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Erika Marie Caramillo
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A STUDY OF THE EFFECTS OF METHYLENE BLUE, SCOPOLAMINE, AND
STRESS ON LEARNING AND MEMORY IN THE ZEBRAFISH

by

Erika Marie Caramillo

A Dissertation
Submitted to the Graduate School
and the Department of Psychology
at The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

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ABSTRACT

A STUDY OF THE EFFECTS OF METHYLENE BLUE, SCOPOLAMINE, AND STRESS ON LEARNING AND MEMORY IN THE ZEBRAFISH

by Erika Marie Caramillo

May 2017

With the ever-increasing aging population, neurodegenerative diseases such as Alzheimer's disease are becoming more prevalent. Owing to such increases in age-related cognitive decline, the need for research into new, effective treatments is more imperative now than ever. The zebrafish is an excellent animal model that can be used to study the potential pharmacological effects of novel cognition-centric treatments. However, more needs to be known about the species and its ability to learn, remember, and the effects certain drugs have on behavior. In this dissertation, I aimed to better understand zebrafish cognition through the testing of three conditions: a known cognitive enhancer (methylene blue; MB), a known inhibitor of memory (scopolamine), and beaker stress, a novel paradigm that will further our understanding of stress on cognitive tasks. Three learning tasks (T-maze, object recognition, and escape learning) were used to elucidate the effects the three conditions had on various types of learning and memory. MB was shown to significantly improve performance in the T-maze when compared to scopolamine-exposed fish. Beaker stress had no significant effect on T-maze performance. In the object recognition task, MB and beaker stress fish exhibited a significant preference for the novel object, thus showing the intended learned behavior. In escape learning, MB exposed fish spent significantly more time away from the aversive stimulus, thus exhibiting learning of the escape response. Scopolamine-exposed fish exhibited a

significant lack of learning as the exposed fish spent significantly more time near the aversive stimulus. Beaker stress exposed fish did not show any significance of learning the behavior in the escape learning task. It can thus be concluded that MB-enhanced learning across all learning tasks. Scopolamine induced amnesia-like effects across all learning tasks. Beaker stress had differing effects dependent upon the learning task. These findings are important in allowing the zebrafish to be used more fully in AD research specifically in regards to screening for new treatments such as MB. The next steps of this project are to determine whether MB influences scopolamine-exposed fish and to further understand the effects of stress on different styles of learning.

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DEDICATION

I would like to dedicate this dissertation to my father, Victoriano “Buddy” Caramillo. Although he was not here to see me through this process, he has always been one of my greatest cheerleaders. He was strict and pushed me to strive for the best, but he was also proud of everything I accomplished, no matter how small. I know that when the time comes for me to walk across the stage, he will be saying, “I’m so proud of you, baby girl, but I still think you should have become a lawyer.”

Additionally, I would like to mention a few of the amazing people who have helped me through the time it took to complete this dissertation. Firstly, I would like to mention my mom and best friend, Regina Caramillo, who gave me sage advice, was always on my side, and made me laugh throughout the four years I was here. I would also like to mention my grandfather, James Marchioni, who always told me the truth, with no sugar coating, when I was upon a big decision. I would also like to acknowledge my husband, Ben Hatch, for being my rock, as cliché as it might sound, for if it was not for him sticking by side through the stress and the happiness, I would not have gotten this far. Finally, I would like to holler at some of the best friends I have ever made. Although I have only known you for four years, I know that we will be terrorizing the scene for years to come. We made it, we did it, and I could not have done it without you.

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LIST OF ABBREVIATIONS

MB	Methylene Blue
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
HPI	Hypothalamus Pituitary Interrenal Axis
CRF	Corticotrophin Releasing Factor
ACTH	Adrenocorticotrophic Hormone
BPA	Bisphenol A
IP	Interperitoneal
HPA	Hypothalamic Pituitary Adrenal
CUS	Chronic Unpredictable Stress
LTP	Long Term Potentiation

CHAPTER I – CONDITIONS

Methylene Blue

Methylene blue (MB) was created as a stain for the textile industry the late 1800s and became the first therapeutic synthetic drug in the history of medicine. MB has been used as a potential treatment for malaria, cyanide poisoning, and schizophrenia (Deutsch et al., 1997; Ohrt et al., 2014; Wendel, 1935). Currently, MB is being researched as a potential therapy for those with memory deficiencies and other cognitive deficits associated with neurodegenerative diseases.

Rodent studies exhibited that low doses of MB enhance cognition in a dose-dependent manner (Riha, Bruchey, Echevarria, & Gonzalez-Lima, 2005). Low doses of MB increase the oxygen consumption of mitochondria whereby it accepts electrons from the oxygen molecules it encounters (Lindahl & Oberg, 1961). The cognitive deficiencies linked to neurodegenerative diseases such as Alzheimer's disease (AD) are associated with the lessened ability of mitochondria to metabolize oxygen, which leads to the inability of the brain to fully utilize the available oxygen (de la Torre, 2004). Due to such metabolizing properties, MB has not only been researched as a treatment for neurodegenerative diseases in humans and rodents, but it is our intention that it will be utilized as a cognitive enhancer in the zebrafish (*Danio rerio*) model.

Overall, however, the effects of MB on cognition in the zebrafish have been inconsistent and conflicting. MB was reported to not prevent the amount of tau phosphorylation or the associated neuronal apoptosis in larval zebrafish (van Bebber, Paquet, Hruscha, Schmid, & Haass, 2010). The abnormal, excessive phosphorylation of tau leads to the development of neurofibrillary tangles that produce some of the cognitive

deficits in AD. Reducing the phosphorylation of the tau protein would arguably reduce the cognitive deficits observed in those with neurodegeneration (Iqbal, Liu, Gong, & Grundke-Iqbal, 2010).

Conflictingly, MB was also found to have neuroprotective properties against the toxicity of TDP-43 in larval zebrafish, a protein linked to the formation of amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease (Vaccaro et al., 2013). With such conflicting information, the ability of MB to not only prevent the onset of cognitive decline but also the potential use of the substance as a therapeutic agent against cognitive decline and the associated mechanisms that influence each type of therapy must be explored more fully. It should be noted that while both studies were completed using larval zebrafish (1-29 dpf), this dissertation utilized adult stage zebrafish (90 dpf-2 years). While larval zebrafish are more sensitive than adult zebrafish to the substances they are exposed to, in this instance, adult zebrafish were more applicable to examine cognitive enhancement and the associated comparison to age-related neurodegenerative diseases like AD (Alsop & Wood, 2011; Spence, Gerlach, Lawrence, & Smith, 2008).

The zebrafish model is a viable comparative animal model for examining the ability of MB to mediate cognition, operationally defined as the process of gaining knowledge and understanding through perception and experience based input, reflection, and the associated behavioral output. The species can be used in a variety of learning and motivation tasks such as the T-maze, object recognition and escape learning, all of which are appropriate tools to measure the differing effects of MB on different types of learning and conditioning. For instance, the T-maze measures spatial learning and memory, a process that relies heavily on the hippocampus (the lateral pallium in the zebrafish;

Colwill, Raymond, Ferreira, & Escudero, 2004; Santana, Rico, & Burgos, 2012). Escape learning measures fear and aversive learning, processes that rely heavily on the amygdala in humans (zebrafish have an amygdala-like structure in the telencephalon; Gerlai, 2013; Perathoner, Cordero-Maldonado, & Crawford, 2016). Finally, the object recognition task measures object recognition memory, a process linked to the rhinal cortex in humans (similar structures are found in the telencephalon of the zebrafish; Fernández & Tendolkar, 2006; Lucon-Xiccato & Dadda, 2014). By utilizing three different types of learning tasks (spatial, escape, and object recognition) this dissertation examined not only whether MB-enhanced overall cognition but also if it was more effective in one of the types of learning.

Such understanding is important because it can exemplify the ability of the zebrafish to screen for cognitive enhancing pharmaceuticals such as MB. Such drugs can potentially be used to treat or prevent AD, and the zebrafish can aid researchers in determining whether such substances have the ability to provide relief from the symptoms of AD. In knowing how the substance acts on different types of learning, we can determine how to best pursue further investigations into drugs like MB as a therapy for AD and other neurodegenerative diseases.

In this document, we aimed to examine the ways in which MB affected zebrafish performance on a variety of learning and memory tasks, specifically the T-maze, escape learning, and object recognition. We had three hypotheses for these tasks. Hypothesis one: fish exposed to MB will have a shorter latency to arm in the T-maze task than fish not exposed to MB. Hypothesis two: in the testing trial of the escape learning task, MB exposed fish will spend more time away from the aversive stimuli when compared to

control. Hypothesis three: fish exposed to MB will spend more time exploring the novel object in the second trial of the object recognition task than control.

Scopolamine

While MB is a proposed cognitive enhancer, there are substances that are used to interfere with normal cognitive processes by inducing amnesia-like states. Namely, researchers use scopolamine, a drug currently used to treat motion sickness, to induce temporary amnesia, which simulates the cognitive symptoms of AD (Bäckman, Jones, Berger, Laukka, & Small, 2004; Ebert & Kirch, 1998). The cognitive declines observed in AD can be due to a decrease in acetylcholine, the death of cholinergic neurons and associated muscarinic and nicotinic receptor sites, and the ultimate degradation of the basal forebrain (Van der Zee & Luiten, 1999). When forming a new memory, acetylcholine is active at both the muscarinic and nicotinic receptor sites while the encoding process is underway (Hasselmo, 2006). Scopolamine temporarily simulates the malfunctioning of the cholinergic system seen in the AD brain. It is an anticholinergic agent that binds to postsynaptic muscarinic receptor sites, blocking the ability of acetylcholine to bind to the receptor, and inhibits the ability of the neurotransmitter to aid in learning and memory retention (Collerton, 1986). Importantly, scopolamine does not change the amount of acetylcholine in the synapse but rather acts exclusively on the receptor sites, which simulates the degradation of the muscarinic receptor sites seen in AD (Deutsch, 1971). The use of scopolamine in research is a viable way to screen for pharmaceuticals that are cholinesterase inhibitors. Cholinesterase inhibitors include those drugs that block the breakdown of acetylcholine in the synapse thus increasing the amount of the neurotransmitter available for transmission. The blocking of acetylcholine

degradation reconciles the lack of acetylcholine that could be caused by AD or via the administration of scopolamine (Čolović, Krstić, Lazarević-Pašti, Bondžić, & Vasić, 2013). If cognition in subjects administered with scopolamine improves after the administration of a cholinesterase inhibitor, it can be assumed that the drug is compensating for the lack of receptor sites by making more of the neurotransmitter available for the receptor sites that are functioning.

Scopolamine has been widely used in zebrafish research to not only induce amnesia-like behaviors but to also examine the cognitive enhancing effects of different substances after the induction of the amnesia-like effects. A variety of tasks are used to examine such cognitive diminishing and enhancing effects. For example, the inhibitory avoidance protocol was used to examine whether caffeine could prevent the cognitive minimizing effects of scopolamine (Blank, Guerim, Cordeiro, & Vianna, 2009; Bortolotto, de Melo, de Paula Cognato, Vianna, & Bonan, 2015). In this experiment, zebrafish were placed into one side (black or white colored floor and wall panels) of a partitioned tank. After a predetermined amount of time, the partition was lifted so the fish could swim to the other side (opposite color) where they were given an electric shock. It was expected that when the fish was put back into the tank, it would actively avoid the colored part of the tank where it was given the electric shock. The addition of scopolamine directly before the experiment altered the normal learning process so the fish would not actively avoid the area in which the shock was received. The intended learned avoidance was not retained. The administration of caffeine before the administration of scopolamine significantly prevented the inhibited aversive memory

processing normally produced by scopolamine. The fish retained the learned avoidance of the side of the tank where it received the electric shock.

Using a similar aversive protocol, quercetin, and rutin, flavonols with antioxidant properties found in plants were also found to prevent the amnesia produced by scopolamine (Richetti et al., 2011). Antioxidants work to protect neurons from the oxidative stress that is seen with AD and other cognition-based pathologies (Benchekroun et al., 2016). Oxidative stress happens when free radicals are created as a result of mitochondrial functioning and there are not enough antioxidants in the body to counteract the creation of the detrimental free radicals (Demirci, Nazroglu, Övey, & Balaban, 2016). Scopolamine increases oxidative stress hence the potential for antioxidants to alleviate the amnesia related to the administration of scopolamine (Hancianu, Cioanca, Mihasan, & Hritcu, 2013).

The Y-maze has been used to explore the effects of the timing of scopolamine administration on zebrafish performance (Cognato et al., 2012). When presented with a Y-maze that has an arm the fish have been exposed to before and one that is novel, the natural inclination of the zebrafish is to spend more time exploring the novel arm. Researchers found that scopolamine administered before the initial training trial of the Y-maze significantly reduced the amount of time the fish spent in the novel arm of the Y-maze in later trials when compared to control. When administered after the initial training trial, however, fish showed no difference in the amount of time spent in the novel arm from the control group. Scopolamine has also been used with other cognitive tasks such as the T-maze and object recognition (Braidà et al., 2014b; Braidà, Ponzoni, Martucci, & Sala, 2014a).

Although scopolamine has already been used with two of the three tasks this dissertation researched, it has not been used previously in an escape learning paradigm with zebrafish. We aimed to use scopolamine with an escape learning task to understand its effects on predator-based aversive fear learning. We also used scopolamine with T-maze and object recognition to expand on what is already known about the substance in conjunction with said tasks.

It should be noted, however, that while scopolamine is known to cause locomotor difficulties at higher doses, the 200 μM dose used in this dissertation does not result in any disruption of movement or visual acuity (Bortolotto et al., 2015; Cognato et al., 2012; Lee et al., 2016; Richetti et al., 2011). This is upheld by previous zebrafish research that states that 200 μM of scopolamine dissolved in the tank water did not disrupt the distance moved or angular velocity of the zebrafish, only cognitive ability, which would indicate that there was no effect on the motor system of the fish (Bortolotto et al., 2015; Cognato et al., 2012; Lee et al., 2016; Richetti et al., 2011). It was also found that scopolamine did not affect visual acuity in rodents or zebrafish that had been exposed to the substance via the tank water or injection (Braida et al., 2014a; Kumar, Talpos, & Steckler, 2015). For instance, in a zebrafish object recognition task, fish that received scopolamine performed at chance and very similar to fish who had not been exposed to the substance upon the probe trial (Braida et al., 2014a). This is also supported by the supposition that the effects of scopolamine on pupil diameter, mydriasis, could allow for the fish to be able to perceive certain stimuli better and might improve visual acuity (Klinkenberg & Blokland, 2010). With this said, we believe that the 200 μM dose of scopolamine does not adversely affect movement or visual acuity.

Using scopolamine to further understand the dynamics of escape learning, spatial learning, and object recognition is important because it can elucidate the potential effects of AD on different types of learning. We had three hypotheses for the outcomes of scopolamine exposure respective to each task. Hypothesis one: fish exposed to scopolamine before performing the escape learning task will not spend more time away from the aversive stimuli in the testing trial compared to control. Hypothesis two: scopolamine exposed fish will not perform better than control fish in the T-maze. They will not learn the conditioned response and will not have shorter latency to arm than control. They will perform at chance. Hypothesis three: fish exposed to scopolamine will not spend more time exploring the novel object in the testing trial when compared to control. They will spend less time around the novel object when compared to control.

Beaker Stress

According to Chrousos (1998), acute stress can be defined as “a state of threatened homeostasis that is re-established by a complex suite of adaptive responses.” Immediately responding to a perceived acute threat is adaptive in function and provides sound survivalist reasoning (Möstl & Palme, 2002). Chronically, however, stress is maladaptive (Schreck, Contreras-Sanchez, & Fitzpatrick, 2001). At the onset of a stressful situation, primary stress responses, such as cortisol release, occur. The primary stress response is followed by secondary stress responses such as changes in metabolism, respiration, and immunity (Mommsen, Vijaya, & Moon, 1999). Behavioral adaptations such as increased arousal, vigilance, and improved cognition are also exhibited (Chrousos, 1998; Tsigos & Chrousos, 2002). At acute levels, these responses are adaptive to a species. When stress is chronic, however, tertiary stress responses manifest, and the

benefits of the stress response morph into possible detriments. Tertiary stress responses are stunted growth, lessened resistance to disease, maladaptive behavior changes, and a reduction in ultimate survival of the organism (Mommsen et al., 1999).

Zebrafish exhibit a stress response similar to that which is seen in humans (Steenbergen, Richardson, & Champagne, 2011). Physical, chemical and other perceived stressors elicit a stress response in zebrafish that evokes the need for adaptive coping (Barton, 2002). In prior studies, locomotion, shoaling behaviors, and exploratory behavior have all been used to measure anxiety in the species (Baiaomonte, Parker, Vinson, & Brennan, 2016; Pagnussat et al., 2013; Schaefer et al., 2015). Freezing, thigmotaxis, and erratic swimming are also noted behavioral markers of anxiety in zebrafish (Maximino et al., 2010).

Novelty of a situation also evokes anxiety in zebrafish and leads to the fish staying at the bottom of the tank and not exploring its new environment (Egan et al., 2009). Aversion of novelty is salient to this dissertation in that the fish would not have been in a beaker prior to testing, so the stress response may have been triggered. Additionally, social confinement and isolation result in a stress response in the zebrafish and other fish species (Davis & Parker, 1990; Shams, Chatterjee, & Gerlai, 2015; Talbot, Pottinger, Smith, & Cairns, 2009). The zebrafish is a species that uses shoaling (group cohesion) as protection against predators and other environmental dangers. When the zebrafish is taken away from its conspecifics, the stress response is triggered in the fish (release of cortisol, erratic swimming, freezing bouts, etc.), and once again, this is salient because the fish was removed from its familiar tank, put into a novel tank, and there were no conspecifics available to shoal with thus potentially increasing the stress response.

Confinement is a noted stress model that elicits a neuroendocrine response in fish (Davis, 2004; Fast, Hosoya, Johnson, & Afonso, 2008). Using cortisol as a marker, the degree of stress can be measured in relation to the severity and longevity of the situation (Carmichael, Tomasso, Simco, & Davis, 1984). Zebrafish, like humans, release cortisol as a physiological response to stress (Barton, 2002; Canavello et al., 2010). While zebrafish have a different stress response axis than mammals, their axis is similar to that of humans (Alsop & Vijayan, 2009). Instead of the HPA axis seen in rodents and humans, the zebrafish has a hypothalamus-pituitary-interrenal (HPI) axis. When presented with a stress-inducing stimulus, the hypothalamus of the zebrafish is activated and releases corticotrophin releasing factor (CRF) that leads to the release of another hormone, adrenocorticotrophic hormone (ACTH), by the pituitary gland. The ACTH then signals the interrenal tissue to release both epinephrine and cortisol (Alsop & Vijayan, 2008). While this axis utilizes different structures in the stress response, cortisol, the corticosteroid secreted in humans, is also secreted in zebrafish (Alsop & Vijayan, 2008). Different from humans, rodents utilize the corticosteroid corticosterone.

Similarities between the human and zebrafish stress models give way to the assertion that zebrafish are a viable and logical comparative stress model (Epstein & Epstein, 2005). In addition to this, the zebrafish model is a practical one as the species is arguably the lowest order vertebrate in which complex human behaviors may be studied (Grunwald & Eisen, 2002). The species also has the benefits of high volume breeding year-round, cost effectiveness, and easy maintenance (Streisinger, Walker, Dower, Knauber, & Singer, 1981).

With this in mind, we tested a new stress induction procedure, the beaker stress protocol, on three cognitive tasks: T-maze, object recognition, and escape learning. Through this procedure, we examined the effects of stress on learning and memory in the zebrafish. Prior to the three tasks, the fish went through the beaker stress procedure, a task comparable to restraint stress in the rodent. From prior research in our lab, it is known that isolation and confinement in the beaker produces a heightened cortisol response in the zebrafish (See Figure 1).

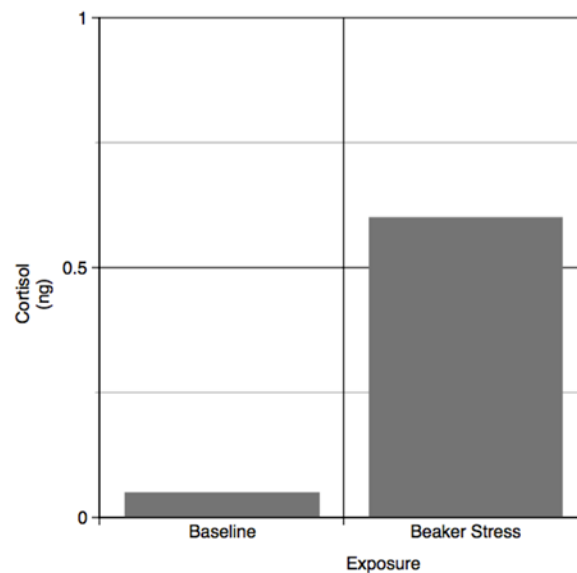


Figure 1. Graph of cortisol in beaker stress exposed fish and baseline

It is also known that in some instances acute stress has a negative effect on learning ability in the zebrafish (Cofiel & Mattioli, 2009; Manuel et al., 2014). The first hypothesis for beaker stress: beaker stress exposed fish will have a longer latency to arm in the T-maze task than control. Hypothesis two: beaker stress exposed fish will spend less time around the novel object in trial two of the object recognition task and more time around the familiar object when compared to control. Hypothesis three: beaker stress exposed fish will spend more time away from the computer screen than control in the

second trial of escape learning. We thus aimed to further understand the effects of confinement stress had on learning in the three different tasks: T-maze, object recognition and escape learning. Additionally, by understanding the effects of stress on the three different types of learning, we aimed to better understand how different environmental factors could affect the symptomology and progression of cognitive based neurodegenerative disorders like AD. If we can better understand how stress affects learning and memory, we can begin to understand how potential confounds like stress (life stress, testing stress, anxiety about the disease itself, etc.) can influence test results with AD treatment studies in humans.

CHAPTER II – SPATIAL LEARNING IN THE ZEBRAFISH

Introduction

Spatial Learning in the Zebrafish

Zebrafish are known to be amenable to many different types of learning. The species not only learns through classical and operant conditioning but more specifically, spatial learning (Fernandes, Rampersad, Luchiari, & Gerlai, 2016; Gerlai, 2016; Valente, Huang, Portugues, & Engert, 2012). Additionally, zebrafish can learn visual discrimination and spatial tasks when tested on the T-maze (Braidia et al., 2014; Karnik & Gerlai, 2012). In a set of three experiments, zebrafish were given the choice of two differently colored arms: in the first experiment, green vs. purple arm, in the second experiment red vs. blue arm, and in the third experiment horizontal vs. vertical black and white stripes arm. In all three experiments, the zebrafish learned a preference for the predetermined visual stimulus that led to a food reward (Colwill et al., 2004). For example, if the fish were consistently rewarded with food for swimming into the green arm as opposed to the purple arm, the fish would spend more time and/or have a shorter latency (time it takes to swim to the arm) to the green arm.

The T-maze has been under-utilized in the zebrafish. The task, however, has recently been used to further examine the mechanisms of action behind learning and memory in the zebrafish, specifically focusing on the nicotinic acetylcholine receptors (Braidia et al., 2014b). It was found that the administration of nicotine improved learning and memory at low doses and caused cognitive deficits at higher doses. Additionally, T-maze has been used to test for learning deficits that may be caused by toxins such as bisphenol A (BPA; Saili et al., 2012). The researchers found that BPA-induced

hyperactive behavior in the zebrafish and thus promoted learning deficits in adulthood due to the disruption of estrogen and the associated neural developmental delays. These studies have expanded on previous visual discrimination findings in the zebrafish. They have also shown the usability of the T-maze to test the effects of various substances on cognition in the species (Colwill et al., 2005).

As such, we utilized the T-maze to further understand the three previous treatments (MB, scopolamine, and beaker stress) on spatial learning. By doing so, we bolstered the usability of the task in quantifying learning and memory abilities in the zebrafish. By understanding how a variety of substances affect zebrafish behaviors in the task, we can not only better understand how such substances can affect human learning but also streamline more efficient learning tasks for future research to test for cognitive deficits in AD in the species.

Methylene Blue and Spatial Learning

MB has been studied as a cognitive-enhancing substance when the cognitive declines are due to a decrease in mitochondrial respiration. Mitochondria are organelles located in the soma of cells such as neurons. Mitochondria create ATP, a source of energy for the cell. In order to be synthesized, ATP requires oxygen that is provided through mitochondrial respiration. Once created, ATP powers the processes of the neuron and allows it to perform tasks such as the transmission that is behind cognition (Henze & Martin, 2003). The potential cognitive enhancement by MB is linked to the ability of the substance to increase mitochondrial respiration and regulate the associated cerebral hypoxia (Fiskum et al., 2008; Gonzalez-Lima, Barksdale, & Rojas, 2014).

Cognition requires energy in the form of ATP. ATP synthesis requires oxygen that is provided by mitochondrial respiration, so if there is more oxygen, there would arguably be more ATP which would allow for more processes like cognition to take place. The MB-based increase seen in mitochondrial respiration is due to the affinity the substance has for mitochondrial tissue oxidases (Visarius, Stucki, & Lauterburg, 1997). Small amounts of MB may donate electrons to cytochrome c, an enzyme found in the mitochondria, which may increase cytochrome oxidase activity and oxygen consumption. In rodents, a small dose of MB caused a 30% increase in cytochrome oxidase activity in the brain (Callaway, Riha, Bruchey, Munshi, & Gonzalez-Lima, 2004). With this said, however, MB has a hormetic dose-response curve. Low doses of MB (0.10 - 0.50 μM), increased cytochrome oxidase activity while high doses of MB (10.0 μM and above) decreased cytochrome oxidase activity (Bruchey & Gonzalez-Lima, 2008). It should be noted that cytochrome oxidase is also found naturally in the zebrafish (Bourdineaud, Rossignol, & Brèthes, 2013; Dabir et al., 2013).

The question then became whether MB would act in a similar cognitive enhancing manner in the zebrafish (Echevarria, Caramillo, & Gonzalez-Lima, 2016). In this study, the T-maze task was used to measure learning acquisition. After training trials using the T-maze, the zebrafish were exposed to four doses of MB (0.10 μM , 0.50 μM , 5.0 μM , and 10.0 μM). The concentrations were based on previous findings in rodents, which used a 0.1-10 μM range in brain homogenates to determine hormetic dose responses in cytochrome oxidase activity (Bruchey & Gonzalez-Lima, 2008). The researchers specifically tested whether the zebrafish would follow the hormetic dose response curve as seen in the rodent literature and hypothesized that they would. This hypothesis was

upheld with the 0.50 μM dose exhibiting the most effective cognitive enhancing effects out of the four doses (Echevarria et al., 2016).

With the present study, we used the optimal 0.50 μM dose of MB in conjunction with the T-maze and examined the effects of the substance on fish that were individually housed as opposed to the group housing seen in the original experiment (Echevarria et al., 2016). The fish were individually housed as it allowed for enhanced ability to monitor individual fish and increased statistical power, which allowed for a more precise understanding of how MB affected spatial learning in the T-maze. By doing so, we intended to further understand the effect of MB on spatial learning, zebrafish as an animal model, and the potential comparisons we could draw between zebrafish and the human model.

Scopolamine and Spatial Learning

While scopolamine has been used in spatial learning tasks such as the T-maze more often than the previous condition, MB, it is still underutilized in zebrafish research as a method to induce amnesia-like effects. Braida et al. (2014a) produced one of the only studies that used scopolamine to induce amnesia-like effects in the zebrafish as a means to study not only spatial learning in the zebrafish but also the effects of nicotine on the known amnesia-inducing effects of scopolamine. In that study, the T-maze was used to test for spatial memory in the zebrafish. The T-maze had favorable habitat objects (shells, grass, etc.) in one of the arms, and the other arm was empty, devoid of any such objects. The latency to swim to the enriched arm was measured. Scopolamine was administered to the zebrafish via intraperitoneal (IP) injection 10 min before administration of nicotine, a nicotinic receptor agonist. Both substances were administered before the initial training

trial of the experiment. Scopolamine blocked the cognitive enhancement normally seen by exposure to nicotine (Braidia et al., 2014a). While scopolamine is a muscarinic antagonist, it also seems to have an effect on antagonizing the facilitation effect seen by the use of nicotine. The effects linked to scopolamine on the muscarinic receptors were greater than the enhancement effects caused by the nicotine on the nicotinic receptors. This is the only study in zebrafish the researcher is aware of that used both scopolamine and the T-maze apparatus.

It should be noted, however, that the effects of scopolamine on spatial learning have been studied in the zebrafish using the Y-maze, a task like the T-maze (Cognato et al., 2012). Scopolamine, in doses of 50, 100, or 200 μM , was administered to the zebrafish via tank water before training in the Y-maze. Those that received the substance exhibited reduced exploration of the novel arm of the Y-maze when compared to those that did not receive the scopolamine. Post training exposure to scopolamine, however, did not affect the exploration of the apparatus (Cognato et al., 2012).

While the substance is not often used in T-maze research in the zebrafish, scopolamine has been widely used in the rodent model to induce amnesiac effects while studying spatial learning. One study investigated the effects scopolamine had on spontaneous alternation in the T-maze (Andriambeloson, Huyard, Poiraud, & Wagner, 2014). Spontaneous alternation in the T-maze is when a rodent alternates which arm of the maze it explores based on which arm it explored in the previous trial, owing to a preference for the more novel option (Deacon & Rawlins, 2006). Such a response uses the working memory (which arm they entered in the previous trial) of the rodent, which involves a muscarinic cholinergic mechanism. Therefore, the use of scopolamine, a

muscarinic antagonist, would be appropriate in the testing of cognitive dysfunction in spatial learning (Rusted & Warburton, 1989). The researchers found that administration of scopolamine 40 min prior to the first trial results in a 25-30% reduction of spontaneous alternation in the T-maze. It was also shown that the AD medications donepezil and galantamine, cholinesterase inhibitors that block the breakdown of synaptic acetylcholine thus allowing for more of the neurotransmitter in the synaptic cleft to potentially bind to receptors, reversed the effects of scopolamine on alternation behaviors (Andriambeloson et al., 2014). A study completed by Moran (1993) also investigated the effects of scopolamine on T-maze alternation and whether the muscarinic antagonist had more effect on working memory or reference memory tasks. It was found that scopolamine dose-dependently disrupted alternation. Additionally, they found that working memory tasks were more sensitive to scopolamine than tasks based on reference memory.

In another rodent study, researchers investigated how scopolamine affected response to change in the T-maze (Lukaszewska, 1993). In this study, response to change was the tendency of the rodent to choose the T-maze arm that had been changed in brightness from one trial to the next, once again exemplifying the necessity of memory in spatial learning and the predilection of the rodent for novelty. Scopolamine was found to significantly reduce the response to change of brightness in the T-maze apparatus. Meaning, the rodent did not significantly choose to explore the arm changed in brightness when compared to an arm that was not changed in brightness.

With the success in the rodent model using both scopolamine and the T-maze as well as the success of using scopolamine and other spatial learning tasks such as the Y-maze, we showed the utility of using scopolamine, the zebrafish, and the T-maze to

demonstrate the use of the species in researching cognitive deficiencies related to neurodegenerative diseases such as AD.

Beaker Stress and Spatial Learning

While the beaker stress protocol has never been used in either the T-maze or any other spatial learning task, the effects of stress itself on such learning has been studied by both rodent and zebrafish researchers alike. In rodents, restraint stress, the effect caused by immobilizing a rodent by a restraint apparatus such as a plastic tube, activates the central noradrenaline systems (Roth et al., 2012). Concurrently with this response, there is also the activation of the stress axis of the rodent, the hypothalamic-pituitary-adrenal (HPA) axis. This activation leads to the release of corticosterones into the body, thus allowing for a rather pronounced stress response both physiologically and behaviorally (Rioja et al., 2004; Rioja et al., 2006; Rioja et al., 2007). The T-maze was used to further understand the effects of the restraint of a rodent in a Plexiglas tube, what could potentially be compared to the zebrafish beaker stress protocol, on the stress responses of the animal (Blanco et al., 2009). Rodents that received the restraint stress had increased anxiety-like behavior, increased escape behavior, and increased passive avoidance of areas that were connected to an electric shock (Graeff, Netto, & Zangrossi, 1998).

Chronic unpredictable stress (CUS) has also been examined using both the T-maze apparatus and the rodent model (Matuszewich, McFadden, Friedman, & Frye, 2014). The researchers studied the effects of 10 days of CUS on spatial recognition memory as the effects of an acute stressor presented once before the initial trial in the T-maze. The acute stressor impaired spatial learning in the rodents. The CUS, however, did not exhibit the same impairment and behaved at baseline. Additionally, the acutely

stressed rodents exhibited heightened levels of corticosterone whereas the CUS rodents exhibited levels consistent with the control group (Matuszewich et al., 2014). This indicates that the type of stressor (acute vs. chronic) not only affects learning behaviors, but it also affects the physiological response seen by stress hormone release in the animals. By using the acute beaker stress protocol, we could see a similar response in the zebrafish, but it is important to understand all aspects of stress to understand how acute stress will affect the performance of the animals and how said performance could potentially change when the type of stress changes.

Due to the similarities in the stress response system between the zebrafish and humans, zebrafish are an excellent model to understand the ways in which stress affects learning in both species. While there is not an abundance of research on this interaction especially specific to the T-maze, researchers have utilized the plus maze, a task like the T-maze, to analyze the effects of stress on spatial learning in the zebrafish (Gaikwad et al., 2011). An acute stressor, alarm pheromone, presented immediately before the last trial of the plus maze task significantly reduced spatial learning.

Confinement stress has been equally untested in the zebrafish. Using a type of confinement stress in which fish were confined to an area of the testing tank that was 8% of the total area of the tank for 1 hr, researchers found that association learning was significantly delayed (Cofiel & Mattioli, 2009). In a study using goldfish, similar effects of isolation stress were found to negatively affect learning when compared to those fish that did not receive the stressor (Laudien, Freyer, Erb, & Denzer, 1986).

With such a lack of knowledge on the effects of stress, specifically confinement stress, on spatial learning in the zebrafish, we aimed to add much-needed insights into the

topic by exploring the effects of the beaker stress protocol on spatial learning in the T-maze. By doing this, we elucidated the effects of stress on learning in not only the zebrafish but also in humans.

Methods

T-Maze Apparatus

The methods for the T-maze were based on previous research by the investigator and were followed with very few changes (Echevarria et al., 2016). A Plexiglas T-maze was filled with 4 L of tank water (height of 82.30 mm) and was used in all stages of the experiment. The water was kept at ~28 C°. The maze consisted of a start box (101.60 mm x 101.60 mm x 101.60 mm) separated from the main arm by a removable Plexiglas insert. From the start box extended the main arm of the maze (101.60 mm x 101.60 mm x 304.80 mm) with two shorter arms (101.60 mm x 101.60 mm x 203.2 mm each) extending from the end of the main arm. At the end of both shorter arms, there was a containment area (101.60 mm x 101.60 mm x 101.6 mm) with removable Plexiglas inserts that allowed the researchers to contain the fish for reward administration (see Figure 2).

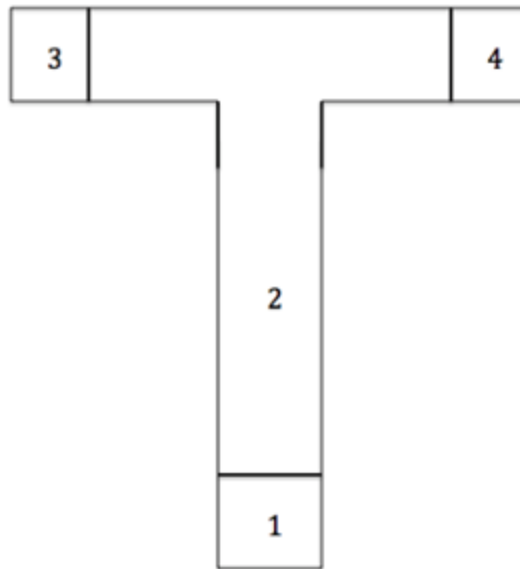


Figure 2. Diagram of the T-Maze apparatus

1, Start Box; 2, Main Arm; 3; Containment Area; 4, Containment Area

The start box and main arm of the maze was lined with opaque white shelving paper to obscure the view of the fish. The shorter arms were made of transparent Plexiglas. Two patterned sleeves (one black and white striped, one black dots on a white background) were fitted onto the shorter arms of the maze during the training days. They were alternated randomly after every trial.

Habituation

To minimize novelty stress, the fish went through 4 days of habituation. During all 4 days, the fish were placed individually into the T-maze, which was open at all points. They were allowed to explore the maze for 1 hr. The fish was food restricted on the last day of habituation (Echevarria et al., 2016).

Training Trials

After the habituation period ended, there were 5 training days and each day followed the same procedure for both the control and experimental groups, respectively. During the training trials, the fish learned to associate a predetermined and randomly assigned pattern (black dots on a white background or black stripes on a white background) with a food reward. Each pattern was placed around one of the two arms of the T-maze during every trial so that one arm had the dotted pattern and the other had the striped background. The placement of the patterned sleeves was alternated randomly after every trial. After filling the maze with 4 L of water, placing the patterned sleeves on the predetermined arm for the trial, randomly assigning one pattern to be the correct choice, and assuring that the Plexiglas dividers were in place to isolate the start box, right arm, and left arm, one fish was netted and placed into the start box of the maze. The fish was allowed to acclimate to the maze in the start box for 10 s. The start box was then opened and the fish was allowed to swim into the main arm of the maze. The fish was given 10 min to make a choice between swimming into the left or right arm. If it swam into the containment area of an arm, the divider for that area was closed; the fish was contained in the end of the arm. The fish had to swim completely into the containment area so that its whole body was past the divider (101.60 mm x 101.60 mm x 101.60 mm) for the experimenter to close the door. If the fish made the predetermined correct choice (either striped or dotted arm), it was given a food reward of live brine shrimp using a dropper (0.05 mL, approx. 25 brine shrimp). The reward was administered to the containment area in which the fish was captured. Other than receiving the food reward, the fish was food deprived for the duration of the training trial days (Echevarria et al., 2016).

If, however, the fish made an incorrect choice, it performed a correction trial in which the fish was placed back into the start box and allowed, once again, to swim through the maze for a maximum of 10 min. The incorrect arm was closed off so the fish could only swim into the correct arm where it was contained by the experimenter-controlled divider and then given the brine shrimp reward. If the fish did not make a choice in the 10 min allotted for either the initial trial or the correction trial, it was considered to have ‘timed-out’, the trial considered void, and the data was not used in statistical analyses.

This procedure was completed four times per fish per day for a total of 20 training trials per fish over the course of the experiment. After each trial of each fish, the water was siphoned out, the maze cleaned, and the water replaced from a reservoir that kept the water at ~28 C°. The latency (time it took to swim to the correct arm) of time to the correct arm was recorded by the experimenter. All behavior was recorded using an overhead camera (Logitech, Tessar 2.0/3.7), which was then coded by the experimenter (Echevarria et al., 2016).

Probe Trial

Following a 12 hr period in its home tank, the fish was then probed individually for learning by using the same procedure as the training trials; however, there were not correction trials for incorrect choices. There was only one day of probing, and each fish was probed four times to test for learning acquisition of the desired behavior, either swimming into the arm of the maze with the dotted sleeve or the striped sleeve. The sleeves on the arms of the T-maze were alternated randomly for each probing trial. An experimenter recorded the latency to the correct arm (Echevarria et al., 2016).

Methylene Blue

As with the method for the T-maze, the administration of MB to the zebrafish were based on previous research published by the investigator (Echevarria, Caramillo, & Gonzalez-Lima, 2016). Immediately after the last trial on the fifth and final day of training, the fish was either exposed to MB mixed with tank water (Sigma-Aldrich, St. Louis, MO, USA; dye content $\geq 82\%$, purity 90%) or a mixture of blue food colorant (Wilton, Woodridge, IL, USA) and tank water that matched the color of the MB dose based on the group to which it was randomly assigned (approximately PMS 294). The experimental group was put individually into 1.5 L of water with the 0.5 μM dose of MB, the dose that was previously found to induce the greatest cognitive enhancement (Echevarria et al., 2016). The control group was put individually into 1.5 L of water mixed with an amount of blue food colorant mixed to match the color of the MB water. The last trial on the fifth and final day was chosen to administer the MB as it is thought that MB acts on the consolidation period of memory storage and follows the protocol seen in rodents (Martinez et al., 1978; Riha et al., 2005). The fish were kept in these tanks for 12 hr per prior MB dosing reports (Riha et al., 2005). After this period of time, they were removed from these tanks, rinsed with fresh tank water, and returned to their home tanks, which contained fresh water, devoid of any drug or colorant.

Scopolamine

Prior to the first trial on each day of the five training trial days, fifteen fish were individually exposed to scopolamine hydrobromide trihydrate (Fisher Scientific, Hanover Park, IL, USA; purity 99%). One hr before the initial trial, the fish were placed individually into their home tank that was filled with 200 μ M of the scopolamine solution dissolved into 1.5 L of tank water. The fish were then allowed to perform the training trials. The control group, also comprised of fifteen fish, was placed into a tank containing 1.5 L of water that had no scopolamine. They were kept in the tank for 1 hr. The fish were then allowed to perform the training trials. After the final trial of each day, the fish were returned to their home tank that was filled with fresh water. On the sixth day, the probe trial day, the fish were not exposed to the scopolamine or the 1 hr control exposure period, but their performance was recorded as in the prior treatment (MB) trials.

Beaker Stress

Prior to the first trial on each day of the five training trial days, 15 fish were individually exposed to a 250 mL beaker filled with 100 mL of fresh tank water. They were kept in this beaker for 15 min. After this time, the fish were then put into the T-maze and went through the training trials as listed above. On the sixth day, the probe trial day, the fish were not exposed to beaker stress, but their performance was recorded as in the prior treatment (MB) trials. The control group, also of fifteen fish, were not placed into the beaker but spent the 15 min before each initial trial in their home tank.

Results

A one-way ANOVA was conducted to determine whether there was a significant difference between the MB ($M = 74.73$, $SD = 55.68$), scopolamine ($M = 76.93$, $SD =$

67.81), and beaker stress (M = 67.53, SD = 55.91) groups in the baseline trial, $F(2, 42) = 0.11$, $p = 0.89$. In confirming that none of the groups differed in the baseline trial, we tested further whether there was a significant difference between the groups in the learning probe trial.

A one-way ANOVA was conducted to determine whether there was a significant difference between any of the groups (MB, scopolamine, or beaker stress) when comparing the change score between the latency to arm in the probe trial and the latency to arm in the baseline trial (probe trial latency – baseline latency = change score). A negative change score would indicate there was an increase in performance. There was marginal significance between groups when comparing the change scores, $F(2, 42) = 2.69$, $p = 0.07$ (See Table 1).

Table 1

T-Maze Comparison Between Groups Using the Change Score

<u>Condition (I)</u>	<u>Condition (II)</u>	<u>Mean Diff.</u>	<u>SE</u>	<u>sig</u>
MB	Scopolamine	-79.87	34.41	.02*
	Beaker Stress	-37.13	34.41	.28
<u>Scopolamine</u>	<u>Beaker Stress</u>	<u>42.73</u>	<u>34.41</u>	<u>.22</u>

Significant Difference at $p < 0.05$

Post hoc tests using an LSD comparison indicated the mean change score for the MB group (M = -27.20, SD = 68.47) was significantly different from the scopolamine group (M = 52.67, SD = 126.98; $p = 0.02$). There were no significant differences between

the MB group and the beaker stress group ($M = 9.93$, $SD = 76.39$; $p = 0.28$) or between the scopolamine group and the beaker stress group ($p = 0.22$).

Additionally, paired samples t-tests were performed to determine whether there was a significant difference between the baseline latency and probe trial latency within the same conditions. There were no significance differences within the MB group $t(14) = 1.54$, $p = 0.14$. The effect size for the MB group ($d = 0.56$) exceeded the boundaries for Cohen's medium effect size. The scopolamine group $t(14) = -1.61$, $p = 0.13$ was not significant but the effect size ($d = .58$) exceeded the boundaries for Cohen's medium effect size. The beaker stress group $t(14) = -0.54$, $p = 0.62$ was not significant, and the effect size ($d = 0.20$) for this group did not exceed the boundaries for Cohen's low effect size.

CHAPTER III - OBJECT RECOGNITION IN THE ZEBRAFISH

Introduction

Object Recognition in the Zebrafish

The study of object recognition in the zebrafish is relatively new with only a handful of researchers exploring the learning behavior in the species. The object recognition task was first delineated in rodents by Ennaceur and Delacour (1988) when they took two objects, one of which they presented to the rodent test subjects on a previous occasion and another object that was unfamiliar, only presented on the probe trial and tested for recognition of the familiar object. They tested whether the animals could distinguish and remember the differences between the two objects. They also studied which object the rodents preferred. Interestingly, the rodents could not only distinguish the two objects from one another via their working memory but also spent significantly more time exploring the novel, unfamiliar object. This finding, that animals generally prefer exploring novel, unfamiliar objects to familiar objects has been widely supported since the inception of the object recognition task in rodents (Hughes, 2007). The preference for the novel object indicates that the animal remembered the familiar object and aimed to explore the object they had never encountered before and is thus a viable measure of learning and memory.

In zebrafish, researchers have begun to create tasks to mimic the object recognition tasks previously used in rodents. Using 2D geometric shapes (square, triangle, circle, and cross) presented on iPod screens placed at opposing sides of the trial tanks, researchers observed the preference of the fish for either the familiar object or the novel object after a variety of time delays (5 min-96 hr). While the results were weak, the

fish generally spent more time exploring the screen that presented the novel object, consistent with those results seen in the rodent model (Braidia et al., 2014a). Such results were corroborated by a study using zebrafish that utilized geometric objects placed in the tank with the fish, not on screens outside the tank (Lucon-Xiccato & Dadda, 2014). The fish were allowed to explore the familiar object for 25 min. After this exposure trial, the fish were then presented with both the familiar object and the novel object after a time delay (2, 6, or 24 hr). The researchers found that the fish spent significantly more time exploring the novel object, regardless of the time delay between the exposure trial and the probe trial

Context and occasion have also been shown to affect the episodic memory and learning associated with the object recognition task (Hamilton et al., 2016). Like the previous studies, when presented with both a familiar object and a novel object in a familiar context, the fish spent significantly more time exploring the novel object in the familiar quadrant of the testing tank. Additionally, when presented with a familiar object in a familiar context but containing a novel area of the tank, the fish spent significantly more time in the novel area of the tank. Such findings demonstrate that the fish have an episodic memory for not only the type of object they have previously witnessed but also where they encountered it and when.

These results, however, have not been upheld by all explorations of object recognition in the zebrafish. Using 3D LEGO® objects instead of the 2D in-tank or computer animated geometric objects, researchers found that the fish spent significantly more time exploring the familiar object over the novel object after 1 and 5 min time delays and no preference for either object after the 10, 15, or 30 min time delays (May et

al., 2015). Due to such varied findings from a variety of researchers as well as the unstreamlined nature of the protocols (objects used, time delays, etc.) we aimed to further elucidate the ways in which the zebrafish learn the object recognition task as well as the boundaries of their episodic memory capabilities. We chose to use a protocol like the LEGO® study in lieu of the geometric shape studies because we found the methods to be more sound, and we agreed with the complexity and familiarity of the objects and the potential influence such features could have on object recognition in the zebrafish.

Methylene Blue and Object Recognition

Until now, there has been no research on the effects of MB on the object recognition task. Additionally, there has been no research on the substance in relation to any episodic memory task in the species. Episodic memories are those that are related to a specific event and include place, time, associated emotions, and other contextual information. With this said, however, there have been a variety of rodent studies that utilized both object recognition tasks, episodic memory tests, and the effect MB has on both. In one study, rodents were pretreated with MB and later injected with ketamine, which induces memory deficits. The rodents were then subjected to the object recognition task in which they were presented with the familiar object and after a 30 min time delay, were presented with the familiar object as well as a novel object (Kandratavicius et al., 2015). The pretreatment with MB significantly increased long-term memory recovery, however, post-treatment with the substance did not show the same recovery.

In another study that used a preventative model of administering MB (rodents were given a dose of MB every day for 3 months prior to testing), researchers found that the substance provided for increased cognitive abilities (Mori et al., 2014). When first

presented with a familiar object and 24 hrs later presented with a novel object, those rodents who were pre-treated with MB had increased object recognition, showing an increase in cognitive abilities and specifically those related to episodic memory.

The effect of post-treatment, or therapeutic administration, with MB on object recognition behaviors has also been tested (Riha et al., 2005). In this study, the rats were presented first with two identical objects for 5 min. They were then removed for a 24 hr time delay after which the rats were injected with a dose of MB and returned to the testing chamber. They were then presented with one of the familiar objects and a novel object, and the amount of time spent exploring the objects was recorded. MB improved memory on the object recognition task in a dose-dependent manner with the 0.50 μM dose exhibiting the most reliable and significant difference in preference. MB treated rodents spent, on average, 10 s more time exploring the novel object than the familiar object.

With both MB pre- and post-treatment exhibiting such significant results in rodent memory enhancement, the ability for the zebrafish to further explore the subject is promising. We used the species to not only expound on the knowledge we have about episodic memory in the zebrafish, but we also aimed to further understand the effects of cognitive enhancers on the processes that underlie the object recognition task.

Scopolamine and Object Recognition

There has been one instance in which scopolamine was used in the object recognition task in zebrafish, which was previously outlined in the manuscript (Braidà, Ponzoni, Martucci, & Sala, 2014a). While there has only been this one instance of using scopolamine-exposed zebrafish in conjunction with the object recognition task, there has

been research into such effects while using the rodent model. In one such study, the rodents were injected with scopolamine and then presented with a set of familiar objects for 3 min (Akkerman et al., 2012). After either a 1 hr or 24 hr time delay, the rodents were then presented with both a familiar object and a novel object, and the amount of time spent around the objects was measured. The rodents injected with scopolamine did not show an exploration deficit above chance, indicative of memory deficits.

In another study, the insular cortex of the rodents was injected with scopolamine after the first trial and the effects on memory consolidation in the object recognition task were examined (Bermudez-Rattoni, Okuda, Roozendaal, & McGaugh, 2005). The insular cortex is known to be involved with taste-based memory, but there is a lack of information on the potential role of the structure in other forms of memory (Bermudez-Rattoni, 2004). Like in the other studies, the rodents were presented with two identical objects to explore. After a 24 hr time delay, the rodents were then presented again with one familiar object and one novel object. The results indicated that post-training injections of scopolamine into the insular cortex presented with a lack of memory consolidation and thus behaviorally, the rodent spent more time exploring the familiar object than the novel object. Not only does this exemplify the ability of scopolamine to affect the learning and memory processes associated with the object recognition task but also the use of the insular cortex in memory consolidation not associated with taste-related memories.

Since scopolamine is a muscarinic cholinergic antagonist that causes amnesia-like effects, researchers have also examined how substances that increase acetylcholine neurotransmission, such as vortioxetine, affect the performance of scopolamine exposed

rats on tasks such as object recognition (Pehrson et al., 2016). The rodents in this study were presented with two identical objects and allowed 5 min to freely explore these objects and were then removed to their home cage for a 1 hr time delay. After this delay, they were moved back to the testing maze and presented with a familiar object and a novel object. The rodents were injected with vortioxetine 1 hr before the initial (familiar objects only) trial and subsequently with scopolamine 55 min before this trial began. Results indicated that vortioxetine reversed the memory deficits usually seen with the administration of scopolamine.

Again, as object recognition is so underutilized in the zebrafish, we aimed to not only use this task more widely in the species but to also assess the effects of scopolamine on the performance of the fish. We streamlined the object recognition task as well as aimed to understand how scopolamine affected memory and learning performance in the task when administered to the tank water of the subject and not injected straight into the brain of the animal. Such expansions and clarifications can not only allow us to further understand the learning and memory capabilities of the zebrafish but can also assist in the elucidation of the effects of neurodegenerative diseases such as AD on working memory using scopolamine as an amnesia-inducing substance in the zebrafish.

Beaker Stress and Object Recognition

As with many of the other tasks and treatments, the availability of previous zebrafish research is negligible, so rodent research must be examined to understand the potential effects of stress on object recognition. For instance, when exposed to cat pheromone, which is used to induce stress, rodents spent more time exploring the familiar than the novel object in an object recognition task than those rodents who were not

exposed to the pheromone (Ozbeyli et al., 2015). When made to perform regular aerobic exercise, however, the rats that were also exposed to the cat pheromone increased the amount of time spent with the novel object when compared to the rodents who did not perform the regular exercise.

Rodent researchers have also explored the effects of restraint stress on rodent performance in the object recognition task (Vargas-López et al., 2015). The rodents were restrained for either 1- or 4 hrs before the initial exploration trial. After the initial familiar object exploration trial, there was a 1- or 24 hr time delay. Rodents who were restrained for 4 hrs before the initial trial and were then tested for object recognition 1 hr later exhibited preference for the familiar object, which shows cognitive deficiencies linked to acute stress. Interestingly, however, neither amount of restraint stress had any effect on object recognition after the 24 hr delay, which would indicate that there is a ceiling on the detrimental effects of the stress on working memory performance. The authors also argued that acute stress did not necessarily impede memory functioning but rather allowed for emotional situations in which the animal preferred to avoid any new, unfamiliar object instead preferring to stay secure by exclusively exploring the familiar, known object.

In another study where rats were restrained in a polypropylene cylinder for 6hrs/day for 21 days with or without the addition of *Lactobacillus helveticus*, bacteria used in the production of cheese, every day, working memory ability was measured using object recognition (Liang et al., 2015). *L. helveticus* in the body is thought to decrease as stressful events occur which then leads to cognitive and emotional dysfunction via the microbiota-gut-brain axis (Borre, Moloney, Clarke, Dinan, & Cryan, 2014; Tannock &

Savage, 1974). In a similar protocol to the other object recognition tasks described in the manuscript, the rodents were presented with the familiar and novel objects after the initial exposure trial at a 3 hr long time delay. After the 21-day restraint stress, rodents who did not receive the *L. helveticus* exhibited memory impairment (spent less time exploring the novel object). Rodents who were administered *L. helveticus* every day had improved memory functioning compared to the control group.

As there is not much information on not only object recognition in the zebrafish, but also the use of the beaker stress protocol, we aimed to use both in conjunction to further understand the underlying mechanisms behind the effects of stress on learning and memory. By understanding this in the zebrafish, we should not only be able to streamline new testing practices but also further understand the biological and behavioral underpinnings of working memory in humans.

Methods

Object Recognition

After a 2-day acclimation period in individual housing, the fish were tested individually in the object recognition task. There were 15 fish per group. Fish were netted and placed into a holding tank (whole tank lined with matte white shelf liner, void of any partitions) for a 5 min habituation period in which they could freely explore the tank without any stimuli present. Immediately following the habituation period, the fish were submitted to Trial 1. In Trial 1, the fish were netted and placed into the testing chamber that was identical to the holding tank with the addition of two identical LEGO® pieces (construction men with red vest and blue pants; see Figure 3), placed side-by-side in the middle of the tank.



Figure 3. Trial 1 Familiar Object Tank

Stars indicate placement of familiar object

These figurines were secured to the bottom of the tank so they could not float or move during testing. They had 10 min to explore the tank. At the end of this exposure, they were placed back into the holding tank for a 30 min time delay. This delay was chosen based on preliminary data that indicated the ability of the fish to significantly retain information in the object recognition task.

Immediately after this 30 min time delay, Trial 2 began. The fish were netted from the holding tank and placed into the middle of the testing chamber that was identical to the holding tank with the following change: one of the familiar objects (construction LEGO® man) was placed at one side of the tank while the other, novel object (heavy metal LEGO® man dressed in all black with black wig) was placed at the other end of the tank, directly across from the familiar object (see Figure 4).



Figure 4. Trial 2 Novel Object Tank

Star indicates placement of familiar object. Circle represents placement of novel object. Objects are randomly assigned between the two locations

The middle of the tank was void of any objects. The placement of these objects was alternated randomly per trial. The fish were then allowed to explore the tank for 10 min. At the end of this period, they were placed back in their home tank and were not tested again.

During both Trial 1 and Trial 2, the fish were recorded via an overhead camera (Logitech, Tesseract 2.0/3.7). These videos were analyzed by the computer program idTracker (Pérez-Escudero et al., 2014) to track where the fish swam and how much time each fish spent around each object. The computer program MATLAB was then used to convert this information for further analysis.

Methylene Blue

The experimental group (15 fish that receive MB) followed the same object recognition procedure listed above, but instead of the previously listed 30 min time delay after Trial 1, the fish were exposed to a 24 hr time delay (12 hr in MB/food colored water

and 12 hr in a fresh watered home tank). The fish were individually exposed to 0.5 μM of MB, the optimal dose previously discovered, mixed with tank water (Sigma-Aldrich, St. Louis, MO, USA; dye content $\geq 82\%$, purity 90%). The control group (15 fish) was individually exposed to a mixture of blue food colorant (Wilton, Woodridge, IL, USA; approx. Pantone PMS 294) mixed with tank water. The experimental group was placed into a tank with 1.5 L of tank water mixed with the 0.5 μM dose of MB. The control group was put into 1.5 L of water mixed with an amount of blue food colorant mixed to match the color of the MB water. The fish were kept in these tanks for 12 hr per MB dosing reports previously listed. After this period, they were removed from these tanks, rinsed with fresh tank water, and returned to their home tanks, which contained fresh water, devoid of any drug or colorant for 12 hr.

After 12 hr in the fresh watered home tank, the fish were then placed into the testing chamber for the Trial 2. The fish were given 10 min to explore the tank and the objects. During both Trial 1 and Trial 2, the fish were recorded via an overhead camera (Logitech, Tessar 2.0/3.7). These videos were then analyzed by the computer program idTracker to track where the fish swam and how much time each fish spent around each object. The computer program MATLAB was used to convert this information for further analysis. Administration of MB was based on research previously completed by the investigator (Echevarria, Caramillo, & Gonzalez-Lima, 2016).

Scopolamine

Prior to the habituation period in the holding tank and Trial 1, 15 fish were individually exposed to scopolamine hydrobromide trihydrate (Fisher Scientific, Hanover Park, IL, USA; purity 99%). One hr before experimentation, the fish were placed

individually into a tank that was filled with 200 μ M of the scopolamine solution dissolved into 1.5 L of tank water. The control group (15 fish) spent 1 hr prior to experimentation in a tank filled with 1.5 L of tank water, void of scopolamine.

Following the scopolamine exposure period, the fish were netted and placed into a holding tank (whole tank lined with matte white shelf liner, void of any partitions) for a 5 min habituation period in which they could freely explore the tank without any stimuli present. At this point, the fish were then subjected to the previously outlined methods in section 3.2.2. The scopolamine-exposed fish were subjected to the 30 min time delay between trials.

During both Trial 1 and Trial 2, the fish were recorded via an overhead camera (Logitech, Tessar 2.0/3.7). These videos were analyzed by the computer program idTracker (Pérez-Escudero, Vincente-Page, Hinz, Arganda, & de Polavieja, 2014) to track where the fish swam and how much time each fish spent around each object. The computer program MATLAB converted this information for further analysis.

Beaker Stress

Prior to experimentation, 15 fish were individually exposed to a 250 mL beaker filled with 100-mL of fresh tank water. They were kept in this beaker for 15 min. The control group (15 fish) stayed in their home tank for this 15 min period.

Following beaker stress, the fish were netted and placed into a holding tank (whole tank lined with matte white shelf liner, void of any partitions) for a 5 min habituation period in which they could freely explore the tank without any stimuli present. After this point, the fish were then subjected to the previously outlined methods

in section 3.2.2. The beaker stress-exposed fish were subjected to the 30 min time delay between trials.

During both Trial 1 and Trial 2, the fish were recorded via an overhead camera (Logitech, Tesser 2.0/3.7). These videos were then analyzed by the computer program idTracker (Pérez-Escudero et al., 2014) to track where the fish swam and how much time each fish spent around each object. The computer program MATLAB was then used to convert this information for further analysis.

Results

Statistical Analyses Methylene Blue

Following the discrimination measures suggested in Akkerman, Prickaerts, Steinbusch, & Blokland (2012), one sample t-tests were conducted to determine which object the fish spent more time exploring. The discrimination measures include D1, D2, and D3 (see Table 2).

Table 2

Measures in the Object Recognition Task

<u>Exploration</u>	<u>Discrimination</u>
	$D_1 = A_3 - B$
$E_{T2} = A_3 + B$	$D_2 = D_1/E_{T2}$
	$D_3 = A_3/E_{T2}$

Exploration in Trial 2 (E_{T2}) was calculated by finding the summation of time spent around the familiar object (A_3) and novel object (B) in Trial 2

Each discrimination measure was compared against a number that represented chance or random exploration. This number was 0 for D1 and D2 and .5 for D3. Comparison of these measures against chance indicated whether the animals discriminated between the familiar object and novel object. A positive measure indicated preference for the familiar object while a negative number indicated preference for the novel object. Significance indicated such discrimination and indicated that the fish remembered the familiar object from the first exposure, and the exploration was not random. We chose to exclusively use D3 as it was found to be the most conservative measure (See Table 3).

Table 3

A Comparison of Object Preference Using D₃ within MB, Scopolamine, and Beaker Stress Conditions

<u>Condition</u>	<u>N</u>	<u>Mean</u>	<u>SD</u>	<u>t</u>	<u>df</u>	<u>sig.</u>
MB	15	0.37	0.11	-4.37	14	.00 *
Scopolamine	15	0.36	0.31	-1.71	14	.11
<u>Beaker Stress</u>	15	0.04	0.10	-18.02	14	.00 *

* Significant Difference at $p < 0.05$

A one sample t-test using the D3 measure indicated that fish exposed to MB significantly preferred the novel object ($M = 0.37$, $SD = 0.11$); $t(14) = -4.37$, $p = 0.00$.

A paired samples t-test was performed to determine whether there was a significant difference between the amount of time spent exploring the familiar object in

Trial 1 and the familiar object in Trial 2 within in the same MB exposed fish, and there was no significant difference ($M = 5.88$, $SD = 41.51$); $t(14) = .55$, $p = 0.59$.

Statistical Analyses Scopolamine

Using the previously listed discrimination measure, a one sample t-test using the D3 measure indicated that there was no significant preference for either the familiar or novel object by fish who were exposed to scopolamine ($M = .36$, $SD = .31$); $t(14) = -1.71$, $p = 0.11$.

A paired samples t-test was performed to determine whether there was a significant difference between the amount of time spent exploring the familiar object in Trial 1 and the familiar object in Trial 2 within in the same scopolamine exposed fish, and there was no significant difference ($M = 9.72$, $SD = 39.96$); $t(14) = 0.94$, $p = 0.36$.

Statistical Analyses Beaker Stress

Using the previously listed discrimination measures, a one sample t-test using the D3 measure indicated that fish exposed to beaker stress significantly preferred the novel object when compared to the familiar object ($M = 0.04$, $SD = 0.10$); $t(14) = -18.02$, $p = 0.00$.

A paired samples t-test was performed to determine whether there was a significant difference between the amount of time spent exploring the familiar object in Trial 1 and the familiar object in Trial 2 within in the same beaker stress exposed fish, and there was no significant difference ($M = 11.42$, $SD = 49.42$); $t(14) = 0.89$, $p = 0.39$.

CHAPTER IV – ESCAPE LEARNING IN THE ZEBRAFISH

Introduction

Escape Learning in the Zebrafish

Unlike T-maze and object recognition, escape learning has been used more extensively to study learning and memory in the zebrafish. Zebrafish exhibit a very clear behavioral fear response when presented with fear-inducing stimuli. For instance, the fish might engage in leaping, diving to the bottom of the tank, increased shoaling, and erratic swimming (swimming in a zig-zagging motion; Gerlai, 1993; Speedie & Gerlai, 2008). In an early study, zebrafish were trained to exhibit fear responses to an originally innocuous stimulus (Hall & Suboski, 1995). By administering an alarm pheromone to the water the same time a red light flashed, the fish displayed fearful behaviors when presented with only the red light, sans alarm pheromone. By exemplifying the ability to be influenced by alarm pheromones and thus be conditioned to associate fear with a neutral stimulus, researchers expanded on the potential use of escape learning in the zebrafish.

In a more recent study, researchers extracted alarm pheromone by making shallow cuts on the skin of recently sacrificed zebrafish (Speedie & Gerlai, 2008). They then added the collected pheromones to the tank water of a live zebrafish and observed the behavioral reactions of the fish. The researchers found that the alarm pheromone elicited a robust fear response that included erratic swimming and increased shoal cohesion in the zebrafish, independent of viewing of a predator fish or other fear-inducing stimuli.

While using pheromones and other olfactory cues does induce the fear response, the use of such chemicals does have some issues we must note. Not only do the tanks have to be thoroughly and methodically cleaned after every trial, but also the pheromones

themselves may not be as stable over time. Due to this, researchers have utilized a protocol in which zebrafish are exposed to live predatory or harmless fish (Bass & Gerlai, 2008). Whether placed inside the same tank or in another tank but still visible to the zebrafish, the predatory fish elicited a significant fear response in the zebrafish (diving to the bottom of the tank, leaping, preferring dark areas of the tank, moving far from the predator fish, etc.) when compared to zebrafish exposed to the harmless fish.

In addition to the use of live predator fish, computer-animated images of such species have also been used successfully to elicit the fear response in the zebrafish (Ahmed, Seguin, & Gerlai, 2011). In tasks such as these, the fish were placed into a testing tank and presented with a computer-animated image of a predatory fish such as the Indian leaf fish, a natural predator of the zebrafish. The presentation of the sympatric fish elicited a robust response from the zebrafish in which it spent significantly more time away from the screen on which the predator image was displayed.

The effectiveness of using a computer animated image of an Indian leaf fish has also been corroborated in another study that examined the effects of different shapes (Indian leaf fish, moving dot, zebrafish, and a bird) on the fear response of the zebrafish (Luca & Gerlai, 2012). The results indicated that the image of the Indian leaf fish elicited a fear response in the zebrafish. Additionally, objects presented on a computer screen above the testing chamber such as the dot and the bird elicited an even more robust fear response than the image of an Indian leaf fish. These images caused the zebrafish to engage in erratic swimming, more time spent on the bottom of the tank, leaping, and decrease of activity.

Such exposure to alarm pheromones and images of predators acts on the zebrafish nervous system in a comparable way to the human fear response. When presented with a fear-inducing stimulus, information is sent from the different sensory systems to the zebrafish equivalent to the amygdala (the seat of the fear response in humans), which is located in the dorsal pallium (Northcutt, 2006). This information is then relayed out to the hypothalamus and other regions involved in the fear response, such as those structures located in the brain stem. The locus coeruleus, periaqueductal grey, and raphe nuclei allow the organism to engage in the fight or flight behavior (Agetsuma et al., 2010; Lillesaar, Stigloher, Tannhauser, Wulliman, & Bally-Cuif, 2009; Ma, 1994). Within the fight-or-flight response is the activation of the HPI axis that increases the amount of cortisol, the stress hormone, in the body of the zebrafish (Alsop & Vijayan, 2008).

With such a robust and observable fear response, the zebrafish is a very viable model that can be used to test the effect of different treatments on the fear response. While alarm pheromone can be used to elicit this response, the use of computer animated images is preferable for its controllability and consistency (speed, size, type of image) as well as not needing to sacrifice fish to obtain the alarm pheromone itself. Additionally, the task can be performed in a time effective manner and the behaviors quantified either via researcher observation or a computer tracking program.

Methylene Blue and Escape Learning

The use of MB and its effects on escape learning has been so far untested in the zebrafish and the rodent model to the knowledge of the researcher. Due to the lack of available research, we delineate the related information available on the influence of MB on the fear response using other fear-related learning tasks such as fear conditioning. One

of the available studies showed that when rodents were injected with MB repeatedly for the 5 days following the extinction period of a fear conditioning paradigm (pairing a tone with a foot shock), they presented with enhanced extinction memory (Gonzalez-Lima & Bruchey, 2004). After being exposed to a fear conditioning paradigm, a rodent would normally freeze when returned to the chamber in which the conditioning took place. Extinction gradually ameliorates this freezing behavior so the rodent begins to explore the chamber normally, as before the conditioning experience. Following the MB exposure, treated rats presented with less freezing behavior than those who were injected with saline, thus showing an enhanced retention of the extinction memory.

Unfortunately, the only studies the researchers are aware of that examine the effect of MB on anything relating to the fear response focus on extinction behaviors and not how the substance specifically modulates cognition related to the escape learning process and not extinction. Due to such a lack of research on MB in conjunction with escape learning, there is an elevated need for more information on such effects. By utilizing the zebrafish model, we aimed to not only learn more about the fear response in the zebrafish but also about the potential memory-enhancing effects of MB on the learning process itself.

Scopolamine and Escape Learning

Scopolamine has yet to be used in researching the fear response in the zebrafish. As such, the rodent model must be examined to understand the effects of muscarinic antagonists on escape learning behaviors. As in zebrafish and humans, the amygdala modulates the fear response, and more specifically, escape learning in rodents (Kapp, Pascoe, & Bixler, 1984). The central nucleus of the amygdala mediates movements

related to the fear response (LeDoux, Iwata, Cicchetti, & Reis, 1988). The muscarinic receptors on the basolateral nucleus in the amygdala are responsible for emotional memory consolidation, which is especially salient in the escape learning response (Muller, Mascagni, Zaric, & McDonald, 2013).

In addition to the amygdala, the hippocampus has also been shown to be instrumental in the encoding and retrieval of fear-related memories (Hasselmo & McClelland, 1999). When a stimulus is to be committed to memory, a mismatch of inputs stimulates the medial septum, which results in increased release of acetylcholine in the hippocampus. Such a release of this neurotransmitter begins the encoding phase of memory (Hasselmo, 1999). Reduced release of acetylcholine in the same areas stimulates memory retrieval.

The effects of scopolamine, a cholinergic antagonist, on such action can be seen in a study in which rodents were administered scopolamine into the hippocampus 10 min before a fear related task (tone/foot shock; Rogers & Kesner, 2004). The rodents were exposed to a contextual retention test (24 hrs after conditioning) and cue retention test (48 hrs after conditioning). The researchers hypothesized that the scopolamine would disrupt contextual conditioning due to the disruption of the encoding of memory while the substance should have no effect on contextual retrieval, and their results upheld their hypothesis.

While we know that scopolamine does influence fear learning with associative tasks (pairing foot shock with tone), the question must now become whether scopolamine influences escape learning when the fear response is elicited by predator exposure. According to some, exposure to a predator increases arousal and allows for greater

encoding and neuronal plasticity, specifically long-term potentiation (LTP; the strengthening of synapses due to repeated activation), which is instrumental in the creation of memories (Dringenberg, Oliveira, & Habib, 2008). In vivo electrophysiological work indicated that LTP was significantly greater when the rodents were in the presence of cat hair (exposure to a predator) and actively avoided the stimuli. When the rodents were administered scopolamine in addition to exposure to the cat hair, the LTP was significantly reduced compared to those who did not receive the substance. Meaning, exposure to a predator and the related fear response can enhance memory and cognitive ability.

In a study in which rats were exposed to a known predator and given scopolamine before said exposure, the natural responses of the rodents were not altered (avoidance of predator, freezing, defensive attack, etc.; Rodgers, Blanchard, Wong, & Blanchard, 1990). However, when the scopolamine was administered during direct exposure to a predator (cat), the rodent spent more time near the cat, exploring the area around the cat, and lessened the fear-related behaviors of the rodent. It should be noted, however, that while these findings were reliable, they were not pronounced enough to say with confidence that there is a significant effect of muscarinic receptors on the fear response. Following the logic these experiments afforded, we aimed to determine the effects of scopolamine on the fear response of the zebrafish in an escape learning paradigm.

Beaker Stress and Escape Learning

Once again, there is no research, to our knowledge, that investigates the connection between stress and the fear response in the zebrafish. Due to this, rodent research must be examined to better understand the potential dynamics between the

condition and the task. When rats are put through chronic restraint stress (6hr/day/21 days) and were then tested using a fear learning paradigm (pairing foot shock with a tone) in which they received the pairing in one context (familiar) for the training trial and then were tested in either the familiar context or a novel context with just the tone (Hoffman, Lorson, Sanabria, Foster-Olive, & Conrad., 2014). Compared to a control, rats who received the chronic restraint stress had enhanced fear acquisition of the tone, decreased fear extinction, and increased fear response to just the familiar context, without tone. In short, rodents who were chronically stressed had enhanced memory abilities.

Neurobiologically underlying this stress influenced fear response is the amygdala. Chronic, repeated stress has been shown to trigger hyperactivity in the amygdala, which can then lead to the development of mood and/or anxiety disorders (Lupien, McEwen, Gunnar, & Heim, 2009; Protopopescu et al., 2005). More specific to fear learning, chronic stress enhances fear based learning, which is controlled by the basolateral amygdala (Pare & Collins, 2000). The pharmacological blocking of stress-induced hyperactivity of the basolateral amygdala via the inhibition of the small conductance channels negates the fear based learning enhancement caused by chronic stress (Atchley, Hankosky, Gasparotto, & Rosenkranz, 2012).

In a paradigm that utilized acute restraint stress, rodents were restrained in a plastic tube for 30 mins prior to the training trial in which a foot shock was given only in that testing chamber (Maldonado, Espejo, Martijena, & Molina, 2013). The rodents were then monitored again in the same testing chamber but without the administration of the foot shock. The researchers recorded freezing behavior, a measure of learned fear response, before the training trial and during the testing trial to compare whether the fear

behavior was successfully learned. Not only did those rats that were acutely restrained have enhanced learned fear response, but substances that block the LTP formation in the amygdala seemed to reverse this effect, reducing the learned fear response.

The validity of using acute restraint stress to induce enhanced fear learning is corroborated by a study that examined whether one, acute exposure to restraint stress was enough to produce similar behavioral effects as seen in those rats who were exposed to chronic restraint stress (Cordero, Venero, Kruyt, & Sandi, 2003). In this study, rodents were exposed to one session of restraint stress that consisted of being confined to a plastic tube for 2 hrs. Two days after this confinement, the rats were then placed into the conditioning chamber in which they received a foot shock. There was also a group that received the foot shock paired with an auditory cue. Either one or seven days after this, the rats were tested for learning acquisition by recording their freezing behaviors. The researchers found that the single exposure to the restraint stressor significantly enhanced fear learning for those rodents who received the context-based conditioning paradigm but not the auditory-based conditioning paradigm. This enhancement was seen after both the one-day and seven-day time delays.

With such findings, we aim to show that acute stress in the form of the beaker stressor can, like in the rodent research, successfully elicit enhanced escape learning in the zebrafish. By subjecting the fish to beaker stress prior to viewing the predator videos, we expected to see a robust increase in fear induced behaviors when placed back in the context of the conditioning trial.

Methods

Predator Exposure Apparatus

The experiment took place in a 4 L tank with green corrugated plastic on three sides and white shelf liner on the bottom to allow for easier filming (see Figure 5).

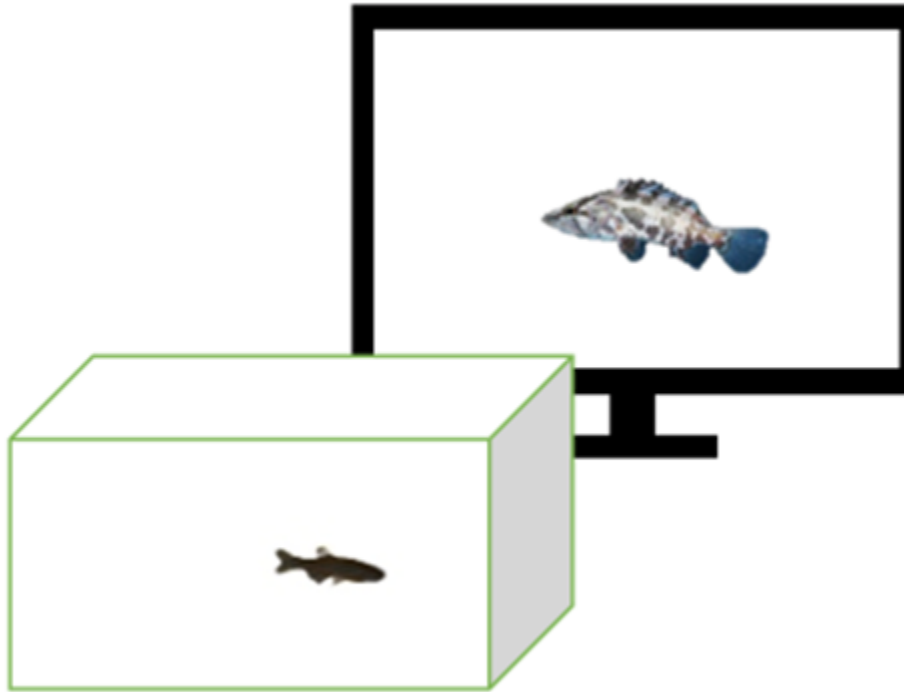


Figure 5. Escape Learning Apparatus

There was a computer monitor (13 in. IBM ThinkVision Flat Panel LCD monitor) flush to the uncovered side of the tank. The monitor was connected to an HP Compaq desktop PC that will run computer software that presents images of predatory fish (Gerlai Lab, Toronto Canada). An image of an Indian leaf fish, a natural predator of the zebrafish, was presented on this screen using software created by the Gerlai lab (Toronto, Canada).

Exposure Procedure

Fifteen experimentally and drug naïve fish per group (control and experimental) were tested once, individually in the escape learning paradigm. The fish went through a two-day acclimation period in their home tanks where they were housed individually. Following this acclimation period, the fish were netted and put into the testing tank individually. The fish were allowed 5 min to habituate to the novel environment in which a blank black screen was presented on the computer monitor. After this habituation period, the fish were automatically (without experimenter manipulation) presented with a computer animation of either a singular Indian Leaf fish or a blank black screen for 5 min. The Indian Leaf fish was presented moving at a set speed of .3cm/s. The behavior of the fish was captured via overhead camera (Logitech, Tessar 2.0/3.7) for later coding. After the 5 min presentation of stimuli, the animation was automatically turned off and reverted to a blank black screen, and the fish was allowed to remain in the tank for 5 min with no stimulus before being netted and returned to the home tank.

The fish was returned to the testing chamber the next day to test for acquisition of the escape learning behavior. On this trial, after the 5 min habituation period, each group (predator exposed and control) viewed a blank black screen for a 5 min period of time. Following this exposure, the fish was allowed to re-acclimate to the tank for 5 min. This trial was recorded via the overhead camera. The fish were returned to their home tanks and were not tested again.

idTracker was used to analyze the videos to quantify how long each fish spent close to computer monitor, how long they spent at the opposite side of the tank from the monitor, their average speed and velocity, and the distance they traveled overall in the tank. MATLAB was used to further translate this information.

Methylene Blue

Immediately after the first trial, 60 fish (15 fish per group; 4 groups: predator MB, predator control, control MB, and control control) were either exposed to MB mixed with tank water (Sigma-Aldrich, St. Louis, MO, USA; dye content $\geq 82\%$, purity 90%) or a mixture of blue food colorant (Wilton, Woodridge, IL, USA) and tank water that matched the color of the 0.5 μM MB dose (approx. Pantone PMS 294). The experimental group was put into 1.5 L of water with the 0.5 μM dose of MB. The control group was put into 1.5 L of water mixed with an amount of blue food colorant mixed to match the color of the MB water. The fish were kept in these tanks for 12 hr per prior MB dosing reports. After this period of time, they were removed from these tanks, rinsed with fresh tank water, and returned to their home tanks, which contained fresh water, devoid of any drug or colorant.

After 12 hr in the fresh watered home tank, the fish went through the procedure listed above for the probe trial. All activity was recorded via an overhead camera and the data was analyzed in the same process detailed above. Administration of MB was based on previous research completed by the investigator (Echevarria, Caramillo, & Gonzalez-Lima, 2016).

Scopolamine

Prior to the exposure trial, 60 fish (15 fish per group; 4 groups: predator scopolamine, predator control, control scopolamine, and control control) were individually exposed to scopolamine hydrobromide trihydrate (Fisher Scientific, Hanover Park, IL, USA; purity 99%). One hr before the initial trial, the predator scopolamine, and control scopolamine fish were placed individually into their home tank that was filled

with 200 μ M of the scopolamine solution dissolved into 1.5 L of tank water. The predator control and control control groups were placed into a tank filled with 1.5 L of tank water sans scopolamine for 1 hr prior to experimentation. After this exposure, the fish were placed into the testing tank. They had a 5 min acclimation period, 5 min exposure to the predator or blank screen, and 5 min re-acclimation period. The fish were placed back into their home tank for 24 hr. The fish were re-tested for the probe trial after the 24 hr period without the scopolamine exposure. Both groups viewed the blank black screen during the probe trial. The fish were recorded throughout.

Beaker Stress

Sixty experimentally naive fish (15 fish per group; 4 groups: predator beaker, predator control, control beaker, and control control) of unknown sex aged 6-9 months were exposed individually to either a 250-mL beaker filled with 100-mL of tank water or kept for that 15 min period of time in their home tank. After the 2-day acclimation to single housing in the blue system, the fish were individually placed into a 250 ml beaker filled with 100 ml of tank water for a 15 min exposure. After this exposure, the fish were placed into the testing tank. They had a 5 min acclimation period, 5 min exposure to the predator or blank screen, and 5 min re-acclimation period. The fish were placed back into its home tank for 24 hr. The fish were re-tested for the probe trial after the 24 hr period without the beaker stress exposure (only 5 min, 5 min, 5 min testing/acclimation). The fish were recorded throughout.

Results

A three-way ANOVA was performed to determine whether there were significant differences on the change scores (time spent away from screen in the probe trial – time

spent away from the screen in the exposure trial = change score; a positive number would indicate learning of the behavior) between the condition received (MB, scopolamine, or beaker stress), whether the condition was received (experimental or control), and whether the predator image was shown on the computer screen during the first day (predator image shown or blank black screen shown; See Table 4).

Table 4

Change Score Comparison Between Condition, Receipt of Condition, and Presentation of Stimuli

	Sum of Squares	df	Mean Square	F	sig
Condition (A)	3.6E + 8	2	1.8E + 8	2.38	.09
Receipt (B)	7.5E + 7	1	7.5E + 7	.98	.32
Presentation (C)	2.7E + 8	1	1.7E + 8	3.52	.06
A x B	4.2E + 8	2	2.1E + 8	2.73	.06
A x C	7.4E + 8	2	3.7E + 8	4.83	.00*
B x C	469201	1	469201	.00	.93
A x B x C	3.7E + 8	2	1.9E + 8	.08	.02*
Error	1.E + 10	168	7.6E + 7		

Significant Difference at $p < 0.05$

There was a statistically significant two way interaction between condition and screen presentation, $F(2, 168) = 4.83$, $p = 0.00$. Additionally, there was a marginally significant main effect on condition, $F(2, 168) = 2.38$, $p = 0.09$ and on screen presentation, $F(1, 168) = 3.52$, $p = 0.06$. There were also marginal interactions between condition and whether the treatment was received, $F(2, 168) = 2.73$, $p = .06$, and a

marginally significant three way interaction of condition, receipt, and screen presentation, $F(2, 168) = 2.45, p = 0.08$.

Specifically, MB had significant main effect with presentation of the stimuli, $F(1, 56) = 3.85, p = 0.05$. T-tests revealed that there was significance for MB grouped fish who were presented with the predator image when both receiving the treatment ($M = 5336.07, SD = 1074.10$), $t(14) = 1.92, p = 0.07$. The effect size ($d = 0.72$) exceeds the boundaries for a medium effect size. There was also significance for MB grouped fish who did not receive the treatment but viewed the predator image ($M = 3569.33, SD = 3866.44$), $t(14) = 3.57, p = 0.00$ (See Table 5).

Table 5

MB Escape Learning Comparison Within Groups Using the Change Score

<u>Condition</u>	<u>Receipt</u>	<u>Presentation</u>	<u>t</u>	<u>df</u>	<u>sig.</u>
MB	Yes	Predator	1.92	14	.07
	Yes	Blank Screen	1.04	14	.31
	No	Predator	3.57	14	.00*
	No	Blank Screen	-.08	14	.93

Significant Difference at $p < 0.05$

T-tests were also used to indicate that there was significance for scopolamine grouped fish when they received the condition and were either presented with the predator image ($M = -3764.10, SD = 7918.78$), $t(14) = -1.84, p = 0.08$, effect size ($d = 0.98$) or the blank black screen ($M = 2570.00, SD = 4434.72$), $t(14) = 2.24, p = .04$ (See Table 6).

Table 6

Scopolamine Escape Learning Comparison Within Groups Using the Change Score

Condition	Receipt	Presentation	t	df	sig
Scopolamine	Yes	Predator	-1.84	14	.08
	Yes	Blank Screen	2.24	14	.04*
	No	Predator	-.09	14	.92
	No	Blank Screen	-.16	14	.87

Significant Difference at $p < 0.05$

Beaker stress had a significant main effect with receipt of the treatment, $F(1, 56) = 3.76$, $p = 0.05$, and screen presentation, $F(1, 56) = 5.76$, $p = 0.02$. T-tests indicated that there was significance for the beaker stress grouped fish when they received the condition but did not view the predator image ($M = -8191.90$, $SD = 14709.80$), $t(14) = -2.15$, $p = 0.04$, and when they did not receive the condition and viewed the predator image ($M = 3930.07$, $SD = 5139.16$), $t(14) = 2.96$, $p = .01$ (see Table 7).

Table 7

Beaker Stress Escape Learning Comparison Within Groups Using the Change Score

Condition	Receipt	Presentation	t	df	sig
Beaker Stress	Yes	Predator	.64	14	.53
	Yes	Blank Screen	-2.15	14	.04*
	No	Predator	2.96	14	.01*
	No	Blank Screen	.54	14	.59

Significant Difference at $p < 0.05$

CHAPTER V – DISCUSSION

In the attempt to further understand the cognitive and behavioral effects different conditions (MB, scopolamine, and stress) have on a variety of tasks (T-maze, object recognition, and escape learning), it was imperative that we examine the different types of learning associated with each task. In the first chapter of this dissertation, we examined the known effects of the three treatments. MB is a potential cognitive enhancer that has been used to treat a wide variety of ailments from malaria to schizophrenia. We aimed to further examine the ability of the substance to enhance cognition across a variety of tasks that focus on different categories of learning. By doing so, we aimed to bolster the ability of the zebrafish to screen for pharmaceuticals that can potentially alleviate the cognitive deficits seen in neurodegenerative diseases like AD.

Scopolamine, however, is quite the opposite from MB as it is a drug used to induce an amnesia-like state. By understanding the ability of scopolamine to interrupt the encoding process of memory across three different learning tasks, we aimed to further understand a potential way to induce AD-like symptoms in the zebrafish. Such an investigation may allow researchers to find and streamline another AD model to screen for potential treatments like MB for AD.

The final treatment that we examined was that of beaker stress. While we know the general effects of acute and chronic stress on learning and memory in rodents and humans, we attempted to understand how the stress condition might influence learning and memory in the zebrafish. By socially isolating and confining the zebrafish to a small amount of water in a novel beaker, we know they express a stress response due to cortisol

assays performed on exposed fish. We were thus interested in how such acute stress affected the different types of learning we examined in this dissertation.

In the following chapters, we examined the learning tasks themselves. Firstly, we delineated the current information about T-maze, a spatial learning task. Based on prior research, we hypothesized that MB would enhance the performance of the MB exposed zebrafish on the T-maze task (lower latency to arm), exemplifying the ability of MB to act on spatial learning and memory (Echevarria, Caramillo, & Gonzalez-Lima, 2016). Our second hypothesis with T-maze was that we believed that scopolamine would decrease performance on the T-maze task (scopolamine exposed fish would have a longer latency to arm; Braida et al., 2014b). Our third hypothesis was that beaker stress exposed fish would perform worse than control on the T-maze task (beaker stress fish would have a longer latency to enter an arm; Cofiel & Mattioli, 2009).

Our results indicated that there was not a significant difference within subjects from baseline to the probe trial, so learning of the spatial behavior was not conclusive. There was, however, a medium effect for both the MB and scopolamine groups, respectively. As such, we conclude that the MB group learned the behavior, and improved their latency to arm (less time to swim to the predetermined correctly patterned arm). The scopolamine group did not learn the behavior and spent more time swimming to an arm than control. Beaker stress did not have significance or an effect, so the behavior was not learned and there was not a significant difference from the baseline trial to the probe trial within fish. With a higher sample size, we would have garnered the power to exhibit the learned behavior significantly. With this said, however, there was marginal significance difference between the three treatment groups. Specifically, there

was a significant difference between the MB and scopolamine groups in latency to arm using the change scores. The MB fish had a significantly lower latency to arm than the scopolamine group. By exhibiting a lower latency to arm, the MB fish arguably remembered the spatial location of a food reward better than the scopolamine-fish. By having a longer latency to arm, the scopolamine group arguably did not remember the spatial location of the food reward as well as the MB-exposed fish. We can thus conclude that MB acts on the mechanisms behind spatial learning and enhances such cognition while scopolamine acts on the mechanisms thereby creating amnesia-like behaviors when tested on the T-maze.

While there was no statistical trend associated with the beaker stress treatment, we believe that the results are still telling. In the least, they show that acute stress in a spatial learning task is neither detrimental nor enhancing and that it perhaps has no effect on learning in this mechanism. Some considerations for future studies would be to increase the subject pool size. We chose 15 zebrafish per group as it is the industry standard, but by increasing the number, we might see greater statistical power that would allow us to see more clearly the comparison of baseline and probe trial latency-to-arm within groups. Ultimately, by understanding how the three conditions affect spatial learning and memory, we have become more confident in not only our testing procedures but also what such conditions might mean for AD research, and the role that MB and stress, in particular, can play on spatial memory in those with the disease.

Deficits in spatial memory for an AD patient can present itself with forgetting where one's keys are located or which street one lives on. Through this experiment, we have shown that MB could have a beneficial effect on spatial memory. If it translates to

humans, we could see AD patients remembering where their keys are or what street they live on for longer. Stress, however, seemed to not play a part in spatial learning and memory, which translates to humans in that perhaps stress related to understanding that one has the disease or the frustration of forgetting things does not have any effect on the ability to remember what one has learned, at least spatially. Through further studies, we aim to elucidate the ways in which MB use can be translated to human research as well as how to use scopolamine as a simulator for AD in the zebrafish.

In the third chapter, we examined the prior research on object recognition. The task we used measured the ability of the fish to recognize objects after a certain time delay, which was able to elucidate object recognition learning and memory. We hypothesized that MB would cause the exposed fish to spend more time around the novel object in trial two of the task, showing they better learned the recognition behavior (Kandratavicius et al., 2015). Per our results, our hypothesis was upheld; MB exposed fish spent significantly more time exploring the novel object than the familiar object in trial two when compared to chance. This indicated that the fish remembered the familiar object from the first trial. For the scopolamine condition, we hypothesized that the scopolamine-exposed fish would perform similarly in trial two of the object recognition task as in trial one. They would not spend significantly more time around the novel object in trial two (Braidia et al., 2014a). Once again, our hypothesis was upheld, and the scopolamine-exposed fish did not spend significantly more time exploring the novel object in trial two of the task. This indicates that the fish did not remember the familiar object from the previous trial. Finally, for beaker stress, we hypothesized that the stress-exposed fish would spend more time exploring the familiar object than the novel object in

the second trial. This was indicative of cognitive deficiencies linked to an acute stressor (Vargas-López et al., 2015). Our hypothesis was partially upheld, as the stress-exposed fish did not spend significantly more time around the novel object, but it did not spend significantly more time around the familiar object either. We can conclude that like scopolamine, stress has a detrimental effect on object recognition learning. By understanding how each treatment affected object recognition learning and memory, we can elucidate how outside factors such as stress can affect the test performance and overall cognitive abilities of a subject. This is important because it allows us, in the future, to be better able to treat the specific problems related to AD and other neurodegenerative diseases.

Object recognition in human AD can be correlated to forgetting a face or forgetting what one's house looks like. In showing that MB had very robust significance in the object recognition task, we can conclude that MB positively affects the ability to remember an object one has seen before and that MB acts on the mechanisms that underlie the object recognition task. This could potentially be used in humans to allow a person to remember an object, whether human or innate, a little while longer. Additionally, we have shown that scopolamine dissolves the ability to remember a previously seen object, and can thus be used to induce an AD-like state in the zebrafish used to test object recognition skills. Additionally, we exhibited that stress had a negative effect on learning in the object recognition task as the fish could not remember the object it had been exposed to in a previous trial. This is indicative of the negative effects stress can have on the cognition of a person. It begs for more effort to be put into alleviating the

stress of AD patients to allow for any cognitive confounds related to stress to be absolved.

In Chapter IV we expounded on predator exposure, a task that utilizes escape learning. It was anticipated that the zebrafish would react differently to the three previously named conditions. Specifically, we hypothesized that MB would enhance escape learning in the zebrafish (experimental fish would perform better than control and the fish would spend more time away from the screen in the second trial). This hypothesis was upheld, as the MB exposed fish spent significantly more time away from the screen than control thus exhibiting the ability of MB to enhance escape learning. Our second hypothesis was that scopolamine would cause a deficit in escape learning in the zebrafish (experimental fish would not spend more time away from the screen than control; Rogers & Kesner, 2004). This hypothesis was also upheld as the scopolamine exposed fish spent significantly less time away from the screen when compared to control, which would indicate that the scopolamine fish did not learn the escape behavior. With this said, however, scopolamine exposed fish who did not view the predator fish in the exposure trial, spent significantly more time away from the screen when compared to control. Finally, we hypothesized that beaker stress would enhance performance in escape learning (experimental fish would spend more time away from the screen in the second trial when compared to control; Maldonado, Espejo, Martijena, & Molina, 2013). This hypothesis was not upheld as the beaker stress fish did not spend more time away from the screen when compared to control. Arguably, it was quite the opposite, and stress obstructed the ability of the fish to learn the escape behavior.

Once again, knowing how MB, scopolamine, and stress affect fear learning, we can begin to understand the underlying mechanisms behind how learning and memory work. This is important because by doing so we can formulate new, more precise models of AD in the zebrafish as well as target certain behaviors with treatments such as MB. Additionally, we might be able to understand potential confounds like stress that could influence our test results. More specifically, fear learning in AD could be associated with any type of high-emotion learning or the ability of a person to remember and be aware of dangerous situations, possibly linked to impulsiveness. Understanding that MB has a positive effect on fear learning, we can now aim to use the substance to enhance the ability of human AD patients to be better able to process emotional learning, be aware of threat, and potentially alleviate emotionally-based deficits related to AD.

Additionally, now that we understand that scopolamine has a negative effect on escape learning, we can use the substance to further understand the effects of an AD-like state on fear learning. Finally, while it is believed that acute stress can enhance fear learning, our results indicated otherwise. Acute stress can perhaps be a detriment to fear learning. This should be considered when conducting research, as testing stress can become a confound to one's results. These findings reiterate the importance of reducing stress in testing as much as possible. One potential confound with this task is the potential that the fish may have been seeing their reflection from the light of the computer screen on the tank walls. To combat this, we will make new task tanks that have matte shelf liner on the inside of the tanks instead of the outside.

In completing this study, we aim to further validate the use of zebrafish as a comparative model for degenerative disease states such as AD. By using a substance,

scopolamine, that has begun to be used to induce an AD-like state in the zebrafish, we were further able to understand the negative effects the disease has on different learning types such as T-maze, object recognition, and escape learning. Additionally, we can continue to examine MB, which can be screened, using zebrafish, as a potential therapeutic and preventative treatment for neurodegenerative diseases like AD. Finally, we can continue to research the more confusing effects of stress on different types of learning. By learning these effects, we can better learn how to combat stress in human AD patients to allow for a better quality of life as well as a longer longevity of their cognitive abilities.

In our future directions, we aim to test how MB can affect the deleterious effects caused by scopolamine and stress. While we know that MB can enhance the cognitive abilities of a healthy brain, can the substance reverse the negative cognitive effects caused by scopolamine and, potentially, stress? By answering these questions, we can further the investigation into MB being used as a treatment for cognitive deficiencies as well as the ability of scopolamine to be used to induce an AD-like state in the zebrafish.

APPENDIX A – IACUC Approval Letters



THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

118 College Drive #5116 | Hattiesburg, MS 39406-0001
Phone: 601.266.6791 | Fax: 601.266.4377 | iacuc@usm.edu | www.usm.edu/iacuc

NOTICE OF COMMITTEE ACTION

The proposal noted below was reviewed and approved by The University of Southern Mississippi Institutional Animal Care and Use Committee (IACUC) in accordance with regulations by the United States Department of Agriculture and the Public Health Service Office of Laboratory Animal Welfare. The project expiration date is noted below. If for some reason the project is not completed by the end of the approval period, your protocol must be reactivated (a new protocol must be submitted and approved) before further work involving the use of animals can be done.

Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 11102703 (Modification dated 10/24/16)
PROJECT TITLE: Zebrafish Learning Acquisition in the T-Maze
PROPOSED PROJECT DATES: 11/2016 – 09/2017
PROJECT TYPE: Modification
PRINCIPAL INVESTIGATOR(S): David Echevarria
DEPARTMENT: Psychology
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Designated Review
PROTOCOL EXPIRATION DATE: September 30, 2017



Jake Schaefer, PhD
IACUC Chair

11/15/2016

Date



THE UNIVERSITY OF
SOUTHERN MISSISSIPPI

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

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PROTOCOL NUMBER: 16052603 (Modification dated 10/24/16)
PROJECT TITLE: Object Recognition in the Zebrafish
PROPOSED PROJECT DATES: 11/2016 - 09/2018
PROJECT TYPE: Modification
PRINCIPAL INVESTIGATOR(S): David Echevarria
DEPARTMENT: Psychology
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Designated Review
PROTOCOL EXPIRATION DATE: September 30, 2018



Jake Schaefer, PhD
IACUC Chair

Date 11/15/2016



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Any significant changes should be brought to the attention of the committee at the earliest possible time. If you should have any questions, please contact me.

PROTOCOL NUMBER: 16052604 (Modification dated 10/24/16)
PROJECT TITLE: Fear Conditioning in Zebrafish
PROPOSED PROJECT DATES: 11/2016 - 09/2018
PROJECT TYPE: Modification
PRINCIPAL INVESTIGATOR(S): David Echevarria
DEPARTMENT: Psychology
FUNDING AGENCY/SPONSOR: N/A
IACUC COMMITTEE ACTION: Designated Review
PROTOCOL EXPIRATION DATE: September 30, 2018



Jake Schaughency, PhD
IACUC Chair

Date 11/15/2016

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