posterior telencephalon, Ant. Optic = anterior optic lobe, Post. Optic = posterior optic lobe, Midbrain = midbrain tegmentum, Brainstem = rhombencephalon. The y-axis equals percentage of c-Fos expression per area (sq. mm). Significant increases in c-Fos expression occurred in the Ant. Telen, Ant. Optic, and Post. Optic regions (p = 0.002, **, 0.0172, ** and 0.0542, *, respectively).

6.5 Discussion

Exposure of mummichog to varying densities of *Pfiesteria shumwayae* and *Chaetoceros concavicornis* resulted in increased c-Fos expression. These increases are an indicator of changes in neuronal activity resulting from stress exposure in these fish. Both HAB species investigated here are proven harmful to fish and are known to cause fish mortality at high cell densities.

Strains of *P. shumwayae* directly attach to fish epithelial tissue such as skin, gills, and olfactory organs, resulting in sloughing of tissue and digestion of cellular components (Burkholder & Glasgow, 1997; Burkholder et al., 2001a; Vogelbein et al., 2002). This strategy of micropredation, through direct contact, has been determined to be the major cause of death in fish bioassays with *P. shumwayae* (Vogelbein et al., 2002; Gordon & Dyer, 2005; Lovko et al., 2003; Burkholder et al., 2001a; Berry et al., 2002). In contrast, no histological alterations were observed in the gills or skin of mummichog from this 2 hour *P. shumwayae*, exposure. This may have resulted from the short exposure period, with longer exposures (> 2 hr) possibly inducing gill and/or skin pathology. In previous research involving fish bioassays with *P. shumwayae*, fish have been viewed as sentinels, and mortality has been the observed endpoint (Berry et al., 2002; Burkholder & Glasgow, 1997; Burkholder et al., 2001a; Burkholder et al., 2001c; Lovko et al., 2003; Noga et al., 1996; Vogelbein et al., 2002). In contrast, this study examined the effect of sublethal exposure of fish to environmentally relevant densities of

P. shumwayae. The ICC method of c-Fos expression (Chapter 5) was used to investigate the sublethal effects of *P. shumwayae* exposure on neuronal activity and stress.

Alterations in fish behavior resulting from exposure to *Pfiesteria* have been observed and documented in both field and laboratory experiments. During bloom events in the Chesapeake Bay and North Carolina estuaries, fish were observed writhing at the surface and showing signs of distress (Burkholder et al., 1995; Magnien, 2001). In addition, some behaviors observed in the field have been documented during laboratory exposures. For example, hybrid tilapia (*Oreochromis* spp.) exposed to *P. shumwayae* (CAAE 101272) displayed hyperactivity followed by periods of inactivity (Gordon et al., 2002). Fish exposed to *P. piscicida* darkened in coloration, displayed a loss in equilibrium combined with periods of hyperactivity and lethargy, and congregated at the bottom of the aquaria (Noga et al., 1996; Lewitus et al., 1995; Burkholder & Glasgow, 1995). Such data suggests that fish may be actively trying to avoid blooms, illustrating behavioral alterations associated with *Pfiesteria* exposure. Although behavioral changes in fishes resulting from *Pfiesteria* exposures have been documented, the neural mechanisms controlling these behaviors remain poorly understood.

Mummichog exposed to *P. shumwayae* (CCMP 2089) in this study exhibited alterations in behavior similar to work conducted by Berry et al. (2002). Fish were observed to be “wavering” and flicking fins, behaviors consistent with response to irritants. In addition to alterations in behavior, mummichogs exposed to *P. shumwayae*, densities as low as 1000 cells/ml displayed significantly greater c-Fos induction in neurons than controls. c-Fos expression is a biomarker of neuronal stress in fish, indicating a neurological effect of *P. shumwayae* exposure. Expression of c-Fos protein

illustrated in this study is similar to mummichog exposed to physical transport stress (Chapter 5). This is the first study, to our knowledge, to describe neurological alterations in fish resulting from sublethal *P. shumwayae* exposure. This work provides an additional, important, and sublethal endpoint of *P. shumwayae* exposure other than mortality and physical damage in fish. These stress mediating alterations, in protein expression, may in addition, begin to elucidate behavioral alterations observed during bloom events and laboratory exposures.

Exposure to *Chaetoceros* has been linked to fish kills through direct physical contact, with blooms as low as 5 cells per milliliter causing gill edema, collapsed pillar cells, and detachment of epithelial cells in fish (Yang & Albright, 1992). Mortality has been attributed to microbial infections due to damaged gill tissue, gill hemorrhage, or suffocation from excess mucus production (Yang & Albright, 1994; Bell, 1961). Gill damage occurs when *Chaetoceros* spines and spinules penetrate the secondary lamellae of the gill leading to hyperplasia, hypertrophy, and partial or complete fusion of secondary lamellae (Yang & Albright, 1992). This increases mucus production followed by hyperplasia and hypertrophy in fish exposed to sublethal concentrations of *Chaetoceros* (Yang & Albright, 1992). Additionally, alterations in physiological parameters include decreases in partial pressure of oxygen, ventilation volume, and oxygen consumption with increases in respiration frequency, haematocrit, lactate, and glucose values. Physical and physiological alterations at the gills may lead to the increased c-Fos expression observed in mummichog exposed to *Chaetoceros*. However, mummichog exposed to *Chaetoceros* demonstrated no alterations in gill from the 2 hour exposure. In this study, the exposure period was short and sublethal, chronic exposures

may induce immune system depression and induce c-Fos expression in mummichog exposed to *Chaetoceros*.

Regions of the mummichog brain in which the most notable and significant increases in c-Fos expression occurred in response to *P. shumwayae* and *C. concavicornis* exposure included the telencephalon and optic lobes. Significant increases in c-Fos expression resulting from *C. concavicornis* densities as low as 1000 cells/ml occurred in the anterior telencephalon and periventricular gray zone of the optic tectum in mummichog. *P. shumwayae* densities as low as 2000 cells/ml. resulted in significant increased c-Fos expression in the anterior telencephalon and observed increases in the anterior optic lobe. Non-significant increases in c-Fos expression were found in the posterior telencephalon and rhombencephalon. However, as *P. shumwayae* and *C. concavicornis* densities increased, c-Fos expression also increased. These increases in the telencephalon and optic tectum of *P. shumwayae* and *C. concavicornis* exposed fish are consistent with changes seen in fish exposed to physical transport stress (Chapter 5).

The cell densities of *P. shumwayae* used for this study are similar to, and in the range of densities recorded from the field and from other laboratory studies. In North Carolina estuaries, densities of *Pfiesteria* can range from 50-35,000 cells/ml, with some samples reaching 100,000 (Burkholder et al., 1995; Glasgow et al., 1995). In the Chesapeake Bay, *Pfiesteria* densities ranged between 300-900 cells/ml in the Pocomoke river in 1997 (Glasgow et al., 2001a). Environmentally relevant densities of *P. shumwayae* range from 300-2500 cells/ml, and laboratory bioassay exposures using *P. shumwayae* (CCMP 2089 and CAAE 101272) have ranged from 15-25,000 cells/ml, with mortality occurring at densities greater than 300 cells/ml (Vogelbein et al., 2002; Gordon

& Dyer, 2005; Lovko et al., 2003; Gordon et al., 2002). In addition, *P. piscicida* densities used for bioassay experiments ranged between 200-4500 cells/ml (Lewitus et al., 1995). *Chaetoceros concavicornis* densities in natural environments fluctuate between 1-10 cells per ml, with most blooms averaging less than 5 cells/ml (Albright et al., 1993). This study exposed mummichog to *C. concavicornis* concentrations ranging from 0 - 4,000 cells/ml and found no mortality under short term exposure concentrations. Concentrations used in this study are one order of magnitude higher than environmental values, but results were used as a model of physical HAB stressor exposure for comparison with *Pfiesteria*.

Increases in c-Fos expression in mummichog exposed to *P. shumwayae* and *C. concavicornis* can have higher-level effects on behavior. Brain regions with increased c-Fos labeling in this study, indicating neuronal stress, are areas known to regulate locomotion, schooling, olfactory, mechanoreception, motor, and auditory functions (Smith, 1982; Wulliman et al., 1996).

Teleost fish such as mummichog, have well developed optic tecta, and cadmium exposure resulted in reduced predation avoidance, resulting in alterations in the ability to school and maintain position in the water column (Sullivan et al., 1978). In addition to retinal information, the optic tectum has afferent and efferent fibers interconnecting areas of the brain anterior and posterior, including the torus semicircularis, which controls motor, lateral line, and auditory functions (Wulliman et al., 1996). Resultant processing of this input produces motor responses required to maintain proper position in the water column as well as position within the school. Therefore, alterations in the optic tectum may explain equilibrium loss and lethargy, after an initial excitatory period, observed in

P. shumwayae exposures. In addition, locomotor behaviors are controlled through motor function in the diencephalon, mesencephalon (torus semicircularis), and medulla. The medial longitudinal fascicle comprises the Mauthner neurons (startle response) as well as descending spinal cord projections (Wulliman et al., 1996). These regions represent the main motor pool and integrative center for skeletal muscle, where sensory information is processed and translated to an appropriate motor response (Smith, 1982). Schooling behaviors, similarly, are controlled by the telencephalon, diencephalon, mesencephalon (optic tectum), and medulla (Smith, 1982). As a result, *P. shumwayae* exposure can elicit changes in several regions of the brain that receive olfactory, motor, auditory, lateral line, and visual stimuli. These alterations lead to changes in locomotion and equilibrium, which have been observed previously in field and laboratory exposures.

This study demonstrated that fish exposed to *P. shumwayae* and *C. concavicornis* result in significantly increased c-Fos expression, a biomarker of neuronal stress. In addition, alterations in behavior observed in *P. shumwayae* exposed mummichogs may be related to neuronal stress in the telencephalon and optic lobes. These regional increases in c-Fos expression may describe higher-level alterations in fish behavior, including general locomotor and schooling behaviors. In addition, the increases in c-Fos expression may explain behavioral alterations, such as surfacing, hyperactivity, and lethargy observed during *Pfiesteria* exposures in the field and laboratory. These results also suggest that c-Fos expression may be used as, a sublethal biomarker of exposure to *P. shumwayae* and *C. concavicornis*, an endpoint other than time to death in fish, and may serve to link behavioral alterations with neuronal stress.

Chapter 7: General Discussion

7.1 Introduction

Sublethal effects of harmful algal bloom (HAB) exposure on fish are currently poorly understood. Knowledge gained from sublethal HAB exposure in fish is highly relevant since non-lethal bloom events occur more frequently than blooms causing major mortality. HAB events occur several times a year in the mid-Atlantic, U.S., with the majority of events not resulting in fish kills. Therefore, information gained from analysis of actual bloom events on individuals and populations of fish remains scarce.

Research over the past 15 years has focused on algal bloom characteristics and effects on human health, with fish mortality serving as a regulatory tool for governmental agencies. Fish populations, however, play an important role in many aquatic and marine ecosystems, and deleterious effects on fish populations can negatively impact these ecosystems by driving them out of balance. Alterations in fish populations could possibly shift predator-prey interactions, energy flow, and community dynamics. Further, fish have been demonstrated to be vectors of bioaccumulated HAB toxins, transferring toxins to higher level carnivores such as marine mammals, shorebirds, and humans (Todd, 1997; Scholin et al., 2000; Lefebvre et al., 2002a; Lefebvre et al., 2001).

This laboratory study investigated the effects of sublethal HAB stress exposures on behavior and brain activity in the mummichog, *Fundulus heteroclitus*. Behavior of fish is difficult to quantify in the field, and the ability to investigate alterations resulting

from HAB exposure in the laboratory may provide a better understanding of how natural ecosystems respond to bloom events. In addition, this study investigated the possibility that alterations in fish behavior resulting from HAB exposure may ultimately be associated with alterations in the central nervous system (CNS). This is the first study, to date, to investigate alterations in behavior and brain activity in an environmentally relevant fish species exposed to sublethal HAB stressors.

7.2 Behavioral Quantification System and Validation

In order to investigate these alterations in behavior and brain activity in mummichog, a behavioral quantification system and a neuronal activity assay were developed. A suite of behavioral endpoints were selected based on observations and previous literature of fish aggregative swimming behavior. A software program was then designed to track groups of fish and was coupled to an existing videography system. This system allowed video capture of thirty minute segments of schools and shoals of mummichog before, during, and after exposure to HAB toxins. One major advantage of this behavioral quantification system was the ability to record for up to one hour continuously at thirty frames per second. This recording ability is a major improvement over existing behavioral analysis systems, which can only record several minutes or seconds continuously. This system has the ability to measure many different types of behaviors in real time such as schooling, shoaling, solitary, and aggregative behaviors. Additionally, it provides the mathematical endpoints of each type of behavior such as nearest neighbor distance and angle, and measures of polarity and cohesion. Previous

behavioral studies have typically reported only two or three behavioral endpoints through time. This novel system can record and quantify upwards of thirty endpoints while maintaining the flexibility to add new endpoints depending on research interests and needs. Initial pilot studies with the system, conducted to determine vessel acclimation time (Gipson, 2001; Kane et al., 2005) and responses to a surrogate toxin, MS-222 (Chapter 4), clearly demonstrate the ability to generate repeatable and quantifiable results of movement analysis.

7.3 HAB-Induced Behavioral Alterations

The ability to quantify alterations in swimming behavior in the laboratory may demonstrate environmentally relevant behavioral consequences of HAB stress exposure. The ability of fish to respond to environmental stimuli, such as predators and noise, is imperative to their survival. Therefore, the behavioral system was designed to evoke startle response and predator avoidance response behaviors. The startle response component of this system supplied a momentary auditory and vibratory stimulus before, during, and after exposure. An overhead visual predator system, composed of a bird model flying over the fish, provided a silent visual stimulus before, during, and after exposure. This allowed characterization of similarities and differences between a visual stimulus and a vibratory stimulus, as well as the ability to document the alterations resulting from exposure. The predator avoidance and startle response systems, when coupled with the swimming behavior software, completed a suite of environmentally

relevant endpoints from which to study behavioral alterations resulting from HAB exposure.

Mummichog exposed to HAB toxins displayed significant alterations in schooling and shoaling behaviors. Exposure to saxitoxin (STX) significantly decreased interactions, schooling, and shoaling behaviors whereas exposure to brevetoxin (PbTx-2) increased these behaviors. These expected results further confirm the known paralytic and excitatory effects of STX and PbTx-2, respectively. In addition, the composition of schools, shoals, and aggregations of exposed groups significantly decreased from 5 to 3 fish after exposure. This is an extremely important finding and describes alterations in group compositions resulting from HAB exposure in addition to the alterations in frequencies of schooling and shoaling behaviors. Groups of fish reducing in size in response to exposure will reduce predator avoidance, foraging success, and swimming efficiency in the wild (Hoare et al., 2000; Pitcher et al., 1988). Twenty-four hours after exposure, groups of fish exposed to both HAB toxins displayed significant decreases in activity and frequency of behaviors compared with pre-exposure activity. Additionally, exposure to both HAB toxins altered startle response and predator response behaviors. These results suggest that sublethal exposure to HAB toxins can have demonstrable effects on swimming and social behaviors, as well as on startle response and predator avoidance behaviors. These alterations can last for at least 24 hours, which can negatively affect groups of mummichog by reducing their ability to maintain schooling, shoaling, and avoidance behaviors in the laboratory. These quantified behavioral changes are relevant to alterations in foraging, predator vigilance, and reproductive behaviors that may occur in the wild.

7.4 HAB-Induced Alterations in Neuronal Activity

Schooling, shoaling, and avoidance behaviors are initiated and regulated through the CNS. Neuronal signaling from the CNS is essential for the proper responses to different stimuli. As such, investigations into possible alterations in brain activity resulting from sublethal HAB stressor exposure in fish were conducted. In order to accomplish this, an immunocytochemistry method used in mammalian research to quantify neuronal activity through protein expression was adapted for fish. c-Fos, the protein product of *c-fos*, an immediate early gene, was quantified in the brains of mummichog as an indicator of changes in neuronal activity and stress. Initially, a simulated transport stress experiment was conducted to confirm that physical stress induces *c-fos* gene expression, and that c-Fos protein expression could be labeled and quantified in several regions of the fish brain. Once increased neuronal c-Fos protein expression was found in several areas of brains from transport stressed mummichog, experiments were then conducted with several HAB chemical and physical stressors. These stressors included PbTx-2, STX, domoic acid (DA), and the HAB species, *Pfiesteria shumwayae* (CCMP 2089) and *Chaetoceros concavicornis* (CCMP 169). PbTx-2, STX, and DA are known neurotoxins produced by HAB species, and the dinoflagellate *P. shumwayae* and the diatom *C. concavicornis* are HAB species known to cause physical stress to fish.

Exposure of mummichog to DA, STX, PbTx-2, *P. shumwayae*, *C. concavicornis*, and transport stress resulted in significant alterations in c-Fos expression. The excitatory

neurotoxins, DA and PbTx-2, and transport stress increased c-Fos expression, while STX significantly inhibited c-Fos expression. Exposure to *P. shumwayae* and *C. concavicornis* increased c-Fos expression in a density dependent relationship. In addition, mummichog brains demonstrated regional differences in c-Fos expression between different stressors. Exposure to *P. shumwayae* and *C. concavicornis* increased c-Fos expression in the telencephalon and optic lobes while physical transport and chemical stressors affected the optic lobes. This is the first study to quantify alterations in c-Fos expression in the telencephalon and mesencephalon of fish. c-Fos expression in these fish may be associated with neuronal stress and alteration in signaling, and can serve as an additional endpoint of sublethal HAB exposure. Changes in c-Fos expression may ultimately be linked to behavioral alterations quantified in the laboratory and mortality during HAB events in the field.

Increases in c-Fos expression observed in *P. shumwayae* exposed fish were similar to the physical transport stress and the diatom *C. concavicornis* exposures (Chapters 5 and 6). Data from this study demonstrates that mummichog exposed to *P. shumwayae* significantly increase stress protein expression similar to other physical stress agents. These results support other studies in which this strain of *P. shumwayae* is stressful to fish through direct physical contact (Lovko et al., 2003; Berry et al., 2002; Vogelbein et al., 2002). It has been found, however, that other strains of *P. shumwayae* may produce a toxin that causes disease and mortality in fish (Burkholder et al., 2001a; Burkholder et al., 2001b). Under different culture and exposure conditions, *P. shumwayae* may produce ichthyotoxic compounds, but data generated from the present

study supports the notion of Vogelbein et al. (2002), that toxicity to fish is through physical contact.

7.5 Effects of HAB-Induced Alterations in Behavior and Brain Activity on Fish Ecology

Quantification of behavior and neurological alterations resulting from sublethal HAB stressor exposure in this laboratory study may further elucidate changes in individual and population dynamics of fish exposed to HAB events in the field. This is the first study to quantify alterations in social behavior of fish exposed to HAB toxins. Further, this research provides laboratory evidence that sublethal HAB exposure to fish alters interactions, swimming behaviors and social dynamics, which may change population demography in the wild during HAB events. Mummichog are an ecologically important species in tidal marsh communities and are likely exposed to sublethal HAB events annually. Sublethal HAB exposure may change group dynamics in aggregative swimming and social behaviors, which may alter predator avoidance, foraging and reproductive success. Alterations in these parameters may have ultimate effects on individual fitness and population dynamics, which may change energy flow and trophic structure in communities. Data from these studies provides a better understanding of how environmentally-realistic concentrations of HAB stressors affect fish behavior and group dynamics.

This study demonstrates that sublethal HAB stressor exposure alters swimming, social, and predator avoidance behaviors and neuronal activity. In addition, alterations in

the CNS of fish exposed to HAB stress may cause alterations in behavior. Alterations in brain activity, and knowledge of specific regions within the brain activated during stress, can provide insights into the control of fish behavior. Results presented here provide a potential link between HAB-induced alterations in behavior and neuronal activity. Changes in c-Fos expression in STX and PbTx-2 exposed fish are similar to behavioral changes, with exposure to STX decreasing behavioral activity and neuronal signaling and PbTx-2 exposure increasing behavioral and neuronal signaling. Such alterations in behavior and CNS activity may change the ability of fish to respond properly to environmental stimuli. Fish exposed to HAB stress in this study exhibited altered swimming and predator avoidance behaviors. This inability to respond to stimuli may have demonstrable effects on the ecosystem, such as fluctuations in prey availability, foraging, and trophic transfer of HAB toxins through the food chain. Ultimately, HAB exposure related changes in neuronal signaling may alter fish behaviors, resulting in individual and population level alterations during HAB events.

7.6 Conclusions and Future Research

This study has demonstrated that exposure to sublethal concentrations of HAB stress can alter behavioral responses and swimming dynamics in mummichog. Additionally, this is the first study to provide a potential link to changes in brain activity with behavioral control in fish exposed to HAB stress. Future studies should quantify alterations in behavior and brain activity in the same fish in order to correlate changes in behavior with brain activity. The behavioral and neurological methods used in this study

were unable to provide such correlations, which could directly link changes in behavior with neuronal activity. Additionally, future research is required to quantify behavioral alterations resulting from sublethal exposures to DA, and *P. shumwayae*, which, due to logistical problems, were unable to be tested. Further, DA and PbTx-2 were not able to be examined in a dose response manner due to many constraints. An examination of dose-response relationships with these two compounds is needed. Nevertheless, data from this study has provided novel information regarding alterations in behavior and brain activity in fish exposed to HABs, and has made significant contributions in the fields of behavioral toxicology and neurology.

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