Spring 5-2017

Anxiety-Like Behaviors and c-Fos Expression in Adult Zebrafish: Effects of Housing Conditions, Alcohol and Caffeine

Adam Douglas Collier
University of Southern Mississippi

ANXIETY-LIKE BEHAVIORS AND C-FOS EXPRESSION IN ADULT ZEBRAFISH:

EFFECTS OF HOUSING CONDITIONS, ALCOHOL, AND CAFFEINE

by

Adam Douglas Collier

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

Approved:

_____
Dr. David Echevarria, Committee Chair
Associate Professor, Psychology

_____
Dr. Alex Flynt, Committee Member
Assistant Professor, Biological Sciences

_____
Dr. Donald Sacco, Committee Member
Assistant Professor, Psychology

_____
Dr. Alen Hajnal, Committee Member
Associate Professor, Psychology

_____
Dr. D. Joe Olmi
Department Chair, Psychology

_____
Dr. Karen S. Coats
Dean of the Graduate School

May 2017

Published by the Graduate School

ABSTRACT

ANXIETY-LIKE BEHAVIORS AND C-FOS EXPRESSION IN ADULT ZEBRAFISH: EFFECTS OF HOUSING CONDITIONS, ALCOHOL, AND CAFFEINE

by Adam Douglas Collier

May 2017

Alcohol abuse is the third largest risk factor for disease world, responsible for an estimated 3.3 million deaths each year. The concomitant ingestion of alcohol and caffeine is hypothesized to increase risk factors associated with alcohol use alone by reducing subjective effects of intoxication. The zebrafish (*Danio rerio*) has recently garnered attention from researchers as an effective pre-clinical in-vivo animal model in behavioral pharmacology research, largely due to small size, low-cost and ease of drug delivery. A number of studies have reported the effects of alcohol and caffeine on zebrafish behavior at a variety of doses. However, the combined effects of alcohol and caffeine have rarely been reported. This study examined the effects of alcohol, caffeine, and alcohol and caffeine combined on anxiety-like behaviors and locomotor behaviors in the novel tank test. Caffeine combined with alcohol produced an antagonistic effect on locomotor behaviors and anxiety-like behaviors compared to alcohol alone. Furthermore, concomitant alcohol and caffeine exposure revealed increased c-Fos protein expression, a biomarker of neuronal activation, in the zebrafish brain region homologous to the mammalian amygdala, the medial pallium. In a separate experiment, zebrafish were housed in enriched or barren environments either isolated or in groups of three for two weeks prior to administration of alcohol and caffeine and novel tank testing to investigate the effect of housing environment on behavior. Overall, the effects of alcohol and

caffeine on zebrafish behavior and the brain in this study are evolutionarily conserved, paralleling findings in rodents and humans and reinforcing the translational relevance of the zebrafish model in behavioral pharmacology research.

ACKNOWLEDGMENTS

The author would like to express his gratitude to his thesis committee chair, and faculty mentor, Dr. David Echevarria, in addition to the other committee members, Dr.'s Alex Flynt, Donald Sacco, and Alen Hajnal, for their constructive feedback and support throughout the duration of this project. The author would especially like to thank Dr. Echevarria for providing excellent guidance throughout the duration of my graduate career. The author also expresses thanks to his colleagues, Kanza Khan, Erika Caramillo, and Haley May, for their invaluable help and support throughout this project.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER I - INTRODUCTION

Alcohol and Caffeine

Substance use disorders are complex and ubiquitous problems characterized by patterns of pathological behavior related to the use of the psychoactive substance (American Psychiatric Association, 2013). Alcohol and caffeine are two such substances that are readily available and frequently used throughout much of the world. Alcohol abuse is associated with over 200 health conditions and is the third largest risk factor for disease globally, responsible for an estimated 3.3 million deaths each year (WHO, 2014). In 2010, the economic cost of alcohol abuse reached about $250 billion dollars in the United States (Sacks, Gonzales, Bouchery, Tomedi, & Brewer, 2015). Despite these devastating effects on public health and the global economy, efficacious pharmacological treatments remain few in number. The development of novel pharmacotherapies for alcohol use disorder will be facilitated by a better understanding of the underlying mechanisms of alcohol in the brain and the factors that are responsible for an individual becoming a compulsive drinker.

Caffeine is comparably a less harmful substance than alcohol. However, caffeine is the most commonly used drug in the world (Winston, 2005) with over 85% of children and adults consuming it regularly, more than 70% of which experience at least one withdrawal symptom following cessation of use (American Psychiatric Association, 2013). Heavy caffeine use, especially in vulnerable individuals, may result in adverse medical and psychological effects such as heart, gastrointestinal, and urinary issues, as well as anxiety, depression, insomnia, irritability and cognitive problems (American Psychiatric Association, 2013; Nawrot et al., 2003). Currently, there is a lack of

consensus among academics and clinicians on whether caffeine use can lead to a clinically defined substance use disorder (Budney, Brown, Griffiths, Hughes, & Juliano, 2013). The *Diagnostic and Statistical Manual of Mental Disorders* (5^{th} ed) has indicated that more research is needed before recognizing caffeine use disorder as a formal condition (American Psychiatric Association, 2013). Therefore, understanding the effects of caffeine on behavior and the brain is critical, and is likely to provide important insights into the addictive potential of caffeine.

The ingestion of alcohol and caffeine simultaneously is hypothesized to increase behavioral and health-related risk factors associated with alcohol use (Heinz, de Wit, Lilje, & Kassel, 2013). Caffeinated alcohol beverages (e.g., alcohol mixed with energy drink) are becoming increasingly popular among younger drinkers, to whom they are predominately marketed towards (O'Brien, McCoy, Rhodes, Wagoner, & Wolfson, 2008; Simon & Mosher, 2007). College students report that caffeinated alcohol beverages are appealing because they increase the onset of intoxication, are stimulatory and have a pleasurable taste (Marczinski, 2011). In laboratory studies, human volunteers administered alcohol mixed with energy drink reported feeling less impaired by the effects of alcohol compared to subjects who consumed alcohol alone, but both groups showed similar deficits in motor coordination and visual reaction time (Ferreira, De Mello, Pompéia, Souza-Formigoni, & Oliveira, 2006; Marczinski & Fillmore, 2006). In an online survey of college students, 19.4% of the sample reported monthly consumption of alcohol mixed with caffeinated drinks and were more likely to report other drug use and engage in high-risk sexual behaviors (Snipes & Benotsch, 2013). In another survey of college students, those who consumed beverages containing alcohol and caffeine in the

last 30 days reported increased instances of binge drinking, serious injury, sexual assault, and drunk driving (O'Brien et al., 2008). Therefore, consuming alcohol mixed with caffeine appears to reduce the subjective perception of intoxication, which may increase the risk of negative alcohol-related consequences. This study employed a vertebrate animal model, the zebrafish, to characterize the effects of alcohol and caffeine on behavior and the brain.

The Zebrafish Animal Model

Rodent models of human brain disorders (e.g., substance use disorder) are primarily employed in an effort to elucidate clinically relevant mechanisms underlying disease pathogenesis but are often impeded by high-cost and experimental inefficiency (Cryan & Holmes, 2005). The zebrafish (*Danio rerio*) has recently garnered attention from researchers as an effective pre-clinical in-vivo animal model of a wide range of human disorders that are highly amenable to experimental, pharmacological, and genetic manipulations (Barros, Alderton, Reynolds, Roach, & Berghmans, 2008; Brennan, 2011; Bruni et al., 2016). A host of favorable and versatile characteristics are inherent to this evolutionary ancient species (Kalueff, Echevarria, & Stewart, 2014b; Kalueff, Stewart, & Gerlai, 2014; Stewart et al., 2015). Generally, zebrafish are a small, low-cost, and genetically tractable aquatic teleost vertebrate that show a high degree of neurochemical, morphological, physiological and genetic similarity to humans (Kalueff, Echevarria, & Stewart, 2014a; Kalueff, Stewart, et al., 2014). For instance, the zebrafish genome has been fully sequenced and is roughly 70% orthologous to the human genome, with zebrafish orthologues corresponding to approximately 82% of disease-related genes in humans (Howe et al., 2013).

Several features of zebrafish neuroendocrine and neurotransmitter systems increase their translational validity even further. Most notably, zebrafish release cortisol as a stress hormone (Canavello et al., 2011; Yeh, Glöck, & Ryu, 2013) and the zebrafish hypothalamic-pituitary-interrenal (HPI) axis is highly homologous to the human hypothalamus-pituitary-adrenal (HPA) axis (Alsop & Vijayan, 2009). As a vertebrate species, zebrafish exhibit substantial neural homology to humans, including the expression of major brain structures, neurotransmitters, receptors, and hormones (Panula et al. 2006, 2010; Alsop and Vijayan 2008). Zebrafish are highly suitable for pharmacological studies, especially given a simple method of drug administration, such as by immersing fish into water with a dissolved concentration of drug to allow it to diffuse through the gills into the bloodstream (Collier, Khan, Caramillo, Mohn, & Echevarria, 2014; Goldsmith, 2004).

Adult zebrafish exhibit a complex behavioral repertoire spanning numerous domains that are relevant to human behavioral disorders, such as learning and memory (Blaser & Vira, 2014; Gerlai, 2016), drug reward (Collier et al., 2014; von Trotha, Vernier, & Bally-Cuif, 2014), social behavior (Gerlai, 2014; Qin, Wong, Seguin, & Gerlai, 2014), and anxiety-related behavior (Gerlai, 2013; Jesuthasan, 2012; Wang et al., 2016). A wide range of experimental paradigms historically employed with rodents have been aquatically converted for zebrafish models to investigate relevant behavioral phenotypes, which tend to be well-conserved in zebrafish compared to their mammalian counterpart (Stewart, Braubach, Spitsbergen, Gerlai, & Kalueff, 2014). For example, zebrafish habituate to novelty over time in the open-field test and their exploratory activity is dependent on the size of the arena and is temporally stable throughout the

testing period, as is seen in rodents (Champagne, Hoefnagels, de Kloet, & Richardson, 2010; Eilam, Dank, & Maurer, 2003; Kalueff, Keisala, Minasyan, Kuuslahti, & Tuohimaa, 2006).

Rats and mice are currently the most commonly employed animals to study normal and abnormal brain functioning. In 2015, 32% of all published neuroscience papers utilized rodent models and less than 1% used all other animal models (e.g., zebrafish) (Keifer & Summers, 2016). However, the rate of zebrafish publications is growing faster than any other model organism and experimental tools and resources are becoming increasingly available (Kalueff, Echevarria, et al., 2014b; Wyatt, Bartoszek, & Yaksi, 2015). Adopting a comparative approach using a variety of alternative animal models to address questions related to the function and dysfunction of behavior and the brain is a critically important strategy (Kalueff, Wheaton, & Murphy, 2007). This increases the ability to identify evolutionarily conserved functions, mechanisms, and targets across model organisms and to translate findings that are relevant to treating human brain disorders. Albeit the zebrafish is a new animal model that still requires validation across multiple domains, the zebrafish has a broad range of advantageous applications and is becoming an increasingly useful animal model for screening the effects of drugs on the brain and behavior.

CHAPTER II – EXPERIMENT 1

The Novel Tank Test

Traditionally, animal models of anxiety are often based on behavioral responses to novel environments (Belzung, 1999; Kurt, Arik, & Celik, 2000). In many taxa, exposure to a novel (and therefore, potentially dangerous) environment often triggers the expression of avoidance-related behaviors in animals that likely serve evolutionarily conserved 'anti-predatory' functions (File, 2001; Sousa, Almeida, & Wotjak, 2006). Novelty exploration is believed to underlie behavioral organization in a new environment and reflect the emotional state of animals (Kallai et al., 2007; Stewart, Gaikwad, Kyzar, & Kalueff, 2012; Treit & Fundytus, 1988). Typical 'spatial' behaviors include total distance traveled, average velocity, and spatial distribution of exploratory activity. Initial exploratory behaviors tend to attenuate over the testing session as animals habituate to novel environments, the impairment of which may be associated with increased anxiety (Champagne et al., 2010; Wong, Elegante, et al., 2010b). Like in rodents, zebrafish novelty-based paradigms and associated behaviors are highly sensitive to exposure to acute and chronic pharmacological manipulations and can, therefore, be used to screen drug effects (Bencan, Sledge, & Levin, 2009; Borsini, Podhorna, & Marazziti, 2002). Accordingly, a number of novelty-based paradigms traditionally developed and used for rodents have been applied to zebrafish behavioral testing.

The novel tank test is a novelty-based paradigm that is unique to zebrafish and other aquatic species and is often used for behavioral phenotyping and testing drug effects. This test is conceptually similar to the open field test used for rodents, but rather than measuring horizontal exploration, the novel tank task primarily measures vertical

exploration (Stewart et al., 2010). The novel tank apparatus typically consists of a narrow tank delineated horizontally into a top and bottom zone (Figure 1).

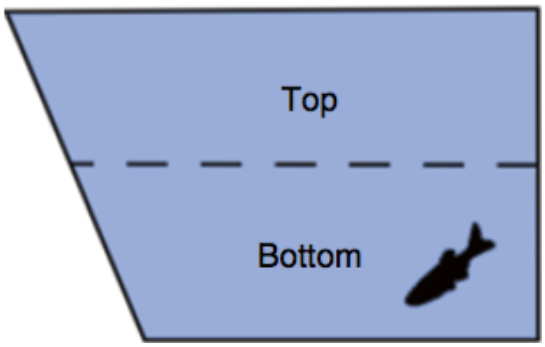

Figure 1. The novel tank test apparatus

A trapezoidal narrow 1.5-gallon housing tank is delineated into two horizontal halves. Side view

Upon exposure to a novel tank apparatus, zebrafish initially exhibit anxiety-like behaviors such, as diving to the bottom of the tank (i.e., geotaxis), reducing exploration, increasing freezing behavior, and increasing erratic movements (Cachat, Stewart, Grossman, Gaikwad, Kadri, Chung, Wu, Wong, Roy, Suciu, et al., 2010). Over the testing, session zebrafish habituate to the novelty of the environment and reduce anxiety-like behaviors. Additionally, exposure to the novel tank test induces stress-related physiological responses, such as elevated cortisol levels, increased breathing and increased heart beat frequency (Kalueff et al., 2016). The novel tank test is an excellent assay for screening anxiotropic (e.g., anxiolytic and anxiogenic) agents, as zebrafish locomotor and anxiety-like behaviors are highly sensitive to such manipulations.

The Effects of Alcohol on Adult Zebrafish

Alcohol (e.g., ethanol) has been one of the most frequently studied substances in adult zebrafish, likely due to the simplicity of drug administration via mixing ethanol

directly into the tank water (see Table 1 for a brief summary of select behavioral and physiological effects of both acute and chronic alcohol in adult zebrafish).

Table 1

Select alcohol effects in adult zebrafish

Alcohol dose	Duration of treatment	Behavioral test or physiological measure	Major effects	Reference
0.2% v/v	14 days continuous exposure	Novel tank test	↓ anxiety ↑ habituation	(Wong, Elegante, et al., 2010a)
0.25% v/v	20 minutes	Conditioned place preference	↑ reward	(Collier et al., 2014)
0.25% v/v	60 minutes	Open field	↑ locomotion	(Gerlai, Lahav, Guo, & Rosenthal, 2000)
0.25% v/v	14 days continuous exposure	Open field	↓ locomotion, indicative of ↑ tolerance	(Gerlai, Lee, & Blaser, 2006)
0.25% v/v	60 minutes	Mirror test	↑ aggression	(Gerlai et al., 2000)
0.25% v/v	60 minutes	c-fos mRNA	↑ galanin c-fos mRNA in hypothalamus ↑orexin c-fos mRNA in hypothalamus	(Sterling, Karatayev, Chang, Algava, & Leibowitz, 2014)
0.3% v/v	5 minutes	Novel tank test	↓ anxiety	(Wong, Elegante, et al., 2010a)
0.3% v/v	5 minutes	Novel tank test	↓ anxiety	(Egan et al., 2009)
0.3% v/v	7 days continuous exposure	Novel tank test	↓ anxiety	(Cachat, Stewart, Grossman, Gaikwad, Kadri, Chung, Wu, Wong, Roy, & Suciu, 2010; Cachat, Stewart, Grossman, Gaikwad, Kadri, Chung, Wu, Wong, Roy, Suciu, et al., 2010; Egan et al., 2009)

Table 1 (continued).

0.3% v/v	12-hour withdrawal after 7 days of continuous exposure	Whole-body cortisol	↑ cortisol	(Cachat, Stewart, Grossman, Gaikwad, Kadri, Chung, Wu, Wong, Roy, Suciu, et al., 2010)
0.50% v/v	60 minutes	Open field	↑ locomotion	(Gerlai et al., 2000)
0.50% v/v	20 minutes	Conditioned place preference	↑ reward	(Collier et al., 2014)
0.50% v/v	10 minutes	Shoaling	↓ shoal cohesion	(Gebauer et al., 2011)
1.00% v/v	60 minutes	Open field	↓ locomotion	(Gerlai et al., 2000)
1.00% v/v	60 minutes	Novel tank test	↑ anxiety	(Gerlai et al., 2000)
1.00% v/v	17 days of 30 min exposure	T-maze	↓ spatial learning performance	(Yang, Kim, Choi, Koh, & Lee, 2003)
1.00% v/v	20 minutes	Open-field	↓ anxiety ↑ locomotion ↑ brain alcohol content	(Rosemberg et al., 2012)
1.00% v/v	20 minutes	Novel tank test	↓ anxiety	(Mathur & Guo, 2011)
1.00% v/v	20 minutes	Light dark test	↓ anxiety	(Mathur & Guo, 2011)
1.00% v/v	60 minutes	Open field	↑ anxiety ↓ locomotion ↑ brain alcohol content	(Rosemberg et al., 2012)
1.00% v/v	60 minutes	Whole-body cortisol	↑ cortisol	(Tran, Chatterjee, & Gerlai, 2015)
1.00 % v/v	8 days of 20 min exposure and 6 days of withdrawal	Novel tank test	↑ anxiety ↓ velocity	(Mathur & Guo, 2011)

Table 1 (continued).

1.00% v/v	20 minutes	Conditioned place preference	↑ reward	(Collier et al., 2014; Kily et al., 2008)

Acute exposure (e.g., a single administration) to ethanol often produces a biphasic response on zebrafish locomotor activity, that is to say, lower doses (e.g., 0.25% and 0.50% v/v) are stimulatory and increase locomotor activity, and higher doses (e.g., 1.00% v/v) are depressive and reduce locomotor activity (Gerlai et al., 2000; Tran, Facciol, & Gerlai, 2016). This biphasic response in zebrafish is similar to the biphasic stimulant and depressant effects of alcohol observed in rodents (Gingras & Cools, 1996; Moore, June, & Lewis, 1993) and experienced by humans (King, Houle, Wit, Holdstock, & Schuster, 2002). Alcohol effects in zebrafish tested in the novel tank test have been shown to be dependent on the duration of ethanol exposure, with 20 minutes of acute exposure to 1.00% v/v ethanol producing anxiolytic-like behaviors and increasing locomotor activity, and 60 minutes of acute exposure to 1.00% v/v ethanol producing anxiogenic-like behaviors and decreasing locomotor activity (Rosemberg et al., 2012). This biphasic response was also reported to correlate with brain alcohol levels in zebrafish, with 60 minutes of 1.00% v/v ethanol administration having resulted in significantly higher brain alcohol levels compared to 20 minutes of 1.00% v/v ethanol (Rosemberg et al., 2012). Blood alcohol levels in zebrafish have been reported to significantly increase following 0.25% and 0.50% v/v ethanol exposure for 60 minutes (Sterling et al., 2014). Another study found acute 30-minute exposure to 0.25% ethanol to reach a pharmacologically relevant blood alcohol concentration of ~0.08 % (Echevarria, Toms, & Jouandot, 2011).

Moderate to high doses of ethanol (e.g., 0.50% and 1.00% v/v) tend to be the most commonly studied doses in zebrafish neurobehavioral research (Mathur, Berberoglu, & Guo, 2011; Tran et al., 2015; Tran & Gerlai, 2013). This proposed study will expose zebrafish to the lower dose of 0.25% v/v for 30 minutes prior to evaluate anxiety-like behaviors and locomotor behaviors in the novel tank test, as this dose is less well characterized and this exposure time results in a relevant blood alcohol concentration of ~0.08%.

The Effects of Caffeine on Adult Zebrafish

Caffeine has been less commonly studied in zebrafish models compared to alcohol. A variety of zebrafish anxiety-like behaviors in the novel tank test are increased following acute caffeine administration, such as increased latency to enter the top half, increased freezing bouts and freezing duration, and decreased average velocity (Table 2) (Cachat, Stewart, Grossman, Gaikwad, Kadri, Chung, Wu, Wong, Roy, & Suciu, 2010; Egan et al., 2009)

Table 2

Select caffeine effects in adult zebrafish

Caffeine dose	Duration of treatment	Behavioral or physiological test	Major effects (compared to control)	Reference
25 mg/L	20 minutes	Novel tank test	↓ velocity	(Ladu, Mwaffo, Li, Macrì, & Porfiri, 2015)
50 mg/L	7 days of 20-minute exposure	Conditioned place preference	↑ reward	Own unpublished observations
50 mg/L and 100 mg/L	60 minutes	Object discrimination task	↓ distance traveled ↑ anxiety	(Santos, Ruiz-Oliveira, Oliveira, Silva, & Luchiari, 2016)

Table 2 (continued).

100 mg/L	15 minutes	Novel tank test	↑ anxiety ↓ habituation	(Wong, Elegante, et al., 2010a)
100 mg/L	5 minutes	Novel tank test	↑ anxiety	(Egan et al., 2009)
100 mg/L	60 minutes	c-Fos protein	↑ c-Fos protein	(Chatterjee, Tran, Shams, & Gerlai, 2015)
250 mg/L	20 minutes	Whole-body cortisol	↑ cortisol	(Cachat, Stewart, et al., 2011)
250 mg/L	20 minutes	Novel tank test	↑ anxiety	(Cachat, Stewart, Grossman, Gaikwad, Kadri, Chung, Wu, Wong, Roy, & Suciu, 2010; Cachat, Stewart, et al., 2011)
250 mg/L	20 minutes	Novel tank test	↑ anxiety ↓ distance traveled ↑ cortisol ↓ average velocity	(Wong, Stewart, et al., 2010)

One study reported that 20-minute exposure to 25 mg/L caffeine decreased average swim velocity (cm/s) compared to control animals and had no effect on time spent in the top half in the novel tank test, although the effects on other anxiety-like behaviors and distance traveled are unreported. Caffeine has largely been studied in adult zebrafish at the doses of 100 mg/L and 250 mg/L administered for ≤ 20 minutes. It is currently unclear whether caffeine has a biphasic effect on zebrafish behavior as alcohol does, as the majority of doses tested have been reported to increase behavioral measures of anxiety and decrease locomotor behaviors, suggestive of a depressive effect. In rodents, low doses of caffeine have a stimulatory effect on locomotor behavior, while

high doses have a depressive effect (Yacoubi et al., 2000). Measures of anxiety behavior in rodents have been reported to consistently increase at all doses tested (Bhorkar, Dandekar, Nakhate, Subhedar, & Kokare, 2014; Jain, Hirani, & Chopde, 2005; Pellow, Chopin, File, & Briley, 1985). This study exposed zebrafish to 25 mg/L caffeine for 30 minutes to characterize this largely unreported dose and duration of exposure on anxiety-like and locomotor behaviors in the novel tank test.

The Effects of Alcohol and Caffeine on Adult Zebrafish

Although the effects of both ethanol and caffeine have been tested individually in zebrafish, the behavioral effects of co-administration of these substances has rarely been reported. One study reported the combined effects of alcohol and caffeine on zebrafish cognitive performance, although this study exposed zebrafish to alcohol or caffeine chronically for 27 days followed by withdrawal of the chronic drug and then acute exposure to alcohol or caffeine for 60 minutes on day 28, thus not administering the drugs simultaneously (Santos et al., 2016). One finding of this study was that zebrafish improved cognitive performance following administration of chronic 0.50% ethanol for 27 days and administration of acute 50 mg/L caffeine on day 28 compared to animals that were administered chronic 0.50% ethanol for 27 days and received no caffeine or ethanol on day 28, indicating that this lower dose of caffeine may have reduced the negative effects of alcohol withdrawal on cognitive performance (Santos et al., 2016).

A recent study reported that 1.00% ethanol increased total distance traveled and decreased the distance to the bottom of the novel tank, and 250 mg/L caffeine *reduced* total distance traveled and decreased the distance to the bottom of the novel tank (Tran et al., 2017). Following co-administration of 1.00% and 250 mg/L caffeine, total distance

traveled and distance to the bottom of the tank resembled that of 250 mg/L caffeine alone, indicative of an antagonistic mechanism. Similarly, in rodents, a high dose of caffeine administered in combination with a high dose of ethanol was found to reduce locomotor activity compared to ethanol alone (Waldeck, 1974). This finding is line with human research suggesting that caffeine's effects may mask some of the effects of alcohol (Ferreira et al., 2006; Marczinski & Fillmore, 2006). However, a low-dose of caffeine administered in combination with a low dose of ethanol was found to increase locomotor activity compared to ethanol alone in rodents, suggestive of an additive effect when low doses of alcohol and caffeine are combined. (Waldeck, 1974). Thus, it is unclear if a low dose of ethanol combined with a low dose of caffeine will have an additive or an antagonistic effect on locomotor behaviors and anxiety-like behaviors in zebrafish. This study exposed zebrafish to 25 mg/L caffeine combined with 25 mg/L ethanol for 30 minutes to characterize the unreported combination of low doses of these substances on anxiety-like and locomotor behaviors in the novel tank test.

Specific Aim 1

Characterize the effects of acute 0.25% alcohol, 25 mg/L caffeine, and 0.25% alcohol and 25 mg/L caffeine combined on locomotor behaviors and anxiety-like behaviors using the novel tank test.

Hypotheses

It was expected that 0.25% v/v ethanol administered for 30 minutes would increase locomotor behaviors and decrease anxiety-like behaviors in the novel tank test. It was also expected that 25 mg/L caffeine administered for 30 minutes would decrease locomotor behaviors and would increase anxiety-like behaviors in the novel tank test. It

was less clear what the effect of 0.25% v/v ethanol combined with 25 mg/L caffeine administered for 30 minutes would be on locomotor and anxiety-like behaviors in the novel tank test, as it was expected that 25 mg/L caffeine would increase anxiety-like behaviors and decrease locomotor behaviors and 0.25% ethanol would decrease anxiety-like behaviors and increase locomotor behaviors.

General Zebrafish Laboratory Housing

All fish were maintained and protocols were carried out according to the Institutional Animal Care and Use Committee (IACUC) of the University of Southern Mississippi, Hattiesburg MS, USA. Adult zebrafish of a randomly bred genetically heterogeneous 'wildtype' strain were obtained from a local distributor (Pet Palace, Hattiesburg MS 39401). All fish acclimated to the laboratory environment for a minimum of 10 days, were housed in groups of 20-25 within 10 L tanks maintained in a circulating system equipped with biological, chemical, and mechanical filtration, aeration, and sterilization by UV light. Ceiling-mounted fluorescent light tubes provided illumination during a 14/10 hour light/dark cycle. Tank water consisted of reverse osmosis deionized (RODI) water supplemented with 60 mg/L dissolved sea salts (Instant Ocean: Blacksburg, VA 24060), and was maintained at ~28 C^{o}. Fish were fed once in the morning with brine shrimp (Premium Grade Brine Shrimp Eggs, Brine Shrimp Direct, Ogden, UT), and once in the afternoon with flake food (Tetra: Blacksburg, VA). All animals were drug and experimentally naïve prior to experimental testing.

Novel Tank Testing Methods

Following ten days of acclimation to the laboratory environment, zebrafish were tested in the novel tank test to evaluate the effects of 0.25% ethanol, 25 mg/L caffeine

and co-administration of 0.25% ethanol and 25 mg/L caffeine on locomotor and anxiety-like behaviors (Table 3). Individual zebrafish were carefully netted from their home tanks of ~20-25 fish and individually placed in 1-liter beakers containing 1 liter of housing system water at ~28° C that was either void of drug or contained the appropriate drug concentration mixed in the water. Each beaker was covered with parafilm to reduce evaporation and prevent fish from jumping out of the beaker. Each beaker was transferred to an adjacent experimental testing room and individually placed within a testing chamber for zebrafish to acclimate to the new environment. Each testing chamber contained the novel tank test apparatus placed flush against a white wall of the chamber to provide contrast, two overhanging fluorescent lights to produce adequate lighting, and a USB web camera pointed horizontally at the novel tank to record behavior (Figure 2). Three fish were tested simultaneously, with one fish being tested per chamber. After 30 minutes in the beaker, zebrafish were carefully netted out and placed directly into a novel tank test apparatus filled with 1.2 liters of system tank water at ~28° C. The experimenter then initiated behavioral recording, gently closed the doors to each testing chamber, and left the experimental room. Zebrafish explored the novel tank test apparatus for 6 minutes and behavior was later evaluated.

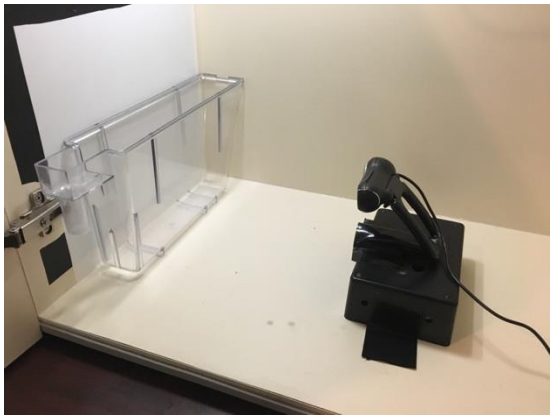

Figure 2. The testing chamber for the novel tank test

Behavioral Analysis

Videos of zebrafish behavior in the novel tank test were recorded using QuickTime for Mac and then decompressed and converted from ".mov" to ".avi" format using MatLab (MathWorks: Natick, MA). Each video file was enhanced using ImageJ software to provide sufficient contrast between each fish and the background of the apparatus. Zebrafish swimming behavior was tracked over the 6 minute testing period and expressed as x and y pixel coordinates using the idTracker program (Pérez-Escudero, Vicente-Page, Hinz, Arganda, & de Polavieja, 2014). Finally, MatLab was used to produce the behavioral measures of interest using the x and y coordinate data previously generated by idTracker. Table 3 contains the locomotor and anxiety-like behavioral measures evaluated using the novel tank test.

Table 3

Novel tank test behavioral measures

Behavioral measure	Definition	↑ Value indicates
Total distance traveled	A measure of locomotor activity, the total distance traversed (e.g., cm) during the testing session.	↑ total distance traveled indicates ↑ hyperactivity

Table 3 (continued).

Average velocity	Magnitude of zebrafish speed, distance (e.g., cm) traveled per second.	↑ average velocity indicates ↑ hyperactivity
Freezing bouts	Number of times spent freezing. Freezing is a complete cessation of movement (except for gills and eyes) for over 3 seconds.	↑ freezing bouts indicates ↑ anxiety
Freezing duration	Total time spent freezing	↑ freezing duration indicates ↑ anxiety
Time in top	The amount of time spent in the top half of the tank during the 6-minute testing session	↑ time in top indicates ↓ anxiety
Latency to enter the top half of the tank	The time it takes for a zebrafish to enter the top half of the tank after being placed in the novel tank test apparatus	↑ latency to enter the top indicates ↑ anxiety

Statistical Analysis

Comparisons were made for each behavioral measure between each of the four drug groups (i.e., control, 0.25% v/v ethanol, 25 mg/L caffeine, 0.25% v/v ethanol and 25 mg/L caffeine combined). If the homogeneity of variance assumption was met, each behavioral measure was analyzed using a one-way ANOVA (factor: drug) to determine if there was a significant overall effect ($p \leq 0.05$) of drug on each behavior. Following a significant overall effect, posthoc Tukey HSD test was used to evaluate significant differences between drug groups for each behavior. If the homogeneity of variance assumption was not met, the Welch's F correction was applied followed by the Games-Howell post hoc test to evaluate significant differences between groups. The accepted level of significance was $p \leq 0.05$. Data is presented as mean ± SEM.

Experiment 1 Results

One-Way ANOVA revealed a significant effect of drug condition on total distance traveled (cm) over the 6 minute testing session, $F(3, 56) = 11.198$, $p < 0.001$. Post-hoc analysis revealed that 0.25% ethanol significantly increased distance traveled compared to control ($p = 0.006$), 25 mg/L caffeine ($p = 0.001$) and 0.25% ethanol + 25 mg/L caffeine ($p \leq 0.001$) (Figure 3).

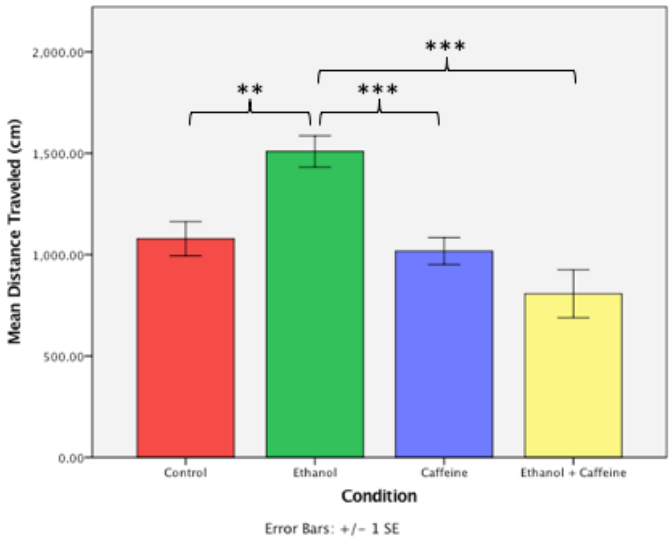

Figure 3. Novel tank test: mean distance traveled

Mean Distance traveled (cm) for control zebrafish and zebrafish administered 0.25% ethanol, 25 mg/L caffeine or 0.25% ethanol + 25 mg/L caffeine. Data expressed as mean (±SEM). **p ≤ 0.01. *** p ≤ 0.001

One-Way ANOVA revealed a significant effect of drug condition on mean swim velocity (cm/s), $F(3, 30.6) = 11.252$, $p < 0.001$. Post-hoc analysis revealed that 0.25% ethanol significantly increased mean swim velocity compared to 25 mg/L caffeine ($p \leq 0.001$) and 0.25% ethanol + 25 mg/L caffeine ($p \leq 0.001$) (Figure 4).

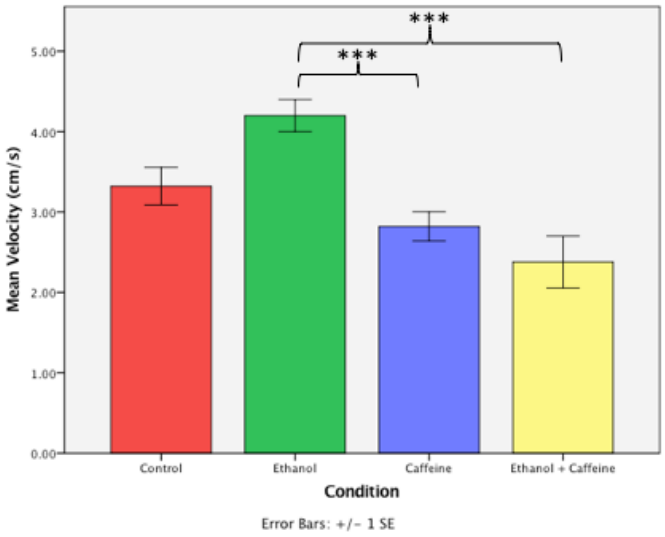

Figure 4. Novel tank test: mean swim velocity

Mean swim velocity (cm/s) for control zebrafish and zebrafish administered 0.25% ethanol, 25 mg/L caffeine or 0.25% ethanol + 25 mg/L caffeine. Data expressed as mean (±SEM). *** $p \leq 0.001$

One-Way ANOVA revealed a significant effect of drug condition on mean freezing bouts, $F(3, 23.3) = 217279.03$, $p \leq 0.001$. Post-hoc analysis revealed that 0.25% ethanol significantly decreased freezing bouts compared to 25 mg/L caffeine ($p = 0.045$) and 0.25% ethanol + 25 mg/L caffeine ($p = 0.027$) (Figure 5)

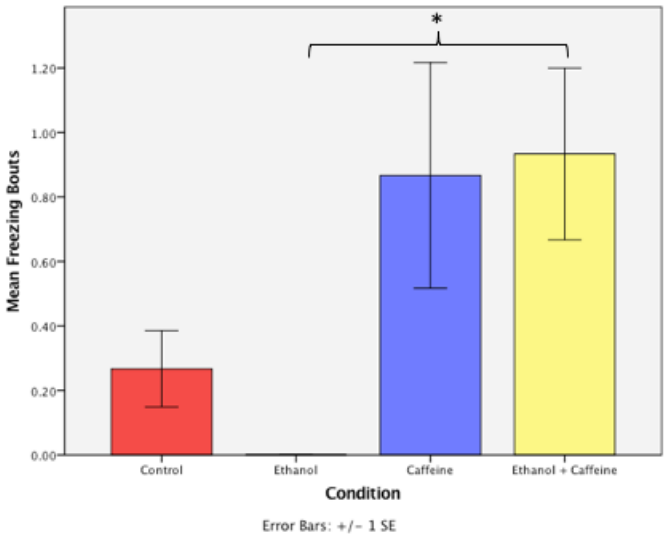

Figure 5. Novel tank test: mean freezing bouts

Mean freezing bouts for control zebrafish and zebrafish administered 0.25% ethanol, 25 mg/L caffeine or 0.25% ethanol + 25 mg/L caffeine. Data expressed as mean (±SEM). *$p \leq 0.05$.

One-Way ANOVA revealed a significant effect of drug condition on mean freezing duration, $F(3, 23.3) = 11658524$, $p \leq 0.001$. Post-hoc analysis revealed that 0.25% ethanol significantly decreased freezing duration compared to 0.25% ethanol + 25 mg/L caffeine ($p = 0.016$) (Figure 6).

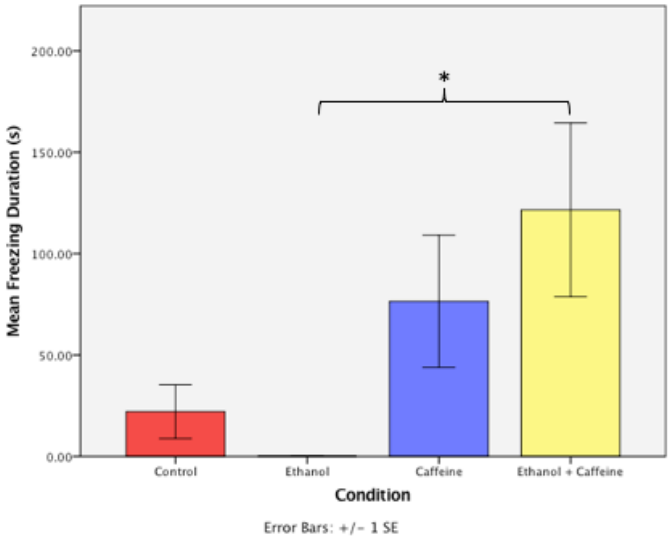

Figure 6. Novel tank test: mean freezing duration

Mean freezing duration (s) for control zebrafish and zebrafish administered 0.25% ethanol, 25 mg/L caffeine or 0.25% ethanol + 25 mg/L caffeine. Data expressed as mean (±SEM). *p ≤ 0.05.

One-Way ANOVA did not reveal a significant effect of drug condition on mean time (s) spent in the top half of the tank, F (3, 29.8) = 1.694, p = 0.190 (Figure 7).

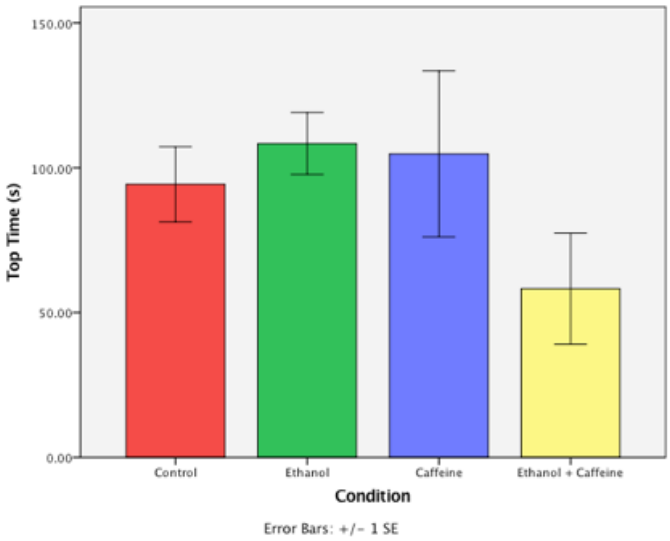

Figure 7. Novel tank test: mean top time

Meantime (s) spent in the top half of the tank for control zebrafish and zebrafish administered 0.25% ethanol, 25 mg/L caffeine or 0.25% ethanol + 25 mg/L caffeine. Data expressed as mean (±SEM).

One-Way ANOVA revealed a significant effect of drug condition on the latency (s) to enter the top half of the tank, F (3, 27.9) = 6.576, p = 0.002. Post-hoc analysis revealed that 0.25% ethanol significantly decreased latency to enter the top half of the tank compared to 0.25% ethanol + 25 mg/L caffeine (p = 0.007) (Figure 8). Table 4 shows descriptive statistics for each drug condition and behavior. Table 4 includes a list of descriptive values for experiment 1

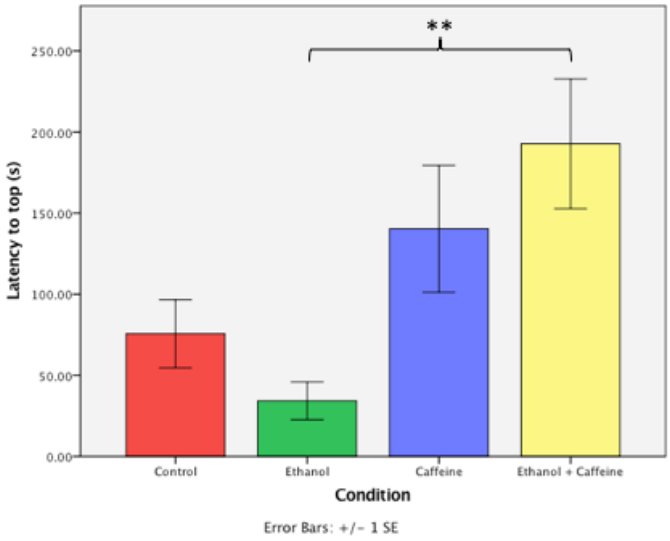

Figure 8. Novel tank test: mean latency to top

Mean Latency to enter the top half of the tank for control zebrafish and zebrafish administered 0.25% ethanol, 25 mg/L caffeine or 0.25% ethanol + 25 mg/L caffeine. Data expressed as mean (±SEM). **p ≤ 0.01.

Table 4

Experiment 1 novel tank test descriptive statistics

	Control			Ethanol			Caffeine			Ethanol+ Caffeine		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	15	1078.24	325.74	15	1509.2	300.81	15	1004.4	249.8	15	807.17	458.67
Velocity (cm/s)	15	3.32	0.91	15	4.2	0.78	15	2.82	0.7	15	2.37	1.25
Freezing Bouts	15	0.27	0.46	15	0.00	0.00	15	0.87	1.36	15	0.93	1.03
Freezing Duration (s)	15	22.05	51.41	15	0.00	0.00	15	76.49	126.39	15	121.61	166.05
Time in Top (s)	15	94.29	50.36	15	108.38	41.47	15	104.78	110.93	15	58.25	74.38
Latency (s)	15	75.56	81.23	15	34.22	44.92	15	140.31	151.61	15	192.71	154.91

CHAPTER III – EXPERIMENT 2

C-Fos Protein

C-Fos is an immediate-early gene (IEG) that is transcribed and translated rapidly in response to neuronal activation (Hoffman, Smith, & Verbalis, 1993; Salierno et al., 2006). As a result, c-fos mRNA and c-Fos protein expression profiles have been utilized by researchers as reliable biomarkers of neuronal activity in various species, including humans (Zhang, Hirsch, Damier, Duyckaerts, & Javoy-Agid, 1992) rodents (Erdtmann-Vourliotis, Mayer, Riechert, & Höllt, 1999; Moreno, Holloway, Albizu, Sealfon, & González-Maeso, 2011; Näkki, Sharp, Sagar, & Honkaniemi, 1996) and zebrafish (Chatterjee et al., 2015; Lau, Mathur, Gould, & Guo, 2011).

For example, elevated whole-brain c-fos mRNA expression in zebrafish has been reported following MDMA and ketamine using PCR (Stewart et al., 2011; Zakhary et al., 2011). Zebrafish administered an acute stressor consisting of 5 minutes of net chasing and 1 minute of air exposure exhibited an upregulation of whole-brain c-fos mRNA expression at 15 and 30 minutes post-stressor, which returned to baseline levels at 60 minutes post-stressor (Pavlidis, Theodoridi, & Tsalafouta, 2015). In-situ hybridization has also been employed to localize expression of c-fos mRNA in the zebrafish brain. For example, when placed in an apparatus containing a light zone and a dark zone, zebrafish avoided the light zone and exhibited increased c-fos mRNA in the medial pallium, the homologous region to the mammalian amygdala (Lau et al., 2011). The amygdala has been reported to be activated during a decision making task in humans as measured by fMRI, suggesting an evolutionary conserved role of this brain area in zebrafish (De Martino, Kumaran, Seymour, & Dolan, 2006).

Although c-fos mRNA has been evaluated in adult zebrafish followed various experimental manipulations, c-Fos protein expression in the zebrafish brain has rarely been investigated. 100 mg/L of acute caffeine administration for 60 minutes upregulated c-Fos protein expression in the central zone of the dorsal telencephalic area, the tectum opticum, and the lateral longitudinal fascicle, but not the dorsal telencaphalic area or the lobus caudalis compared to control animals (Chatterjee et al., 2015). It is unclear what the roles of these brain areas are on zebrafish caffeine responses, although this study demonstrates that acute caffeine administration differentially affected c-Fos protein expression across brain regions.

The Limbic System

The limbic system in the mammalian brain is made up of a number of interconnected brain areas (e.g., the amygdala, hippocampus and hypothalamus) that regulate motivated behaviors such as eating, reproduction, fleeing and fighting (Isaacson, 1982). It has been hypothesized that these limbic structures are integral in mediating the behavioral responses towards naturally rewarding stimuli (e.g., food) and that this system becomes "hijacked" during drug addiction (Kauer & Malenka, 2007; Kelley & Berridge, 2002). Given that the limbic system mediates motivated behaviors necessary for survival (e.g., eating), these systems are evolutionary ancient and well conserved across species. It has been reported that acute amphetamine administration increased c-Fos protein expression in the zebrafish medial pallium and the lateral pallium, two zebrafish brain areas homologous to the mammalian amygdala and hippocampus, respectively (von Trotha et al., 2014). Another study found that 60 minute administration of a dose equivalent 0.25% v/v ethanol upregulated galanin mRNA in the ventral zone of the

paraventricular nucleus of the zebrafish hypothalamus, which is a neuropeptide believed to mediate alcohol intake (Barson & Leibowitz, 2016; Lawrence, Cowen, Yang, Chen, & Oldfield, 2006; Sterling et al., 2014). Figures 9 and 10 illustrate the location of the adult zebrafish medial pallium (Dm), lateral pallium (Dl), and the ventral zone of the periventricular nucleus of the hypothalamus.

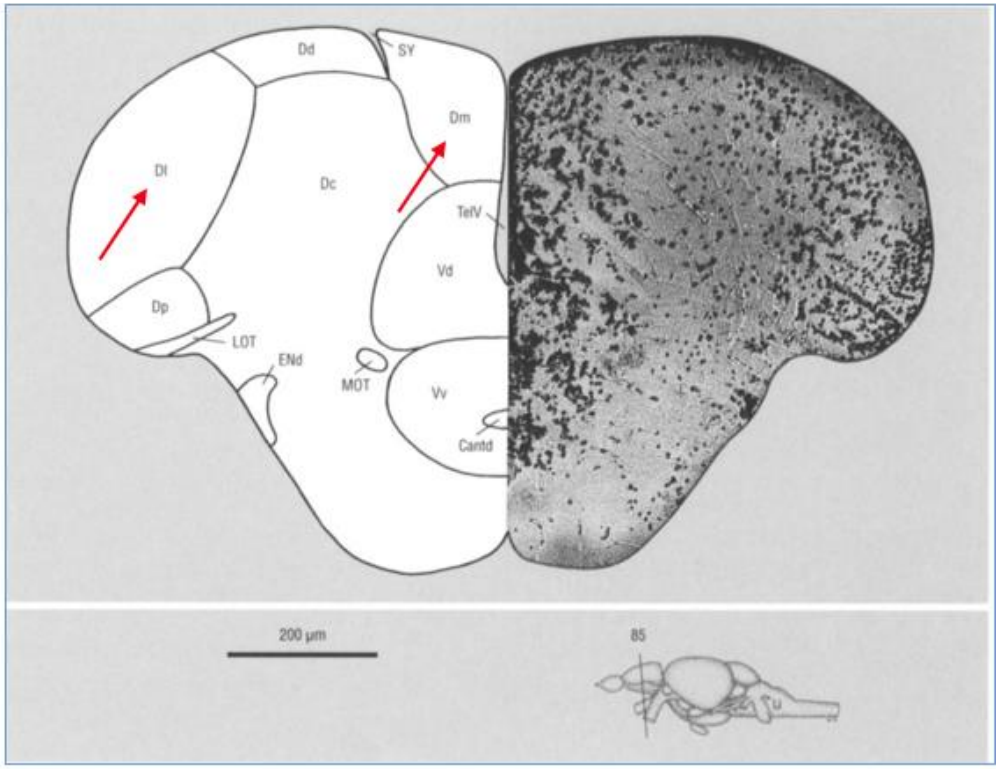

Figure 9. The adult zebrafish medial and lateral pallium

A brain section illustrating the adult zebrafish medial pallium (Dm) and lateral pallium (Dl), homologous to the mammalian amygdala and hippocampus, respectively. Image from (Wulliman, Rupp, & Reichert, 2012)

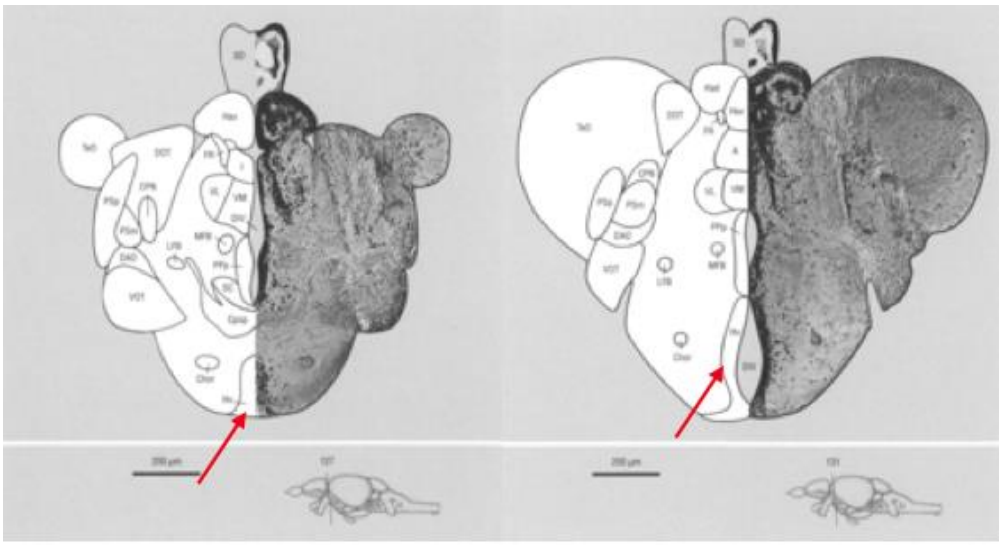

Figure 10. The adult zebrafish paraventricular hypothalamus

Two brain sections illustrating the location of the adult zebrafish paraventricular hypothalamus. Images from (Wulliman et al., 2012)

Brain areas in the limbic system are also implicated in mediating anxiety in humans (Etkin & Wager, 2007; Shin, Rauch, & Pitman, 2006). Increased amygdala activity measured by fMRI was observed in human subjects viewing images of faces with fearful expressions and was found to correlate with reported levels of anxiety (Somerville, Kim, Johnstone, Alexander, & Whalen, 2004). Furthermore, a meta-analysis of brain imaging studies in patients with posttraumatic stress disorder, social anxiety disorder, and specific phobias found greater activity in the amygdala in patients with each of these disorders compared to healthy subjects (Etkin & Wager, 2007). In rats, c-Fos protein was reported to be upregulated in various regions of the hypothalamus and amygdala while avoidance an electrical shock and being placed into a novel environment (Duncan, Knapp, & Breese, 1996). In another study, c-fos mRNA and c-Fos protein were reported to be upregulated in the periventricular nucleus of the hypothalamus of rats (Ogilvie, Lee, & Rivier, 1998). Furthermore, c-Fos protein was upregulated in the periventricular nucleus of the hypothalamus and the amygdala of rats administered

alcohol (Singewald, Salchner, & Sharp, 2003). In summary, brain areas of the limbic system (e.g., the amygdala, the hippocampus and the hypothalamus) are implicated in the response to drugs and to anxiety-inducing stimuli (e.g., novelty), and c-Fos protein expression is a viable biomarker to evaluate neuronal activity in these areas.

Specific Aim 2

Characterize the neuroanatomical correlates of acute 30 minute administration of 0.25% alcohol, 25 mg/L caffeine, and 0.25% alcohol and 25 mg/L caffeine combined following novel tank testing via expression profiles of the immediate-early gene (IEG) c-Fos protein in the medial pallium (e.g., amygdala), the lateral pallium (e.g., hippocampus), and the periventricular nucleus of the hypothalamus.

Hypothesis

It was expected that acute administration of 0.25% v/v ethanol, 25 mg/L caffeine, and 0.25% v/v ethanol and 25 mg/L combined would differentially increase c-Fos protein expression in the medial pallium, the lateral pallium, and the periventricular nucleus of the hypothalamus in the zebrafish brain.

C-Fos Immunohistochemistry Methods

Four groups were evaluated for c-Fos protein levels in the brain, with 3 animals in each group. Groups consisted of zebrafish from the four groups tested previously in the novel tank test. After novel tank testing, zebrafish were netted out of the NTT tank, placed on a paper towel, and decapitated just posterior to the gills. Heads were then placed into plastic containers containing 4% paraformaldehyde/1X PBS for 12 hours at 4° C. To cryoprotect the tissue before freezing, heads were sequentially placed in 10% sucrose for 2 hours, 20% sucrose for 4 hours, and 30% sucrose for 24 hours. Zebrafish

heads were then placed into plastic molds, covered in OCT, and frozen by submerging the molds into a bath of dry ice and 100% ethanol. Zebrafish heads were then sectioned at 20 μm using a Tissue-Tek Cyro3 Cryostat at ~ -28° C.

Tissue sections were transferred onto Fisherbrand Tissue Path Superfrost Plus slides and fixed immediately with 4% paraformaldehyde/1X PBS for 7 minutes. Sections were then transferred to 1X PBS following fixation, washed two times in 1X PBS for 10 minutes each, and then permeabilized in 1 X PBS containing 0.1% triton for 30 minutes. To block non-specific binding, sections were incubated in 5% goat serum in PBS containing 0.1% triton for 30 minutes. Sections were incubated overnight at 4° C in the primary anti c-Fos polyclonal antibody (Santa Cruz Biotechnology, CA) 1:200 in PBS containing 5% goat serum. On the following day, sections were washed with PBS containing 0.1% triton 3 times for 10 minutes each and then blocked for 30 minutes in PBS containing 0.1% triton and 5% goat serum. Sections were then incubated with Alexa Fluor 488, the secondary fluorescent antibody (Abcam, MA), diluted 1:200 in PBS containing 0.1% triton and 5% goat serum for 2 hours at room temperature in the dark. Sections were then washed with PBS containing 0.1% triton for 5 minutes, 3 times, and then counterstained with the DNA stain DAPI. Slides were mounted with FisherBrand coverslips and immunoreactive cells were be imaged using a Nikon Fluorescence Eclipse 80i microscope. Brain areas of interest were identified based on surrounding landmarks and by referencing a zebrafish brain atlas (Wulliman et al., 2012). Photomicrographs were taken for each brain area of interest across conditions. The researcher manually quantified immunoreactive cells while blind to the experimental conditions of each respective slide.

Statistical Analysis

Comparisons were made for C-Fos immunoreactive cells present in the hypothalamus, medial pallium and lateral pallium between each of the four drug groups (i.e., control, 0.25% v/v ethanol, 25 mg/L caffeine, 0.25% v/v ethanol and 25 mg/L caffeine combined). If the homogeneity of variance assumption was met, each comparison was analyzed using a one-way ANOVA (factor: drug) to determine if there was a significant overall effect ($p \leq 0.05$) of drug on the number of immunoreactive c-Fos cells. Following a significant overall effect, posthoc Tukey HSD test was used to evaluate significant differences between drug groups for each behavior. If the homogeneity of variance assumption was not met, the Welch's F correction was applied followed by the Games Howell post hoc test to evaluate significant differences between groups. The accepted level of significance was $p \leq 0.05$. Data is presented as mean ± SEM.

Experiment 2 Results

One-way ANOVA did not reveal a significant effect of drug treatment on c-Fos protein expression in the periventricular nucleus of the hypothalamus, $F(3, 11) = 0.349$, $p = 0.79$ (Figure 11).

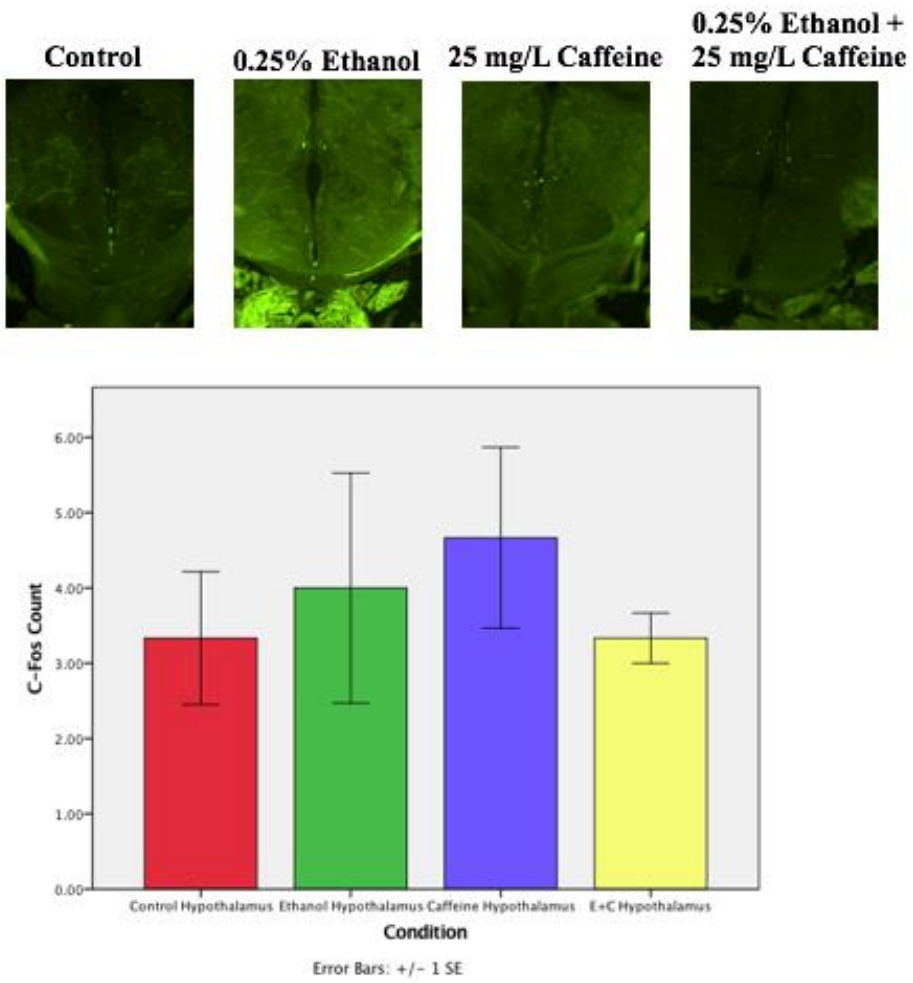

Figure 11. C-Fos expression in the zebrafish hypothalamus

Images taken of zebrafish brain sections containing the hypothalamus. The green colored row depicts c-Fos protein expression as represented by greater fluorescent expression. The graph represents the number of immunoreactive c-Fos-positive cells present in the hypothalamus of control zebrafish and those administered 0.25 % ethanol, 25 mg/L caffeine, and 0.25% ethanol combined with 25 mg/L caffeine. Red = control, green = 0.25% ethanol, blue = 25 mg/L caffeine, yellow = 0.25% ethanol and 25 mg/L caffeine combined

There was a significant effect of drug treatment on c-Fos protein expression in the medial pallium, $F(3, 11) = 15.25$, $p \leq 0.001$. Post-hoc analysis revealed that 0.25% ethanol combined with 25 mg/L caffeine (E+C) significantly increased c-Fos expression compared to control ($p = 0.04$). The difference between 0.25% ethanol combined with 25

mg/L caffeine (E+C) and 0.25% ethanol was marginally significant (p = 0.056) (Figure 12).

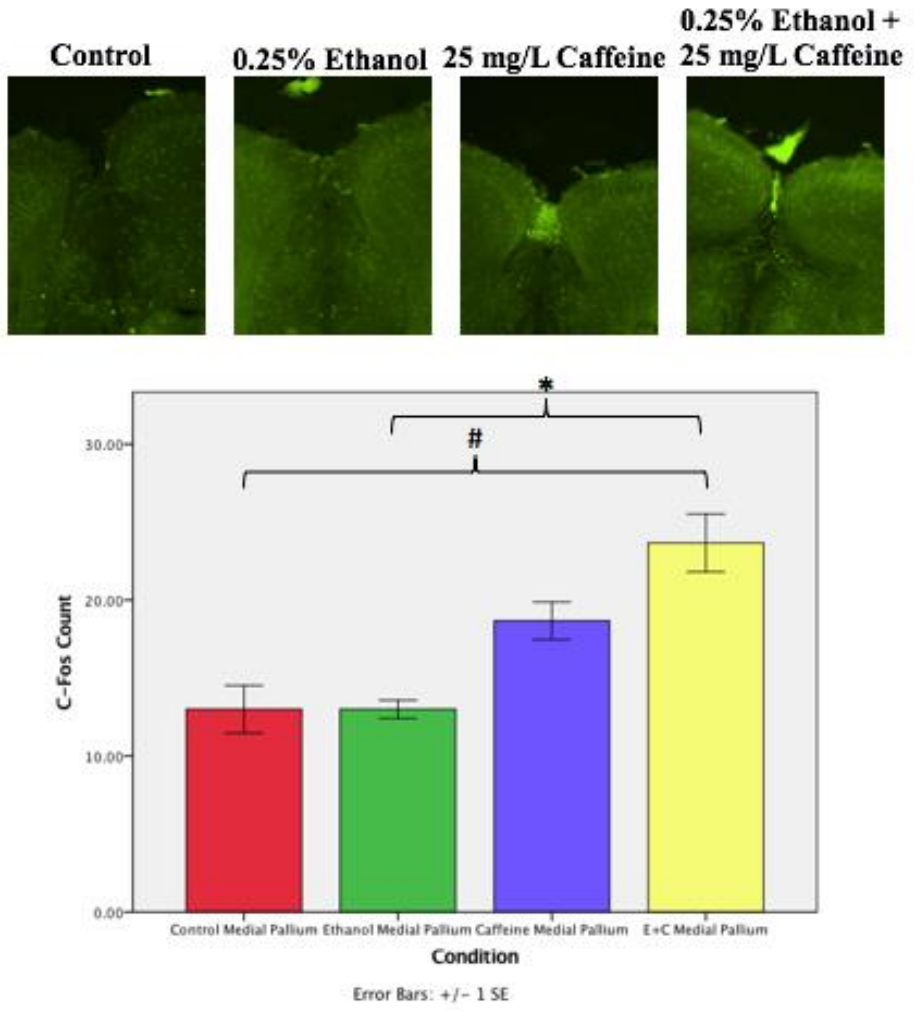

Figure 12. C-Fos expression in the zebrafish medial pallium

Figure 12. Images taken of zebrafish brain sections containing the Medial Pallium. The green row depicts c-Fos protein expression as represented by greater fluorescent expression. The graph represents the number of immunoreactive c-Fos-positive cells present in the Medial Pallium of control zebrafish and those administered 0.25 % ethanol, 25 mg/L caffeine, and 0.25% ethanol combined with 25 mg/L caffeine. Red = control, green = 0.25% ethanol, blue = 25 mg/L caffeine, yellow = 0.25% ethanol and 25 mg/L caffeine combined. # $p \leq 0.06$, *$p \leq 0.05$

One-way ANOVA did not reveal a significant effect of drug treatment on c-Fos protein expression in the lateral pallium, $F(3, 11) = 2.94$, $p = 0.10$ (Figure 13). Table 5

shows descriptive statistics for c-Fos protein expression in each brain area for each drug condition.

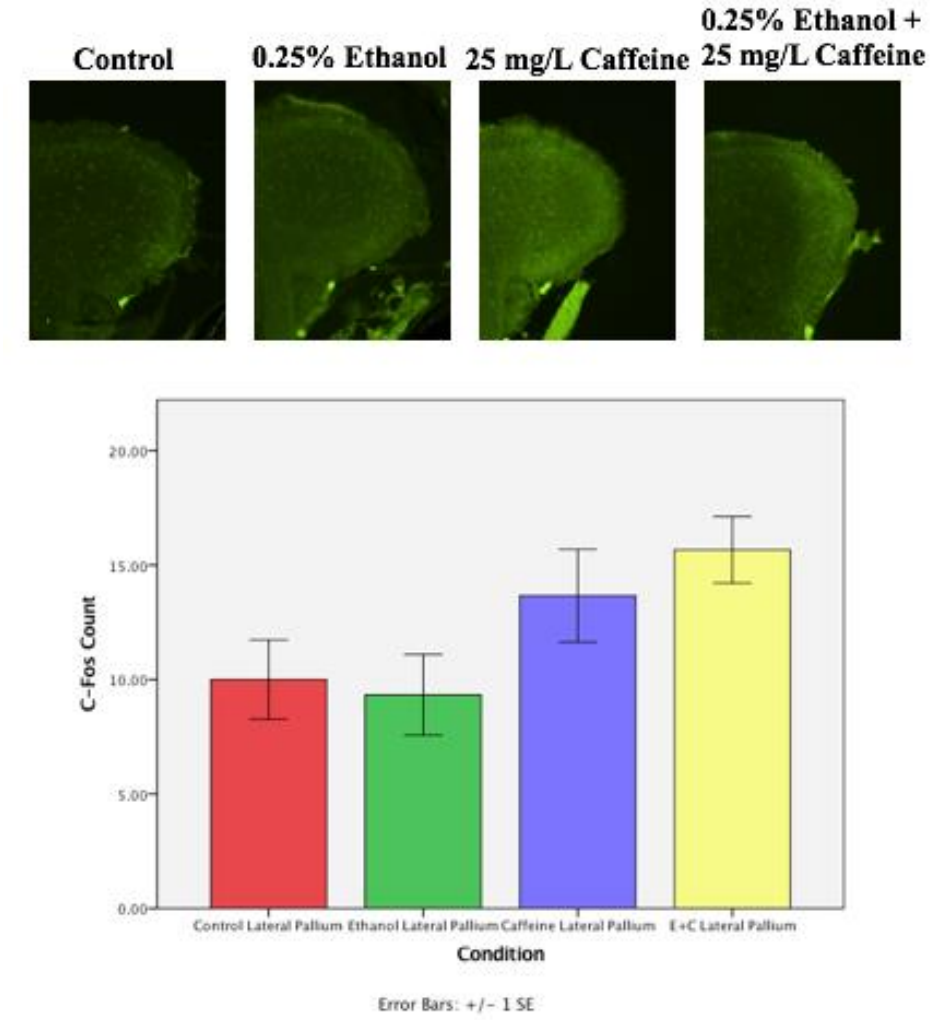

Figure 13. C-Fos expression in the zebrafish lateral pallium

Images taken of zebrafish brain sections containing the lateral pallium. The green row depicts c-Fos protein expression as represented by greater fluorescent expression. The graph represents the number of immunoreactive c-Fos-positive cells present in the lateral pallium of control zebrafish and those administered 0.25 % ethanol, 25 mg/L caffeine, and 0.25% ethanol combined with 25 mg/L caffeine. Red = control, green = 0.25% ethanol, blue = 25 mg/L caffeine, yellow = 0.25% ethanol and 25 mg/L caffeine combined

Table 5

Experiment 2: C-Fos protein expression descriptive statistics

	Control			Ethanol			Caffeine			Ethanol+ Caffeine		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Hypot-halamus	3	3.33	1.52	3	4.0	2.65	3	4.67	2.08	3	3.33	0.57
Medial Pallium	3	13	2.65	3	13	1.00	3	18.66	2.08	3	23.66	3.21
Lateral Pallium	3	10	3.00	3	9.33	3.05	3	13.66	3.51	3	15.66	2.51

CHAPTER IV – EXPERIMENT 3

Social Isolation and Environmental Enrichment

Social isolation in humans is reported to be a significant risk factor for morbidity and mortality (Cacioppo & Hawkley, 2003). Adults who reported to feel socially isolated reported higher levels of anxiety, negative affect, perceived stress, lower levels of optimism, happiness and life satisfaction compared to non-isolated adults (Cacioppo et al., 2000; Cacioppo et al., 2002). Social isolation has been shown to produce a variety of deleterious effects on non-human animal wellbeing. For instance, socially isolated baboons living freely in the wild showed elevated cortisol levels compared to non-socially isolated baboons (Sapolsky, Alberts, & Altmann, 1997). Rats socially isolated for 1 week from conspecifics show increased stress hormone (i.e., corticosterone) levels and delayed neurogenesis in the hippocampus following exercise compared to group-housed rats (Kempermann, Gast, & Gage, 2002). Anxiety-like behaviors in rats produced by caffeine administration were increased in animals that had been individually housed (Sudakov, Medvedeva, Rusakova, & Figurina, 2001). Furthermore, socially isolated fruit flies (*Drosphila melanogaster*) have been reported to have decreased lifespans (Ruan & Wu, 2008).

Zebrafish are a highly social species that prefer to spend time in proximity to conspecifics and naturally form cohesive mixed-sex groups called shoals, with visual exposure to conspecifics having been employed as a rewarding stimulus in studies of associative learning (Al-Imari & Gerlai, 2008; Engeszer, Ryan, & Parichy, 2004; Saverino & Gerlai, 2008). In zebrafish, animals that were individually housed with no visual or olfactory cues from conspecifics for two weeks have been reported to display

reduced anxiety-like behaviors in the novel tank test, both in control fish and those administered 1.00% v/v ethanol for 20 minutes, compared to group-housed fish (Parker, Millington, Combe, & Brennan, 2012). These individually housed zebrafish also had significantly lower whole-body cortisol levels compared to fish housed in groups of ten (Parker et al., 2012). The fact that individually housed zebrafish showed reduced anxiety-like behaviors in the novel tank test compared to group-housed fish may be attributable to habituation to being socially isolated, as fish are tested individually in the novel tank test.

Another study found that zebrafish individually housed in a narrow tank similar to the novel tank apparatus before testing were reported to not display geotaxis or changes in swim velocity, although these effects were observed in fish housed in a wider tank (Bencan et al., 2009). This was likely the result of habituation to the narrow tank dimensions of the apparatus employed in the novel tank test and reduction of its novelty. The high cortisol levels reported in group housed fish may have been the result of the establishment of dominant-subordinate relationships characterized by patterns of chasing and biting by dominant fish (Larson, O'Malley, & Melloni, 2006; Oliveira, Silva, & Simoes, 2011). Both dominant and subordinate zebrafish housed in pairs for two hours showed increased cortisol levels compared to individually housed fish, indicating that this dominance hierarchy is likely stressful for each fish involved (Pavlidis et al., 2013). Overall, the effects of social isolation and group housing on anxiety-like behaviors have not been well characterized in zebrafish, especially regarding drug responses. Investigating these differences will be a valuable contribution to the zebrafish field, as laboratories engage in different practices in zebrafish housing (e.g., individual housing vs. group housing) prior to behavioral testing.

Environmental enrichment, often defined as living within a naturalistic environment and spatially complex environment containing functionally relevant stimuli has been reported to improve animal welfare (Van Praag, Kempermann, & Gage, 2000; Young, Lawlor, Leone, Dragunow, & During, 1999). For example, in rodents, environmental enrichment typically includes a large area covered with bedding material along with various forms of stimulation such as exercise wheels, toys, and tunnels, as well as the presence of conspecifics (Rampon et al., 2000). Mice living in an enriched environment for 10 months show increased neurogenesis in the hippocampus, improved learning and increased habituation to a novel environment compared to control animals (Kempermann et al., 2002). In a separate study, rats were housed for 9 weeks in an enriched environment, alone or in groups of three, or in a barren environment void of stimuli, alone or in groups of three (Schrijver, Bahr, Weiss, & Würbel, 2002). Overall, it was found that rats housed in enriched environments, either in isolation or in groups of three, showed increased habituation to novelty and improved spatial learning and memory, and rats isolated in barren environments showed the highest levels of anxiety-like behaviors and increased locomotor behavior. No differences in the stress hormone corticosterone were found across any conditions.

Environmental enrichment in fish has been reported to improve overall welfare and has been achieved in a similar manner as in rodent studies, as for example, by adding environmental stimuli to the tank (e.g., gravel, stones, plants) and adding contact and interactions with conspecifics (Näslund & Johnsson, 2014). For instance, providing a piece of wood in the laboratory housing of brown trout reduced instances of aggression between conspecifics (Gustafsson, Greenberg, & Bergman, 2012) and the presence of

areas to seek shelter reduced cortisol levels in Atlantic salmon (Näslund et al., 2013). Zebrafish individually housed in an enriched environment consisting of gravel and artificial plants for one week have been reported to show reduced locomotor activity and increased neurogenesis in the forebrain, as measured by the expression of proliferating cell nuclear antigen (PCNA), compared to zebrafish individually housed in a barren environment (von Krogh, Sørensen, Nilsson, & Øverli, 2010).

In another study, zebrafish raised in an enriched environment showed increased time spent in the light area of the light-dark test, indicative of reduced anxiety (Maximino, de Brito, de Mattos Dias, Gouveia, & Morato, 2010). When placed in a tank divided into an enriched environment compartment containing gravel and artificial plants and a barren environment compartment, and zebrafish were given the option to spend time in either environment, a strong preference was observed for the enriched environment (Schroeder, Jones, Young, & Sneddon, 2014). This study also compared the preference to spend time in a compartment containing a floating plant or a submerged plant, and it was found that zebrafish had a preference for the floating plant compartment. Although the effects of social isolation and environmental enrichment on zebrafish locomotor behaviors and anxiety behaviors have been reported, it is not clear what the effects of these conditions are on drug responses.

This experiment employed four housing conditions, consisting of social isolation in a barren environment (IB), social isolation in an enriched environment (IE), social housing of 3 fish in a barren environment (SB) and social housing of 3 fish in an enriched environment (SE). This experiment also evaluated four drug conditions of control, 0.25% v/v ethanol, 25 mg/L caffeine and 0.25% v/v ethanol and 25 mg/L combined.

Specific Aim 3

Characterize how two weeks of social isolation in a barren or enriched environment affects locomotor behaviors and anxiety-like behaviors in the novel tank test (Table 3) compared to zebrafish housed in groups of three in a barren or enriched environment in control animals and in zebrafish administered acute 0.25% alcohol, 25 mg/L caffeine, and 0.25% alcohol and 25 mg/L caffeine combined.

Hypotheses

It was expected that zebrafish socially isolated in an enriched environment would show the greatest decrease in locomotor behaviors and anxiety behaviors in the novel tank test in all drug conditions compared to all other housing conditions. It was also expected that zebrafish individually and group-housed within an enriched environment would show decreased locomotor behaviors and anxiety behaviors in the novel tank test in all drug conditions compared to zebrafish individually and group-housed in a barren environment.

Housing Condition Methods

Following acclimation to the laboratory environment for 10 days, zebrafish were removed from their 10-liter group housing tanks and were housed for 14 days either individually in a barren (IB) or enriched (IE) 0.8 gallon tank, or housed in groups of three in a barren (SB) or enriched (SE) 2.5 gallon tank (Figures 14 and 15). All tanks were equipped with a 50-watt Tetra aquarium heater (Tetra: Blacksburg, VA) and Elite underwater mini filter (Hagen: Baie d'Urfé, QC). All tanks were covered on the interior with opaque blue shelf liner, sealed along the bottom of the tank with silicone aquarium sealant, and covered along the top of the tank with perforated black mesh to prevent

zebrafish from jumping out. Barren housing conditions consisted of the respective tank, blue shelf liner, aquarium filter, and heater. Blue shelf liner was selected due to blue being one of the most common colors of housing tank inserts and tank lids in commercial zebrafish housing systems (e.g., Pentair Aquatic Eco-Systems: Apopka, FL), which is employed in the Zebrafish Behavioral Neuroscience Laboratory at USM. Enriched housing conditions included each of the aforementioned components, in addition to a mixture of black and brown aquarium gravel, several larger gray stones and green artificial plants submerged just under the surface of the water to provide shelter. Following 14 days of housing in their respective environment, zebrafish were administered either 0.25% v/v ethanol, 25 mg/L caffeine or 0.25% v/v ethanol and caffeine for 30 minutes and tested in the novel tank test to evaluate locomotor and anxiety-like behaviors using novel tank testing methods and behavioral analysis as previously discussed.

Figure 14. Individual housing tanks

Individual barren (IB) and individual enriched (IE) tanks used to house one zebrafish consisted of a volume of 0.8 gallons that were 7 " L x 7 " W x 7 " H

Figure 15. Social housing tanks

Social barren (SB) and social enriched (SE) tanks used to house three zebrafish consisted of a volume of 2.5 gallons that were 6.25 " L x 12.25 " W x 8.25 " H

Statistical Analysis

The interaction and main effects of drug and housing conditions on each anxiety-like behavior and locomotor behavior (Table 3) was evaluated using a 4 x 4 factorial ANOVA with "drug" (four levels, control, 0.25% ethanol, 25 mg/L caffeine and 0.25% ethanol and 25 mg/L caffeine combined) and "housing condition" (four levels, individual barren (IB), individual enriched (IE), social barren (SB) and social enriched (SE)) as between-subject factors. Following a significant interaction effect, simple effects analysis was conducted to investigate the interaction effect by examining the effect of drug on behavior at each level of environment. Due to ANOVA having been reported as insensitive in detecting interaction effects (Wahlsten, 1990), in addition, to sample sizes being unequal across groups, Hochberg's GT2 posthoc tests were used to evaluate differences between all 16 groups across the factors of drug and housing condition for

each behavior even when interaction terms were found to be non-significant. The accepted level of significance was $p \leq 0.05$. Data is presented as mean ± SEM.

Experiment 3 Results

Table 6 shows the results of the 4x4 factorial ANOVA for the main effects of "drug" and "housing condition", and the "drug" x "housing condition" interaction for each behavior. There were significant main effects of drug on distance traveled (cm), $F(3, 128) = 3.14$, $p = 0.028$, freezing duration (s), $F(3, 128) = 6.95$, $p < 0.001$, and latency to enter the top half of the tank (s), $F(3, 128) = 4.21$, $p = 0.007$. There were also significant main effects of housing condition on distance traveled (cm) $F(3, 128) = 4.68$, $p = 0.004$, average velocity (cm/s), F (3, 128) = 3,35, $p = 0.021$, and freezing duration (s), $F(3, 128) = 4.49$, $p = 0.005$. There was a significant interaction effect between the type of drug administration and housing environment on freezing duration (s), $F(9, 128) = 5.00$, $p < 0.001$. This indicates that the effect of drug condition on freezing duration was different for zebrafish depending on the housing condition. An analysis of simple effects showed that zebrafish administered 0.25% ethanol and 25 mg/L caffeine combined and housed in an individual enriched (IE) environment showed significantly greater freezing duration (s) compared to zebrafish administered ethanol and caffeine and housed in individual barren (IB) ($p \leq 0.001$), social barren (SB) ($p \leq 0.001$) or social enriched (SE) ($p \leq 0.001$) environments.

Table 6

Experiment 3: Two-way factorial ANOVA results

Behavior	Drug	Housing Condition	Drug x Housing Condition
Distance traveled (cm)	**F (3, 128) = 3.14, p = 0.028**	**F (3, 128) = 4.68, p = 0.004**	F (9, 128) = 0.85, p = 0.57
Average velocity (cm/s)	F (3, 128) = 2.44, p = 0.067	**F (3, 128) = 3,35, p = 0.021**	F (9, 128) = 1.05, p = 0.41
Freezing bouts	F (3, 128) = 1.05, p = 0.380	F (3, 128) = 1.16, p = 0.328	F (9, 128) = 0.85, p = 0.57
Freezing duration (s)	**F (3, 128) = 6.95, p < 0.001**	**F (3, 128) = 4.49, p = 0.005**	**F (9, 128) = 5.00, p <0.001**
Time in top (s)	F (3, 128) = 1.93, p = 0.128	F (3, 128) = 2.05, p = 0.110	F (9, 128) = 1.77, p = 0.08
Latency to enter the top half of the tank (s)	**F (3, 128) = 4.21, p = 0.007**	F (3, 128) = 1.95, p = 0.124	F (9, 128) = 1.04, p = 0.42

Text bolded and underlined indicates statistical significance of $p \leq 0.05$

Figures describing the results of this experiment are reserved for conditions in which there was a statistically significant main effect or interaction. Figures describing non-significant findings in this experiment are located the appendices, along with tables of descriptive values for each condition. Post-hoc tests did not reveal any significant differences in control zebrafish or those that received 0.25% ethanol for any behavior across housing conditions. Post-hoc tests revealed a significant difference in mean distance traveled (cm) in zebrafish administered 25 mg/L caffeine between individual barren (IB) and social enriched (SE) housing conditions ($p = 0.043$). No significant differences were revealed for other behaviors across housing conditions (Figure 16).

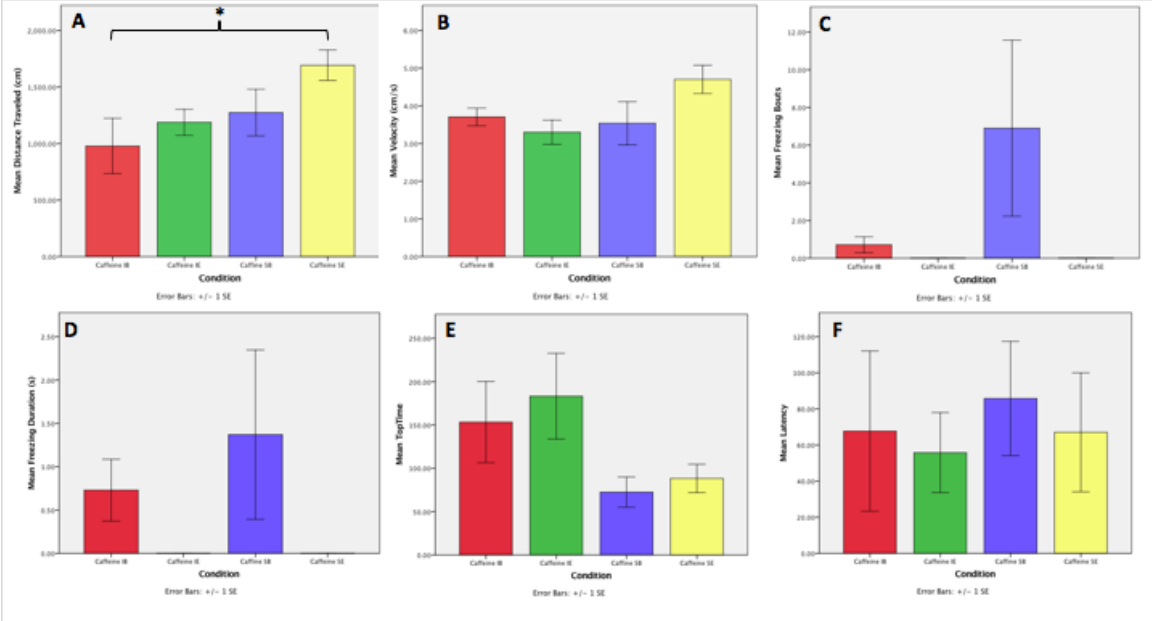

Figure 16. Effects of housing conditions and 25 mg/L caffeine on anxiety-like behaviors

Red = IB, Green = IE, Blue = SB, Yellow = SE. A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank. *p ≤ 0.05.

Post-hoc tests revealed a significant difference in mean freezing duration (s) in zebrafish housed in an individual enriched (IE) environment and administered 0.25% ethanol combined with 25 mg/L caffeine compared to zebrafish housed in individual barren (IB) ($p = 0.015$), social barren (SB) ($p = 0.042$), and social enriched (SE) ($p = 0.011$) housing conditions. No significant differences were revealed for other behaviors across housing conditions (Figure 17).

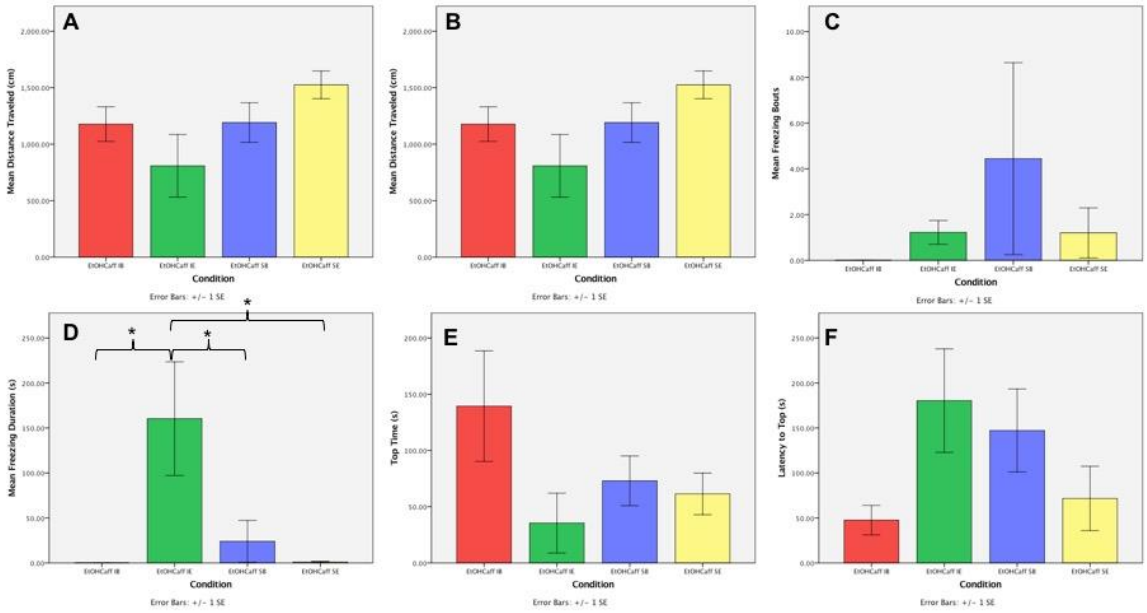

Figure 17. Effects of housing conditions and 0.25% ethanol combined with 25 mg/L caffeine on anxiety-like behaviors.

Red = IB, Green = IE, Blue = SB, Yellow = SE. A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank. *$p \leq 0.05$.

Post-hoc tests did not reveal any significant differences between each drug condition and zebrafish behavior for animals housed in an individual barren (IB) environment. Post-hoc tests revealed a significant difference in mean freezing duration (s) in zebrafish housed in an individual enriched (IE) environment and administered 0.25% ethanol combined with 25 mg/L caffeine compared to control ($p = 0.018$), ethanol ($p = 0.023$), and caffeine-treated groups ($p = 0.017$). No significant differences were revealed for other behaviors across housing conditions (Figure 18).

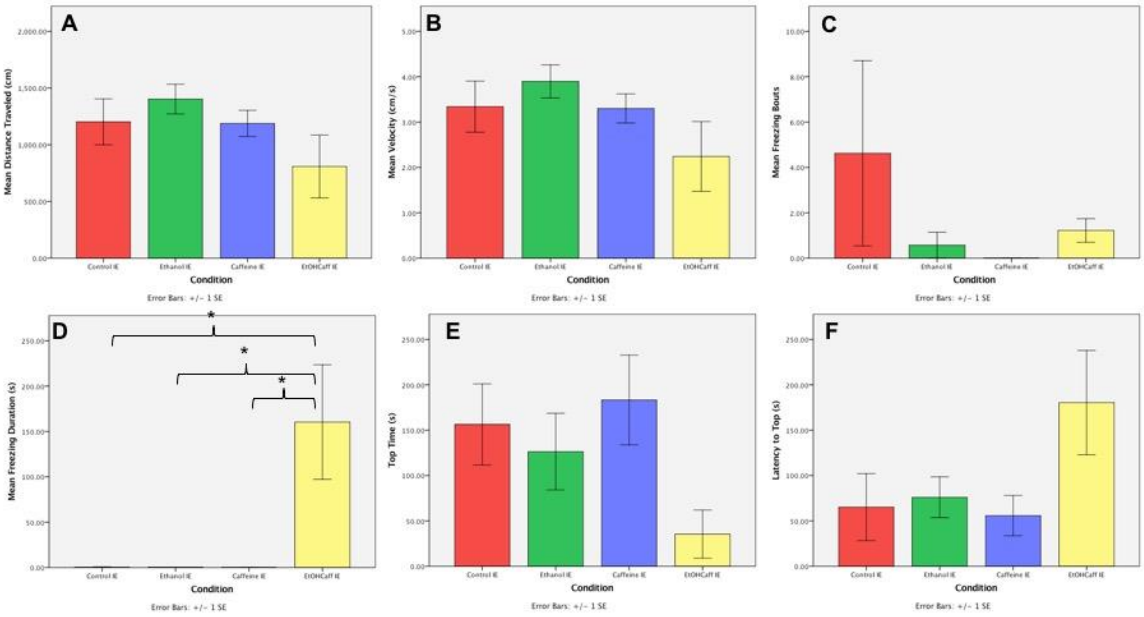

Figure 18. Effects of 0.25% ethanol, 25 mg/L caffeine and 0.25% ethanol and 25 mg/L combined on anxiety-like behaviors in zebrafish housed in an individual enriched (IE) environment.

Red = control, Green = ethanol, Blue = caffeine, Yellow = ethanol and caffeine (E+C). A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank. *$p \leq 0.05$.

Post-hoc tests revealed a significant difference in latency to enter the top half of the tank (s) in zebrafish housed in a social barren (SB) environment and administered 0.25% ethanol combined with 25 mg/L caffeine compared to control ($p = 0.018$), ethanol ($p = 0.023$), and caffeine-treated groups ($p = 0.025$). No significant differences were revealed for other behaviors across housing conditions (Figure 19).

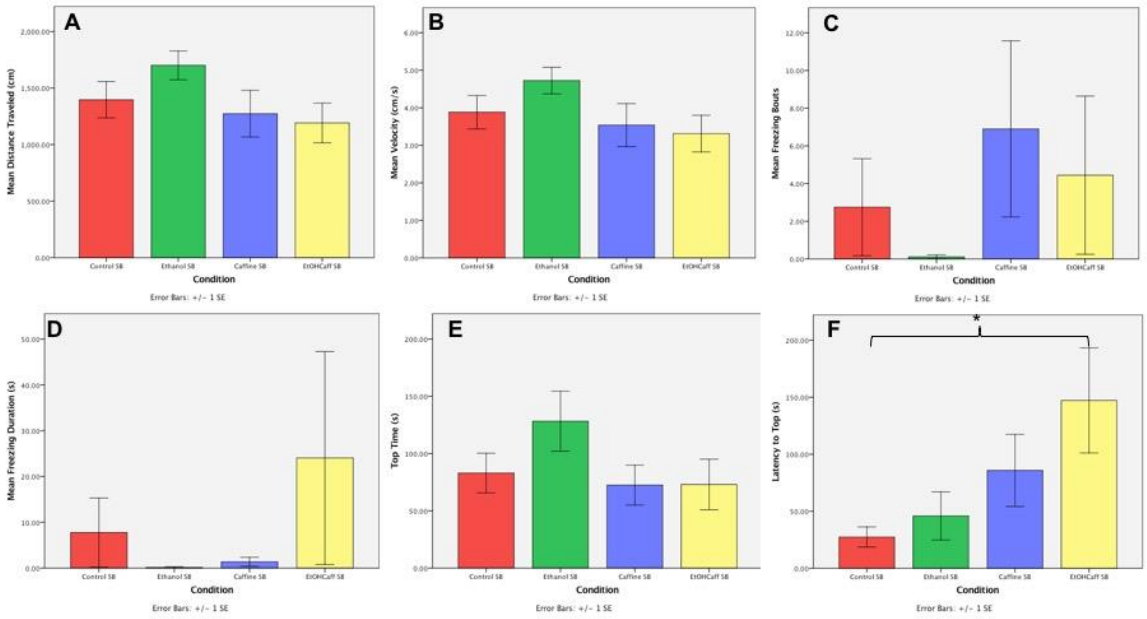

Figure 19. Effects of 0.25% ethanol, 25 mg/L caffeine and 0.25% ethanol and 25 mg/L caffeine combined on anxiety-like behaviors in the novel tank test in zebrafish housed in a social barren (SB) environment

Red = control, Green = ethanol, Blue = caffeine, Yellow = ethanol and caffeine (E+C). A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank. *$p \leq 0.05$.

Post-hoc tests did not reveal any significant differences between each drug condition and zebrafish behavior for animals housed in a social enriched (SE) environment.

CHAPTER V – DISCUSSION

The pathological use of alcohol is associated with over 200 health conditions and is a tremendous burden to the global economy, responsible for over $250 billion in economic costs in 2010 within the United States alone (Sacks et al., 2015; WHO, 2014). Although caffeine is comparably a less harmful substance, when combined with alcohol it increases behavioral and health-related risk factors associated with alcohol use alone, likely by reducing the subjective perception of intoxication and the depressant effects of alcohol (Ferreira et al., 2006; O'Brien et al., 2008). Discovering the underlying mechanisms (e.g., behavioral and neural) of drug action is fundamental to treatment, reversal, and prevention of substance use disorders (Duman, Heninger, & Nestler, 1994; Nestler, 2013).

In experiment 1, this study employed the adult zebrafish to investigate the effects of 0.25% v/v ethanol, 25 mg/L caffeine, and 0.25% v/v ethanol and 25 mg/L caffeine combined on locomotor behaviors and anxiety-like behaviors in the well-validated novel tank test (Cachat, Canavello, et al., 2011; Stewart et al., 2014). Administration of 0.25% ethanol alone was found to increase the mean distance traveled over the 6 minute testing session compared to control zebrafish and those administered 25 mg/L caffeine and 0.25% ethanol and 25 mg/L caffeine combined, suggestive of a stimulatory effect. Similarly, ethanol alone increased mean swim velocity compared to zebrafish administered caffeine alone and ethanol and caffeine combined. These findings are in line with the stimulant effects of alcohol reported at low doses in zebrafish (Gerlai et al., 2000), rodents (Gingras & Cools, 1996) and humans (King et al., 2002). Interestingly, the effects of alcohol and caffeine combined on distance traveled and swim velocity

resembled that of caffeine alone, indicative of an antagonistic effect of caffeine on the stimulatory effects of alcohol, which is in line with previous findings of zebrafish co-administered high doses of alcohol and caffeine (Tran et al., 2017). Caffeine alone did not affect distance traveled or swim velocity compared to control subjects, suggesting that 30 minute administration of 25 mg/L caffeine did not have a stimulatory or depressant effect. This finding is not consistent with a previous report of 25 mg/L caffeine reducing total distance traveled compared to control animals (Ladu et al., 2015). Administration of ethanol and caffeine combined increased freezing bouts and increased freezing duration compared to ethanol alone and largely resembled the effects of caffeine alone. Interestingly, there were no significant effects of any drug condition on time spent in the top of the novel tank, although administration of ethanol combined with caffeine increased the latency to enter the top half of the tank indicative of an anxiety-like response. Overall, these findings corroborate reports in humans that the ingestion of alcohol (i.e., ethanol) and caffeine together antagonized some effects alcohol alone (Ferreira et al., 2006; Marczinski, 2011).

In experiment 2, the expression of the immediate-early gene c-Fos protein was evaluated following administration of 0.25% ethanol, 25 mg/L caffeine and 0.25% ethanol and 25 mg/L caffeine combined, and novel tank testing, in the medial pallium (amygdala), the lateral pallium (hippocampus) and the periventricular nucleus of the hypothalamus of the zebrafish brain. These three brain areas are implicated in mediating responses to psychoactive drugs and to anxiety-inducing stimuli (Duncan et al., 1996; Ogilvie et al., 1998; Somerville et al., 2004). The effects of these drug conditions on c-Fos expression have not been described in zebrafish. Comparing c-Fos expression

profiles in each brain region of interest across drug conditions helps provide a useful characterization of how each experimental manipulation is acting on the zebrafish brain.

No differences were observed in c-Fos protein expression in the periventricular nucleus of the hypothalamus or the lateral pallium (hippocampus). This lack of an observed effect may be attributable to a lack of sensitivity of these brain areas to the low doses of substances or the low sample size employed in this study. However, c-Fos protein expression was significantly increased in the medial pallium (amygdala) of zebrafish administered 0.25% ethanol and 25 mg/L caffeine combined compared to zebrafish administered ethanol alone. These findings are in line with the behavioral differences produce by administration of ethanol and caffeine combined compared to ethanol alone in the novel tank test (i.e., increased distance traveled, increased velocity, increased latency to enter the top half of the novel tank). Thus, it may be that the increased neuronal activation in the medial pallium is in part mediating these behavioral effects.

Increased c-Fos protein expression in the zebrafish medial pallium has been reported following both acute administration of amphetamine and during drug-seeking behavior towards amphetamine following an associative conditioning procedure (i.e., conditioned place preference) (von Trotha et al., 2014). This suggests that the function of the zebrafish medial pallium (amygdala) is comparable to the function of the mammalian amygdala in mediating the effects of drugs on behavior (Koob, 2009; Koob & Nestler, 1997). The mammalian amygdala is also reported to mediate anxiety. For instance, in humans, increased amygdala activity measured by fMRI was observed in human subjects viewing images of faces with fearful expressions and was found to correlate with

reported levels of anxiety (Somerville et al., 2004). Furthermore, a meta-analysis of brain imaging studies in patients with posttraumatic stress disorder, social anxiety disorder, and specific phobias found greater activity in the amygdala in patients with each of these disorders compared to healthy subjects (Etkin & Wager, 2007). Therefore, increased latency to enter the upper half of the novel tank, suggestive of anxiety, in zebrafish administered ethanol and caffeine may be partly attributable to increased c-Fos expression in the medial pallium.

In an effort to effectively assess the behavioral effects of experimentally administered drugs, it is imperative that there is a baseline understanding of the effects of housing conditions on zebrafish behavior. Zebrafish laboratories often engage in different practices regarding the housing of zebrafish prior to experimental testing, with some labs keeping zebrafish in group housing, and others keeping them in individual housing to track the behavior of an individual over time. Standard laboratory zebrafish housing tanks are often barren and void of environmentally enriching stimuli, such as gravel and plants providing shelter. Experiment 3 characterized the effects of two weeks of housing in one of four conditions (i.e., individual barren (IB), individual enriched (IE), social barren (SB), social enriched (SE)) on anxiety-like behaviors and locomotor behaviors in the novel tank test in control animals, as well as in animals administered 0.25% ethanol, 25 mg/L caffeine, and 0.25% ethanol and 25 mg/L caffeine combined.

Due to the large number of groups and behaviors evaluated in experiment 3, only significant findings will be described here. Zebrafish housed in a SE environment and administered caffeine alone showed increased distance traveled compared to zebrafish housed in an IB environment and administered caffeine. Thus, this effect is attributable to

the difference in housing conditions. The fact that zebrafish were housed in an enriched environment in groups of 3 and then individually removed a placed in the barren novel tank test may partly explain this effect, although no other differences were observed between housing conditions in zebrafish administered caffeine. Zebrafish that were administered ethanol and caffeine combined and housed in an IE environment exhibited significantly longer freezing duration compared to zebrafish housed in an IB, SB and SE environment and administered ethanol and caffeine. Similarly, zebrafish housed in an IE environment and administered both ethanol and caffeine showed significantly longer freezing duration than zebrafish housed in an IE environment in all other drug conditions. Again, this difference may be attributable to the discrepancy between the housing environment and the novel tank test environment, although no other behaviors were significantly different across housing conditions in zebrafish administered ethanol and caffeine combined or across drug conditions in zebrafish housed in an IE environment.

Zebrafish housed in a SB environment and administered ethanol and caffeine combined showed significantly greater latency to enter the top half of the novel tank compared to zebrafish housed in an SB environment in the control condition, indicative of increased anxiety. This finding closely parallels the finding in experiment 1 of increased latency to enter the top half of the novel tank in zebrafish administered caffeine and ethanol compared to ethanol alone and lends further support that the combination of these drugs at low doses increase anxiety-like behaviors. Overall, there was a lack of significant differences in behavior across drug and environmental conditions in this experiment, which may be attributable to relatively low sample sizes (n = 7-12). This experiment would benefit from increasing sample sizes to n =15 as in experiment 1 to

more accurately assess the main effects and interactions of drug and environment on locomotor and anxiety-like behaviors. Unfortunately, it was not feasible to characterize c-Fos expression profiles in response to each housing condition combined with each drug condition due to the large number (i.e., 16) of groups in the experiment.

The field of zebrafish research has evolved greatly beyond its origins in genetics and developmental biology, partly due to the growing appreciation of zebrafish as advantageous neurobehavioral models in comparison to rodent models, largely due to the low-cost, ease of handling and small size of this aquatic species. Studying the effects of psychoactive drugs on zebrafish is a recent enterprise in comparison to rodents, and there is thus a lack of information available regarding drug absorption rates (Klee, Ebbert, Schneider, Hurt, & Ekker, 2011). One potential limitation of this overall study pertains to methods of drug delivery. The most commonly employed method of administration is via submersion in a bath solution containing a concentration of the drug to be absorbed by the gills, skin, and mouth. Zebrafish are known to absorb most water-soluble drugs administered in this manner, but the degree of uptake can vary among individuals (Best & Alderton, 2008). Zebrafish may be administered compounds by injection (e.g., intraperitoneal), which has been reported to be a more precise method of drug delivery, although injections will reduce the rate of experimental throughput and may be stress inducing (Kokel & Peterson, 2008).

Although the effects of ethanol immersion on blood alcohol content and brain alcohol content has been described for multiple doses and durations of exposure (Echevarria et al., 2011; Sterling et al., 2014), no studies have been reported regarding the uptake of caffeine in the zebrafish brain. However, as caffeine has been found to alter

zebrafish behavior in the novel tank test across multiple doses and to increase c-Fos protein expression in multiple brain areas, it is likely that caffeine is crossing the zebrafish blood-brain-barrier (Chatterjee et al., 2015; Wong, Stewart, et al., 2010). Although zebrafish have similar CNS structure to humans and possess all the major mammalian neurotransmitters, there are undoubtedly very large differences in animal physiology. For instance, two forms of the serotonin transporter, SERT A and B, are found in zebrafish and not in mammals or humans (Norton, Folchert, & Bally-Cuif, 2008; Wang, Takai, Yoshioka, & Shirabe, 2006) (Wang et al. 2006; Norton et al. 2008). Furthermore, as there are notable differences in neuronal architecture between zebrafish and mammals, the underlying mechanisms and behavioral effects associated with drug action are likely to differ to some degree (Eddins, Petro, Williams, Cerutti, & Levin, 2009). Although the zebrafish brain and behavior are not homologous to that of mammals, neuroanatomy, neurochemistry, and physiology is generally conserved across vertebrates, mediating many of the same behaviors and establishing the use of zebrafish as an alternative animal model to mitigate limitations of rodent models (McCammon & Sive, 2015a, 2015b; Stewart et al., 2015).

In summary, the results of this study reinforce the translational relevance of the zebrafish model in behavioral pharmacology research. The effects of alcohol and caffeine on zebrafish behavior and the brain are evolutionarily conserved, paralleling findings in rodents and humans. This study contributes to the zebrafish field and informs future research that aims to employ this valuable animal model to better understand the underlying mechanisms contributing to the pathogenesis of substance use disorders and in the development of novel therapies.

APPENDIX A – Experiment 3 Additional Figures

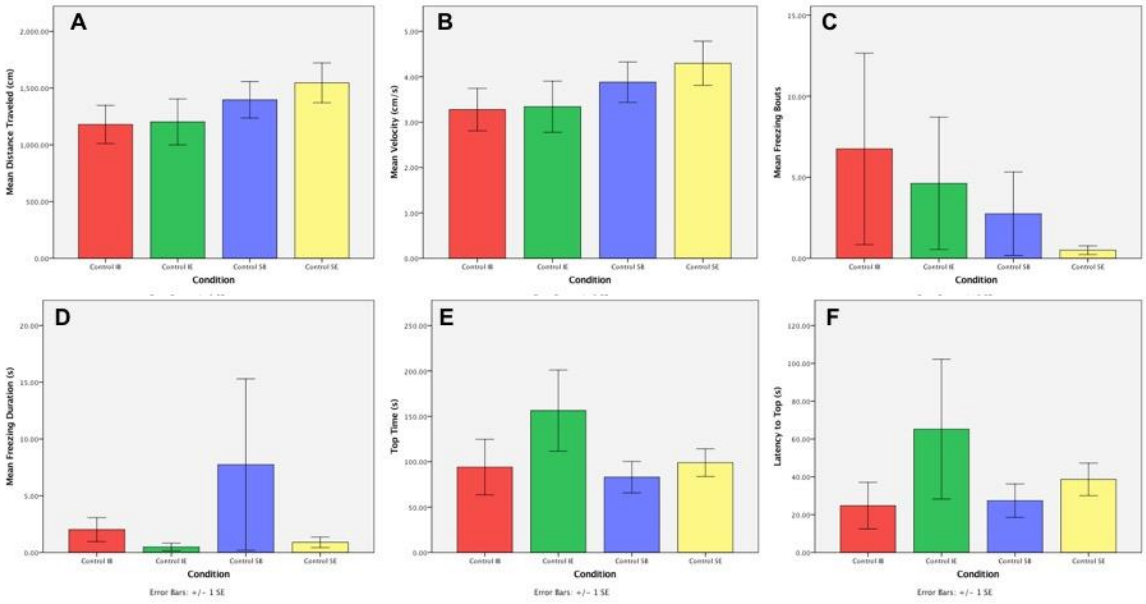

Figure A1. . Effects of housing conditions on anxiety-like behaviors in control zebrafish in the novel tank test.

Red = IB, Green = IE, Blue = SB, Yellow = SE. A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank.

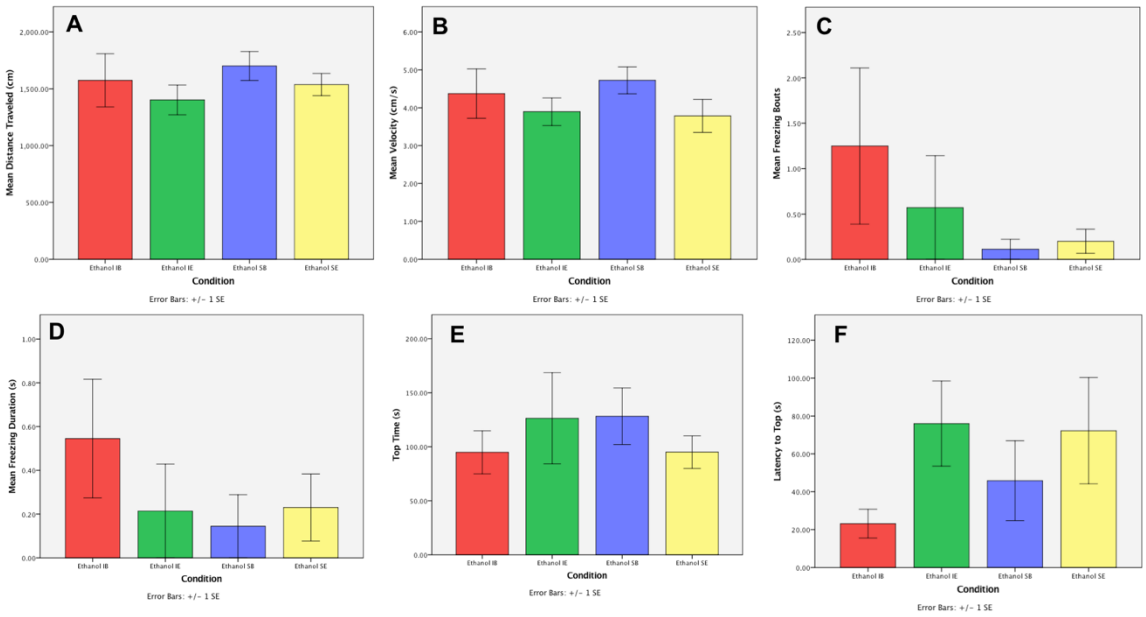

Figure A2. . Effects of housing conditions on anxiety-like behaviors in the novel tank test in zebrafish administered 0.25% ethanol.

Red = IB, Green = IE, Blue = SB, Yellow = SE. A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank.

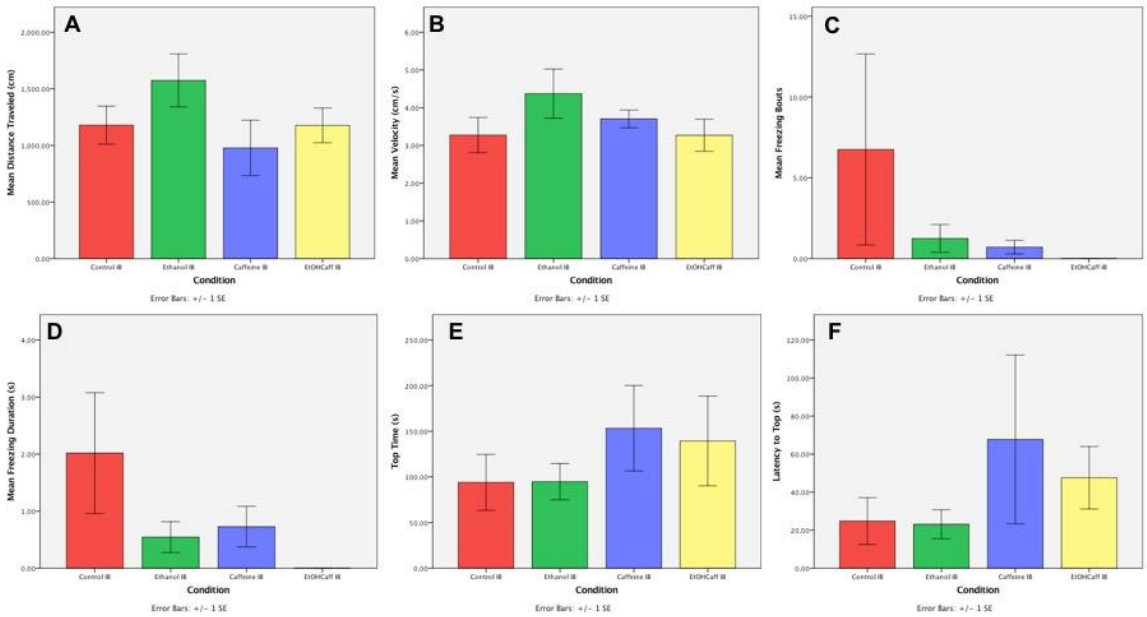

Figure A3. Effects of 0.25% ethanol, 25 mg/L caffeine and 0.25% ethanol and 25 mg/L caffeine combined on anxiety-like behaviors in the novel tank test in zebrafish housed in an individual barren (IB) environment.

Red = control, Green = ethanol, Blue = caffeine, Yellow = ethanol and caffeine (E+C). A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank.

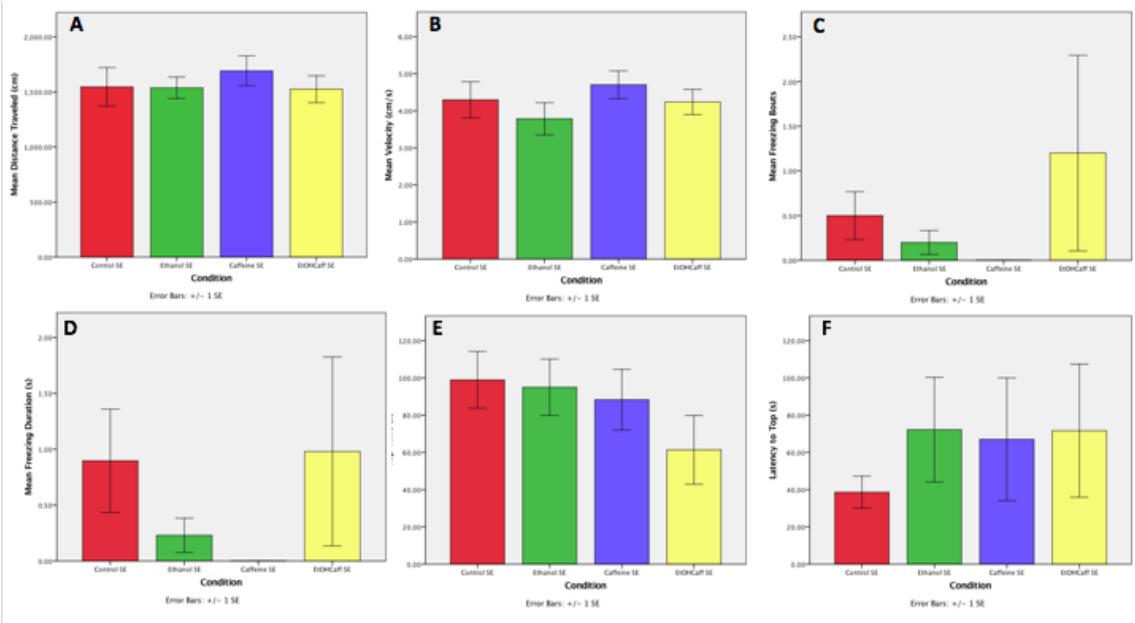

Figure A4. . Effects of 0.25% ethanol, 25 mg/L caffeine and 0.25% ethanol and 25 mg/L caffeine combined on anxiety-like behaviors in the novel tank test in zebrafish housed in a social enriched (SE) environment

Red = control, Green = ethanol, Blue = caffeine, Yellow = ethanol and caffeine (E+C). A) Mean distance traveled (cm). B) Mean swim velocity (cm/s). C) Mean freezing bouts. D) Mean freezing duration (s). E) Time in the top half of the tank (s). F) Latency to enter the top half of the tank.

APPENDIX B – Experiment 3 Additional Tables

Table A1.

Experiment 3: Novel Tank Test Descriptive Statistics for Control Zebrafish in Each Housing Condition

	Control IB			Control IE			Control SB			Control SE		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	8	1179.35	475.11	8	1202.33	573.14	12	1396.79	555.82	12	1546.81	554.1
Velocity (cm/s)	8	3.27	1.32	8	3.34	1.59	12	3.88	3.34	12	4.29	4.30
Freezing Bouts	8	6.75	16.7	8	4.6	11,55	12	4.6	8.92	12	0.50	0.85
Freezing Duration (s)	8	2.01	2.99	8	0.49	0.94	12	7.7	26.17	12	0.89	1.46
Time in Top (s)	8	94.07	86.5	8	156.3	126.47	12	82.96	60.00	12	98.98	48.31
Latency (s)	8	24.77	24.78	8	65.2	104.56	12	27.39	30.59	12	38.66	27.17

Table A2.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Administered 0.25% Ethanol in Each Housing Condition

	Ethanol IB			Ethanol IE			Ethanol SB			Ethanol SE		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	8	1574.69	661.78	7	1402.60	346.88	9	1701.03	382.45	10	1538.95	308.08
Velocity (cm/s)	8	4.37	1.83	7	3.89	0.96	9	4.72	1.06	10	3.78	1.37
Freezing Bouts	8	1.25	2.43	7	0.57	1.51	9	0.11	0.33	10	0.20	0.42
Freezing Duration (s)	8	0.54	0.77	7	0.21	0.56	9	0.14	0.43	10	0.23	0.48
Time in Top (s)	8	94.83	56.28	7	126.36	111.71	9	95.02	47.75	10	95.02	47.75
Latency (s)	8	23.14	21.52	7	76.02	59.5	9	45.87	63.36	10	72.24	88.77

Table A3.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Administered 25 mg/L Caffeine in Each Housing Condition

	Caffeine IB			Caffeine IE			Caffeine SB			Caffeine SE		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	7	978.41	647.57	8	1187.96	326.14	10	1273.54	652.37	11	1692.66	446.88
Velocity (cm/s)	7	3.70	.62097	8	3.30	0.90	10	3.53	1.812	11	4.70	1.24
Freezing Bouts	7	0.71	1.11	8	0.00	0.00	10	6.90	14.77	11	0.00	0.00
Freezing Duration (s)	7	0.73	0.94	8	0.00	0.00	10	1.37	3.091	11	0.00	0.00
Time in Top (s)	7	153.34	123.98	8	183.27	139.94	10	72.46	55.20	11	88.29	54.13
Latency (s)	7	67.70	117.36	8	55.81	62.72	10	85.80	100.05	11	67.06	109.32

Table A4.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Administered 0.25% Ethanol Combined with 25 mg/L Caffeine in Each Housing Condition

	Ethanol + Caffeine IB			Ethanol + Caffeine IE			Ethanol + Caffeine SB			Ethanol + Caffeine SE		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	8	1177.34	432.99	9	808.61	831.28	9	1191.44	526.24	10	1525.66	386.76
Velocity (cm/s)	8	3.27	1.2	9	2.25	2.30	9	3.31	1.46	10	4.23	1.07
Freezing Bouts	8	0.00	0.00	9	1.22	1.56	9	4.44	12.59	10	1.20	3.46
Freezing Duration (s)	8	0.00	0.00	9	160.30	189.45	9	24.03	69.75	10	0.98	2.67
Time in Top (s)	8	139.38	138.9	9	35.42	79.70	9	72.98	66.24	10	61.39	58.49
Latency (s)	8	47.55	46.51	9	180.37	172.49	9	147.22	138.21	10	71.66	113.11

Table A5.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Housed in an Individual Barren Environment Across Drug Conditions

	Control IB			Ethanol IB			Caffeine IB			Ethanol + Caffeine IB		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	8	1179.35	475.11	8	1574.69	661.78	7	978.41	647.57	8	1177.34	432.99
Velocity (cm/s)	8	3.27	1.32	8	4.37	1.83	7	3.70	.62097	8	3.27	1.2
Freezing Bouts	8	6.75	16.7	8	1.25	2.43	7	0.71	1.11	8	0.00	0.00
Freezing Duration (s)	8	2.01	2.99	8	0.54	0.77	7	0.73	0.94	8	0.00	0.00
Time in Top (s)	8	94.07	86.5	8	94.83	56.28	7	153.34	123.98	8	139.38	138.9
Latency (s)	8	24.77	24.78	8	23.14	21.52	7	67.70	117.36	8	47.55	46.51

Table A6.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Housed in an Individual Enriched Environment Across Drug Conditions

	Control IE			Ethanol IE			Caffeine IE			Ethanol + Caffeine IE		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	8	1202.33	573.14	7	1402.60	346.88	8	1187.96	326.14	9	808.61	831.28
Velocity (cm/s)	8	3.34	1.59	7	3.89	0.96	8	3.30	0.90	9	2.25	2.30
Freezing Bouts	8	4.6	11,55	7	0.57	1.51	8	0.00	0.00	9	1.22	1.56
Freezing Duration (s)	8	0.49	0.94	7	0.21	0.56	8	0.00	0.00	9	160.30	189.45
Time in Top (s)	8	156.3	126.47	7	126.36	111.71	8	183.27	139.94	9	35.42	79.70
Latency (s)	8	65.2	104.56	7	76.02	59.5	8	55.81	62.72	9	180.37	172.49

Table A7.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Housed in a Social Barren Environment Across Drug Conditions

	Control SB			Ethanol SB			Caffeine SB			Ethanol + Caffeine SB		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	12	1396.79	555.82	9	1701.03	382.45	10	1273.54	652.37	9	1191.44	526.24
Velocity (cm/s)	12	3.88	3.34	9	4.72	1.06	10	3.53	1.812	9	3.31	1.46
Freezing Bouts	12	4.6	8.92	9	0.11	0.33	10	6.90	14.77	9	4.44	12.59
Freezing Duration (s)	12	7.7	26.17	9	0.14	0.43	10	1.37	3.091	9	24.03	69.75
Time in Top (s)	12	82.96	60.00	9	95.02	47.75	10	72.46	55.20	9	72.98	66.24
Latency (s)	12	27.39	30.59	9	45.87	63.36	10	85.80	100.05	9	147.22	138.21

Table A8.

Experiment 3: Novel Tank Test Descriptive Statistics for Zebrafish Housed in a Social Enriched Environment Across Drug Conditions

	Control SE			Ethanol SE			Caffeine SE			Ethanol + Caffeine SE		
	n	M	SD	n	M	SD	n	M	SD	n	M	SD
Distance Traveled (cm)	12	1546.81	554.02	10	1538.95	308.08	11	1692.66	446.88	10	1525.66	386.76
Velocity (cm/s)	12	4.29	4.30	10	3.78	1.37	11	4.70	1.241599	10	4.23	1.07
Freezing Bouts	12	0.50	0.85	10	0.20	0.42	11	0.00	0.00	10	1.20	3.46
Freezing Duration (s)	12	0.89	1.46	10	0.23	0.48	11	0.00	0.00	10	0.98	2.67
Time in Top (s)	12	98.98	48.31	10	95.02	47.75	11	88.29	54.13	10	61.39	58.49
Latency (s)	12	38.66	27.17	10	72.24	88.77	11	67.06	109.32	10	71.66	113.11

APPENDIX C – 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:	**16081902**
PROJECT TITLE:	**Effects of housing and environmental conditions on behavioral and neuroendocrine phenotypes in adult zebrafish**
PROPOSED PROJECT DATES:	**08/2016 – 09/2018**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**David Echevarria**
DEPARTMENT:	**Psychology**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2018**

Jake Schaefer, PhD
IACUC Chair

9/6/2016
Date

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:	**17020902**
PROJECT TITLE:	**Effects of alcohol and caffeine on anxiety-like behaviors and c-Fos protein expression in adult zebrafish**
PROPOSED PROJECT DATES:	**02/2017 – 09/2019**
PROJECT TYPE:	**New**
PRINCIPAL INVESTIGATOR(S):	**David Echevarria**
DEPARTMENT:	**Psychology**
FUNDING AGENCY/SPONSOR:	**N/A**
IACUC COMMITTEE ACTION:	**Full Committee Approval**
PROTOCOL EXPIRATON DATE:	**September 30, 2019**

Jake Schaefer, PhD
IACUC Chair

2/24/2017
Date

REFERENCES

Al-Imari, L., & Gerlai, R. (2008). Sight of conspecifics as reward in associative learning in zebrafish (Danio rerio). *Behavioural Brain Research, 189*, 216-219. doi:10.1016/j.bbr.2007.12.007

Alsop, D., & Vijayan, M. (2009). The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event. *General and Comparative Endocrinology, 161*, 62-66. doi:10.1016/j.ygcen.2008.09.011

American Psychiatric Association. (2013). *Diagnostic and statistical manual of mental disorders* (5 ed.). Washington, DC.

Barros, T. P., Alderton, W. K., Reynolds, H. M., Roach, a. G., & Berghmans, S. (2008). Zebrafish: an emerging technology for in vivo pharmacological assessment to identify potential safety liabilities in early drug discovery. *British Journal of Pharmacology, 154*, 1400-1413. doi:10.1038/bjp.2008.249

Barson, J. R., & Leibowitz, S. F. (2016). Hypothalamic neuropeptide signaling in alcohol addiction. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 65*, 321-329.

Belzung, C. (1999). Measuring rodent exploratory behavior. *Techniques in the Behavioral and Neural Sciences, 13*, 738-752.

Bencan, Z., Sledge, D., & Levin, E. D. (2009). Buspirone, chlordiazepoxide and diazepam effects in a zebrafish model of anxiety. *Pharmacology Biochemistry and Behavior, 94*, 75-80.

Best, J. D., & Alderton, W. K. (2008). Zebrafish: An in vivo model for the study of neurological diseases. *Neuropsychiatric Disease and Treatment, 4*, 567-576.

Bhorkar, A. A., Dandekar, M. P., Nakhate, K. T., Subhedar, N. K., & Kokare, D. M. (2014). Involvement of the central melanocortin system in the effects of caffeine on anxiety-like behavior in mice. *Life Sciences, 95*(2), 72-80.

Blaser, R., & Vira, D. (2014). Experiments on learning in zebrafish (Danio rerio): A promising model of neurocognitive function. *Neuroscience and Biobehavioral Reviews, 42,* 224-231.

Borsini, F., Podhorna, J., & Marazziti, D. (2002). Do animal models of anxiety predict anxiolytic-like effects of antidepressants? *Psychopharmacology, 163*(2), 121-141.

Brennan, C. H. (2011). Zebrafish behavioural assays of translational relevance for the study of psychiatric disease. *Reviews in the Neurosciences, 22,* 37-48. doi:10.1515/RNS.2011.006

Bruni, G., Rennekamp, A. J., Velenich, A., McCarroll, M., Gendelev, L., Fertsch, E., . . . Evron, T. (2016). Zebrafish behavioral profiling identifies multitarget antipsychotic-like compounds. *Nature Chemical Biology*.

Budney, A. J., Brown, P. C., Griffiths, R. R., Hughes, J. R., & Juliano, L. M. (2013). Caffeine Withdrawal and Dependence: A Convenience Survey Among Addiction Professionals. *Journal of caffeine research, 3*, 67-71. doi:10.1089/jcr.2013.0005

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., . . . Suciu, C. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols, 5*(11), 1786-1799.

Cachat, J., Stewart, A., Grossman, L., Gaikwad, S., Kadri, F., Chung, K. M., . . . Kalueff, A. V. (2010). Measuring behavioral and endocrine responses to novelty stress in adult zebrafish. *Nature Protocols, 5,* 1786-1799. doi:10.1038/nprot.2010.140

Cachat, J., Stewart, A., Utterback, E., Hart, P., Gaikwad, S., Wong, K., . . . Kalueff, A. V. (2011). Three-dimensional neurophenotyping of adult zebrafish behavior. *PloS One, 6*(3), e17597.

Cachat, J. M., Canavello, P. R., Elegante, M. F., Bartels, B. K., Elkhayat, S. I., Hart, P. C., . . . Kalueff, A. (2011). Modeling Stress and Anxiety in Zebrafish. In A. V. Kalueff & J. M. Cachat (Eds.), *Zebrafish Models in Neurobehavioral Research* (Vol. 52, pp. 73-88): Humana Press.

Cacioppo, J. T., Ernst, J. M., Burleson, M. H., McClintock, M. K., Malarkey, W. B., Hawkley, L. C., . . . Hugdahl, K. (2000). Lonely traits and concomitant physiological processes: the MacArthur social neuroscience studies. *International Journal of Psychophysiology, 35*(2), 143-154.

Cacioppo, J. T., & Hawkley, L. C. (2003). Social isolation and health, with an emphasis on underlying mechanisms. *Perspectives in Biology and Medicine, 46*(3), S39-S52.

Cacioppo, J. T., Hawkley, L. C., Crawford, L. E., Ernst, J. M., Burleson, M. H., Kowalewski, R. B., . . . Berntson, G. G. (2002). Loneliness and health: Potential mechanisms. *Psychosomatic Medicine, 64*(3), 407-417.

Canavello, P. R., Cachat, J. M., Beeson, E. C., Laffoon, A. L., Grimes, C., Haymore, W. A., . . . Elkhayat, S. I. (2011). Measuring endocrine (cortisol) responses of zebrafish to stress *Zebrafish neurobehavioral protocols* (pp. 135-142): Springer.

Champagne, D. L., Hoefnagels, C. C., de Kloet, R. E., & Richardson, M. K. (2010). Translating rodent behavioral repertoire to zebrafish (Danio rerio): relevance for stress research. *Behavioural Brain Research, 214*(2), 332-342.

Chatterjee, D., Tran, S., Shams, S., & Gerlai, R. (2015). A Simple Method for Immunohistochemical Staining of Zebrafish Brain Sections for c-fos Protein Expression. *Zebrafish, 12*(6), 414-420.

Collier, A. D., Khan, K. M., Caramillo, E. M., Mohn, R. S., & Echevarria, D. J. (2014). Zebrafish and conditioned place preference: a translational model of drug reward. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 55*, 16-25. doi:10.1016/j.pnpbp.2014.05.014

Cryan, J. F., & Holmes, A. (2005). The ascent of mouse: advances in modelling human depression and anxiety. *Nature reviews Drug discovery, 4*(9), 775-790.

De Martino, B., Kumaran, D., Seymour, B., & Dolan, R. J. (2006). Frames, biases, and rational decision-making in the human brain. *Science, 313*(5787), 684-687.

Duman, R. S., Heninger, G. R., & Nestler, E. J. (1994). Molecular Psychiatry Adaptations of Receptor-Coupled Signal Transduction Pathways Underlying Stress-and Drug-Induced Neural Plasticity. *The Journal of nervous and mental disease, 182*(12), 692-700.

Duncan, G. E., Knapp, D. J., & Breese, G. R. (1996). Neuroanatomical characterization of Fos induction in rat behavioral models of anxiety. *Brain Research, 713*(1), 79-91.

Echevarria, D. J., Toms, C. N., & Jouandot, D. J. (2011). Alcohol-induced behavior change in zebrafish models. *Reviews in the Neurosciences, 22*, 85-93. doi:10.1515/RNS.2011.010

Eddins, D., Petro, A., Williams, P., Cerutti, D. T., & Levin, E. D. (2009). Nicotine effects on learning in zebrafish: the role of dopaminergic systems. *Psychopharmacology, 202*(1-3), 103-109. doi:10.1007/s00213-008-1287-4

Egan, R. J., Bergner, C. L., Hart, P. C., Cachat, J. M., Canavello, P. R., Elegante, M. F., . . . Kalueff, A. V. (2009). Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. *Behavioural Brain Research, 205*, 38-44. doi:10.1016/j.bbr.2009.06.022

Eilam, D., Dank, M., & Maurer, R. (2003). Voles scale locomotion to the size of the open-field by adjusting the distance between stops: a possible link to path integration. *Behavioural Brain Research, 141*(1), 73-81.

Engeszer, R. E., Ryan, M. J., & Parichy, D. M. (2004). Learned social preference in zebrafish. *Current Biology, 14*(10), 881-884.

Erdtmann-Vourliotis, M., Mayer, P., Riechert, U., & Höllt, V. (1999). Acute injection of drugs with low addictive potential (Δ9-tetrahydrocannabinol, 3,4-methylenedioxymethamphetamine, lysergic acid diamide) causes a much higher c-fos expression in limbic brain areas than highly addicting drugs (cocaine and morphine). *Molecular Brain Research, 71*, 313-324. doi:10.1016/S0169-328X(99)00207-7

Etkin, A., & Wager, T. D. (2007). Functional neuroimaging of anxiety: a meta-analysis of emotional processing in PTSD, social anxiety disorder, and specific phobia. *American Journal of Psychiatry*.

Ferreira, S. E., De Mello, M. T., Pompéia, S., Souza-Formigoni, D., & Oliveira, M. L. (2006). Effects of energy drink ingestion on alcohol intoxication. *Alcoholism: Clinical and Experimental Research, 30*(4), 598-605.

File, S. E. (2001). Factors controlling measures of anxiety and responses to novelty in the mouse. *Behavioural Brain Research, 125*(1), 151-157.

Gebauer, D. L., Pagnussat, N., Piato, Â. L., Schaefer, I. C., Bonan, C. D., & Lara, D. R. (2011). Effects of anxiolytics in zebrafish: similarities and differences between benzodiazepines, buspirone and ethanol. *Pharmacology Biochemistry and Behavior, 99*(3), 480-486.

Gerlai, R. (2013). Antipredatory behavior of zebrafish: adaptive function and a tool for translational research. *Evolutionary psychology : an international journal of evolutionary approaches to psychology and behavior, 11*, 591-605.

Gerlai, R. (2014). Social behavior of zebrafish: From synthetic images to biological mechanisms of shoaling. *Journal of Neuroscience Methods*. doi:10.1016/j.jneumeth.2014.04.028

Gerlai, R. (2016). Learning and memory in zebrafish (Danio rerio). *Methods in Cell Biology*.

Gerlai, R., Lahav, M., Guo, S., & Rosenthal, a. (2000). Drinks like a fish: zebra fish (Danio rerio) as a behavior genetic model to study alcohol effects. *Pharmacology, biochemistry, and behavior, 67*, 773-782.

Gerlai, R., Lee, V., & Blaser, R. (2006). Effects of acute and chronic ethanol exposure on the behavior of adult zebrafish (Danio rerio). *Pharmacology, biochemistry, and behavior, 85*, 752-761. doi:10.1016/j.pbb.2006.11.010

Gingras, M. A., & Cools, A. R. (1996). Analysis of the biphasic locomotor response to ethanol in high and low responders to novelty: a study in Nijmegen Wistar rats. *Psychopharmacology, 125*(3), 258-264.

Goldsmith, P. (2004). Zebrafish as a pharmacological tool: the how, why and when. *Curr Opin Pharmacol, 4*(5), 504-512. doi:10.1016/j.coph.2004.04.005

Gustafsson, P., Greenberg, L. A., & Bergman, E. (2012). The influence of large wood on brown trout (Salmo trutta) behaviour and surface foraging. *Freshwater Biology, 57*(5), 1050-1059.

Heinz, A. J., de Wit, H., Lilje, T. C., & Kassel, J. D. (2013). The combined effects of alcohol, caffeine, and expectancies on subjective experience, impulsivity, and risk-taking. *Experimental and Clinical Psychopharmacology, 21*(3), 222.

Hoffman, G. E., Smith, M. S., & Verbalis, J. G. (1993). c-Fos and related immediate early gene products as markers of activity in neuroendocrine systems. *Frontiers in Neuroendocrinology, 14*, 173-213. doi:10.1006/frne.1993.1006

Howe, K., Clark, M. D., Torroja, C. F., Torrance, J., Berthelot, C., Muffato, M., . . . Stemple, D. L. (2013). The zebrafish reference genome sequence and its relationship to the human genome. *Nature, 496*, 498-503. doi:10.1038/nature12111

Isaacson, R. L. (1982). *Limbic system*: Wiley Online Library.

Jain, N., Hirani, K., & Chopde, C. (2005). Reversal of caffeine-induced anxiety by neurosteroid 3-alpha-hydroxy-5-alpha-pregnane-20-one in rats. *Neuropharmacology, 48*(5), 627-638.

Jesuthasan, S. (2012). Fear, anxiety, and control in the zebrafish. *Developmental Neurobiology, 72,* 395-403. doi:10.1002/dneu.20873

Kallai, J., Makany, T., Csatho, A., Karadi, K., Horvath, D., Kovacs-Labadi, B., . . . Jacobs, J. W. (2007). Cognitive and affective aspects of thigmotaxis strategy in humans. *Behavioral Neuroscience, 121*(1), 21.

Kalueff, A. V., Echevarria, D., & Stewart, A. M. (2014a). Gaining translational momentum: More zebrafish models for neuroscience research. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. doi:10.1016/j.pnpbp.2014.01.022

Kalueff, A. V., Echevarria, D. J., Homechaudhuri, S., Stewart, A. M., Collier, A. D., Kaluyeva, A. A., . . . Wang, J. (2016). Zebrafish neurobehavioral phenomics for aquatic neuropharmacology and toxicology research. *Aquatic Toxicology, 170*, 297-309.

Kalueff, A. V., Echevarria, D. J., & Stewart, A. M. (2014b). Gaining translational momentum: More zebrafish models for neuroscience research. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. doi:10.1016/j.pnpbp.2014.01.022

Kalueff, A. V., Keisala, T., Minasyan, A., Kuuslahti, M., & Tuohimaa, P. (2006). Temporal stability of novelty exploration in mice exposed to different open field tests. *Behavioural Processes, 72*(1), 104-112.

Kalueff, A. V., Stewart, A. M., & Gerlai, R. (2014). Zebrafish as an emerging model for studying complex brain disorders. *Trends in Pharmacological Sciences, 35*(2), 63-75.

Kalueff, A. V., Wheaton, M., & Murphy, D. L. (2007). What's wrong with my mouse model? Advances and strategies in animal modeling of anxiety and depression. *Behavioural Brain Research, 179*, 1-18. doi:10.1016/j.bbr.2007.01.023

Kauer, J. A., & Malenka, R. C. (2007). Synaptic plasticity and addiction. *Nature Reviews Neuroscience, 8*(11), 844-858.

Keifer, J., & Summers, C. H. (2016). Putting the "Biology" Back into "Neurobiology": The Strength of Diversity in Animal Model Systems for Neuroscience Research. *Frontiers in Systems Neuroscience, 10*, 69.

Kelley, A. E., & Berridge, K. C. (2002). The Neuroscience of Natural Rewards: Relevance to Addictive Drugs. *Journal of Neuroscience, 22*, 3306-3311.

Kempermann, G., Gast, D., & Gage, F. H. (2002). Neuroplasticity in old age: Sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Annals of Neurology, 52*(2), 135-143.

Kily, L. J. M., Cowe, Y. C. M., Hussain, O., Patel, S., McElwaine, S., Cotter, F. E., & Brennan, C. H. (2008). Gene expression changes in a zebrafish model of drug dependency suggest conservation of neuro-adaptation pathways. *The Journal of experimental biology, 211*, 1623-1634. doi:10.1242/jeb.014399

King, A. C., Houle, T., Wit, H., Holdstock, L., & Schuster, A. (2002). Biphasic alcohol response differs in heavy versus light drinkers. *Alcoholism: Clinical and Experimental Research, 26*(6), 827-835.

Klee, E. W., Ebbert, J. O., Schneider, H., Hurt, R. D., & Ekker, S. C. (2011). Zebrafish for the study of the biological effects of nicotine. *Nicotine & tobacco research :*

official journal of the Society for Research on Nicotine and Tobacco, 13, 301-312. doi:10.1093/ntr/ntr010

Kokel, D., & Peterson, R. T. (2008). Chemobehavioural phenomics and behaviour-based psychiatric drug discovery in the zebrafish. *Briefings in functional genomics & proteomics, 7*, 483-490. doi:10.1093/bfgp/eln040

Koob, G. F. (2009). Brain stress systems in the amygdala and addiction. *Brain Research, 1293*, 61-75.

Koob, G. F., & Nestler, E. J. (1997). The neurobiology of drug addiction. *The Journal of neuropsychiatry and clinical neurosciences, 9*, 482-497.

Kurt, M., Arik, A. C., & Celik, S. (2000). The effects of sertraline and fluoxetine on anxiety in the elevated plus-maze test. *Journal of Basic and Clinical Physiology and Pharmacology*, *11*(2), 173-180.

Ladu, F., Mwaffo, V., Li, J., Macrì, S., & Porfiri, M. (2015). Acute caffeine administration affects zebrafish response to a robotic stimulus. *Behavioural Brain Research, 289*, 48-54. doi:10.1016/j.bbr.2015.04.020

Larson, E. T., O'Malley, D. M., & Melloni, R. H. (2006). Aggression and vasotocin are associated with dominant–subordinate relationships in zebrafish. *Behavioural Brain Research, 167*(1), 94-102.

Lau, B. Y. B., Mathur, P., Gould, G. G., & Guo, S. (2011). Identification of a brain center whose activity discriminates a choice behavior in zebrafish. *Proceedings of the National Academy of Sciences of the United States of America, 108*, 2581-2586. doi:10.1073/pnas.1018275108

Lawrence, A. J., Cowen, M. S., Yang, H. J., Chen, F., & Oldfield, B. (2006). The orexin system regulates alcohol-seeking in rats. *British Journal of Pharmacology, 148*(6), 752-759.

Marczinski, C. A. (2011). Alcohol mixed with energy drinks: consumption patterns and motivations for use in US college students. *International Journal of Environmental Research and Public Health, 8*(8), 3232-3245.

Marczinski, C. A., & Fillmore, M. T. (2006). Clubgoers and their trendy cocktails: implications of mixing caffeine into alcohol on information processing and subjective reports of intoxication. *Experimental and Clinical Psychopharmacology, 14*(4), 450.

Mathur, P., Berberoglu, M. a., & Guo, S. (2011). Preference for ethanol in zebrafish following a single exposure. *Behavioural Brain Research, 217*, 128-133. doi:10.1016/j.bbr.2010.10.015

Mathur, P., & Guo, S. (2011). Differences of acute versus chronic ethanol exposure on anxiety-like behavioral responses in zebrafish. *Behavioural Brain Research, 219*, 234-239. doi:10.1016/j.bbr.2011.01.019

Maximino, C., de Brito, T. M., de Mattos Dias, C. A. G., Gouveia, A., & Morato, S. (2010). Scototaxis as anxiety-like behavior in fish. *Nature Protocols, 5*(2), 209-216.

McCammon, J. M., & Sive, H. (2015a). Addressing the Genetics of Human Mental Health Disorders in Model Organisms. *Annual review of genomics and human genetics, 16*, 173-197.

McCammon, J. M., & Sive, H. (2015b). Challenges in understanding psychiatric disorders and developing therapeutics: a role for zebrafish. *Disease Models and Mechanisms, 8*(7), 647-656.

Moore, T. O., June, H. L., & Lewis, M. J. (1993). Ethanol-induced stimulation and depression on measures of locomotor activity: effects of basal activity levels in rats. *Alcohol, 10*(6), 537-540.

Moreno, J. L., Holloway, T., Albizu, L., Sealfon, S. C., & González-Maeso, J. (2011). Metabotropic glutamate mGlu2 receptor is necessary for the pharmacological and behavioral effects induced by hallucinogenic 5-HT2A receptor agonists. *Neuroscience Letters, 493,* 76-79. doi:10.1016/j.neulet.2011.01.046

Näkki, R., Sharp, F. R., Sagar, S. M., & Honkaniemi, J. (1996). Effects of phencyclidine on immediate early gene expression in the brain. *Journal of Neuroscience Research, 45,* 13-27. doi:10.1002/(SICI)1097-4547(19960701)45:1<13::AID-JNR2>3.0.CO;2-K

Näslund, J., & Johnsson, J. I. (2014). Environmental enrichment for fish in captive environments: effects of physical structures and substrates. *Fish and Fisheries*.

Näslund, J., Rosengren, M., Del Villar, D., Gansel, L., Norrgård, J. R., Persson, L., . . . Kvingedal, E. (2013). Hatchery tank enrichment affects cortisol levels and shelter-seeking in Atlantic salmon (Salmo salar). *Canadian Journal of Fisheries and Aquatic Sciences, 70*(4), 585-590.

Nawrot, P., Jordan, S., Eastwood, J., Rotstein, J., Hugenholtz, A., & Feeley, M. (2003). Effects of caffeine on human health. *Food Additives and Contaminants, 20,* 1-30. doi:10.1080/0265203021000007840

Nestler, E. J. (2013). The origins of molecular psychiatry. *Journal of molecular psychiatry, 1*(3), 1-2.

Norton, W. H. J., Folchert, A., & Bally-Cuif, L. (2008). Comparative analysis of serotonin receptor (HTR1A/HTR1B families) and transporter (slc6a4a/b) gene expression in the zebrafish brain. *The Journal of comparative neurology, 511*, 521-542. doi:10.1002/cne.21831

O'Brien, M. C., McCoy, T. P., Rhodes, S. D., Wagoner, A., & Wolfson, M. (2008). Caffeinated cocktails: energy drink consumption, high-risk drinking, and alcohol-related consequences among college students. *Academic Emergency Medicine, 15*(5), 453-460.

Ogilvie, K. M., Lee, S., & Rivier, C. (1998). Divergence in the expression of molecular markers of neuronal activation in the parvocellular paraventricular nucleus of the hypothalamus evoked by alcohol administration via different routes. *Journal of Neuroscience, 18*(11), 4344-4352.

Oliveira, R. F., Silva, J. F., & Simoes, J. M. (2011). Fighting zebrafish: characterization of aggressive behavior and winner–loser effects. *Zebrafish, 8*(2), 73-81.

Parker, M. O., Millington, M. E., Combe, F. J., & Brennan, C. H. (2012). Housing conditions differentially affect physiological and behavioural stress responses of zebrafish, as well as the response to anxiolytics. *PloS One, 7*(4), e34992.

Pavlidis, M., Digka, N., Theodoridi, A., Campo, A., Barsakis, K., Skouradakis, G., . . . Tsalafouta, A. (2013). Husbandry of zebrafish, Danio rerio, and the cortisol stress response. *Zebrafish, 10*(4), 524-531.

Pavlidis, M., Theodoridi, A., & Tsalafouta, A. (2015). Neuroendocrine regulation of the stress response in adult zebrafish, Danio rerio. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*. doi:10.1016/j.pnpbp.2015.02.014

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open: closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *Journal of Neuroscience Methods, 14*(3), 149-167.

Pérez-Escudero, A., Vicente-Page, J., Hinz, R. C., Arganda, S., & de Polavieja, G. G. (2014). idTracker: tracking individuals in a group by automatic identification of unmarked animals. *Nature methods, 11*, 743-748. doi:10.1038/nmeth.2994

Qin, M., Wong, A., Seguin, D., & Gerlai, R. (2014). Induction of social behavior in zebrafish: live versus computer animated fish as stimuli. *Zebrafish, 11*(3), 185-197.

Rampon, C., Jiang, C. H., Dong, H., Tang, Y.-P., Lockhart, D. J., Schultz, P. G., . . . Hu, Y. (2000). Effects of environmental enrichment on gene expression in the brain. *Proceedings of the National Academy of Sciences, 97*(23), 12880-12884.

Rosemberg, D. B., Braga, M. M., Rico, E. P., Loss, C. M., Córdova, S. D., Mussulini, B. H. M., . . . Dias, R. D. (2012). Behavioral effects of taurine pretreatment in zebrafish acutely exposed to ethanol. *Neuropharmacology, 63*(4), 613-623.

Ruan, H., & Wu, C.-F. (2008). Social interaction-mediated lifespan extension of Drosophila Cu/Zn superoxide dismutase mutants. *Proceedings of the National Academy of Sciences, 105*(21), 7506-7510.

Sacks, J. J., Gonzales, K. R., Bouchery, E. E., Tomedi, L. E., & Brewer, R. D. (2015). 2010 national and state costs of excessive alcohol consumption. *American Journal of Preventive Medicine, 49*(5), e73-e79.

Salierno, J., Snyder, N., Murphy, A., Poli, M., Hall, S., Baden, D., & Kane, A. (2006). Harmful algal bloom toxins alter c-Fos protein expression in the brain of killifish, Fundulus heteroclitus. *Aquatic Toxicology, 78*(4), 350-357.

Santos, L. C., Ruiz-Oliveira, J., Oliveira, J. J., Silva, P. F., & Luchiari, A. C. (2016). Irish coffee: Effects of alcohol and caffeine on object discrimination in zebrafish. *Pharmacology Biochemistry and Behavior, 143*, 34-43.

Sapolsky, R. M., Alberts, S. C., & Altmann, J. (1997). Hypercortisolism associated with social subordinance or social isolation among wild baboons. *Archives of General Psychiatry, 54*(12), 1137-1143.

Saverino, C., & Gerlai, R. (2008). The social zebrafish: behavioral responses to conspecific, heterospecific, and computer animated fish. *Behavioural Brain Research, 191*(1), 77-87.

Schrijver, N. C., Bahr, N. I., Weiss, I. C., & Würbel, H. (2002). Dissociable effects of isolation rearing and environmental enrichment on exploration, spatial learning and HPA activity in adult rats. *Pharmacology Biochemistry and Behavior, 73*(1), 209-224.

Schroeder, P., Jones, S., Young, I. S., & Sneddon, L. U. (2014). What do zebrafish want? Impact of social grouping, dominance and gender on preference for enrichment. *Laboratory Animals*, 0023677214538239.

Shin, L. M., Rauch, S. L., & Pitman, R. K. (2006). Amygdala, medial prefrontal cortex, and hippocampal function in PTSD. *Annals of the New York Academy of Sciences, 1071*(1), 67-79.

Simon, M. R., & Mosher, J. F. (2007). *Alcohol, energy drinks, and youth: a dangerous mix*: Marin Institute.

Singewald, N., Salchner, P., & Sharp, T. (2003). Induction of c-Fos expression in specific areas of the fear circuitry in rat forebrain by anxiogenic drugs. *Biological Psychiatry, 53*(4), 275-283.

Snipes, D. J., & Benotsch, E. G. (2013). High-risk cocktails and high-risk sex: examining the relation between alcohol mixed with energy drink consumption, sexual behavior, and drug use in college students. *Addictive Behaviors, 38*(1), 1418-1423.

Somerville, L. H., Kim, H., Johnstone, T., Alexander, A. L., & Whalen, P. J. (2004). Human amygdala responses during presentation of happy and neutral faces: correlations with state anxiety. *Biological Psychiatry, 55*(9), 897-903.

Sousa, N., Almeida, O., & Wotjak, C. (2006). A hitchhiker's guide to behavioral analysis in laboratory rodents. *Genes, Brain and Behavior, 5*(s2), 5-24.

Sterling, M. E., Karatayev, O., Chang, G.-Q., Algava, D. B., & Leibowitz, S. F. (2014). Model of voluntary ethanol intake in zebrafish: Effect on behavior and hypothalamic orexigenic peptides. *Behavioural Brain Research*. doi:10.1016/j.bbr.2014.09.024

Stewart, A., Kadri, F., DiLeo, J., Min Chung, K., Cachat, J., Goodspeed, J., . . . Kalueff, A. V. (2010). The Developing Utility of Zebrafish in Modeling Neurobehavioral Disorders. *International Journal of Comparative Psychology, 23*.

Stewart, A., Riehl, R., Wong, K., Green, J., Cosgrove, J., Vollmer, K., . . . Kalueff, A. V. (2011). Behavioral effects of MDMA ('ecstasy') on adult zebrafish. *Behavioural Pharmacology, 22,* 275-280. doi:10.1097/FBP.0b013e328345f758

Stewart, A. M., Braubach, O., Spitsbergen, J., Gerlai, R., & Kalueff, A. V. (2014). Zebrafish models for translational neuroscience research: from tank to bedside. *Trends in Neurosciences, 37,* 264-278. doi:10.1016/j.tins.2014.02.011

Stewart, A. M., Gaikwad, S., Kyzar, E., & Kalueff, A. V. (2012). Understanding spatio-temporal strategies of adult zebrafish exploration in the open field test. *Brain Research, 1451,* 44-52. doi:10.1016/j.brainres.2012.02.064

Stewart, A. M., Ullmann, J. F. P., Norton, W. H. J., Parker, M. O., Brennan, C. H., Gerlai, R., & Kalueff, A. V. (2015). Molecular psychiatry of zebrafish. *Molecular Psychiatry, 20,* 2-17. doi:10.1038/mp.2014.128

Sudakov, S., Medvedeva, O., Rusakova, I., & Figurina, I. (2001). Effect of short-term and chronic caffeine intake on rats with various anxiety level. *Bulletin of Experimental Biology and Medicine, 132*(6), 1177-1179.

Tran, S., Chatterjee, D., & Gerlai, R. (2015). An integrative analysis of ethanol tolerance and withdrawal in zebrafish (Danio rerio). *Behavioural Brain Research, 276,* 161-170.

Tran, S., Facciol, A., & Gerlai, R. (2016). Alcohol-induced behavioral changes in zebrafish: The role of dopamine D2-like receptors. *Psychopharmacology,* 1-10.

Tran, S., Fulcher, N., Nowicki, M., Desai, P., Tsang, B., Facciol, A., . . . Gerlai, R. (2017). Time-dependent interacting effects of caffeine, diazepam, and ethanol on zebrafish behaviour. *Progress in Neuro-Psychopharmacology and Biological Psychiatry, 75*, 16-27.

Tran, S., & Gerlai, R. (2013). Time-course of behavioural changes induced by ethanol in zebrafish (Danio rerio). *Behavioural Brain Research, 252*, 204-213. doi:10.1016/j.bbr.2013.05.065

Treit, D., & Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacology Biochemistry and Behavior, 31*(4), 959-962.

Van Praag, H., Kempermann, G., & Gage, F. H. (2000). Neural consequences of enviromental enrichment. *Nature Reviews Neuroscience, 1*(3), 191-198.

von Krogh, K., Sørensen, C., Nilsson, G. E., & Øverli, Ø. (2010). Forebrain cell proliferation, behavior, and physiology of zebrafish, Danio rerio, kept in enriched or barren environments. *Physiology and Behavior, 101*(1), 32-39.

von Trotha, J. W., Vernier, P., & Bally-Cuif, L. (2014). Emotions and motivated behavior converge on an amygdala-like structure in the zebrafish. *The European journal of neuroscience*. doi:10.1111/ejn.12692

Wahlsten, D. (1990). Insensitivity of the analysis of variance to heredity-environment interaction. *Behavioral and Brain Sciences, 13*(01), 109-120.

Waldeck, B. (1974). Ethanol and caffeine: a complex interaction with respect to locomotor activity and central catecholamines. *Psychopharmacologia, 36*(3), 209-220.

Wang, Y., Li, S., Liu, W., Wang, F., Hu, L.-F., Zhong, Z.-m., . . . Liu, C.-F. (2016). Vesicular Monoamine Transporter 2 (Vmat2) knockdown elicits anxiety-like behavior in zebrafish. *Biochemical and Biophysical Research Communications*.

Wang, Y., Takai, R., Yoshioka, H., & Shirabe, K. (2006). Characterization and expression of serotonin transporter genes in zebrafish. *The Tohoku journal of experimental medicine, 208*, 267-274.

WHO. (2014). *Global status report on alcohol and health*. World Health Organization.

Winston, A. P. (2005). Neuropsychiatric effects of caffeine. *Advances in Psychiatric Treatment, 11*, 432-439. doi:10.1192/apt.11.6.432

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., . . . Kalueff, A. V. (2010a). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research, 208*(2), 450-457. doi:10.1016/j.bbr.2009.12.023

Wong, K., Elegante, M., Bartels, B., Elkhayat, S., Tien, D., Roy, S., . . . Kalueff, A. V. (2010b). Analyzing habituation responses to novelty in zebrafish (Danio rerio). *Behavioural Brain Research, 208*, 450-457. doi:10.1016/j.bbr.2009.12.023

Wong, K., Stewart, A., Gilder, T., Wu, N., Frank, K., Gaikwad, S., . . . Kalueff, A. V. (2010). Modeling seizure-related behavioral and endocrine phenotypes in adult zebrafish. *Brain Research, 1348*, 209-215. doi:10.1016/j.brainres.2010.06.012

Wulliman, M. F., Rupp, B., & Reichert, H. (2012). *Neuroanatomy of the zebrafish brain: a topological atlas*: Birkhäuser.

Wyatt, C., Bartoszek, E. M., & Yaksi, E. (2015). Methods for studying the zebrafish brain: past, present and future. *European Journal of Neuroscience, 42*(2), 1746-1763.

Yacoubi, M. E., Ledent, C., Ménard, J. F., Parmentier, M., Costentin, J., & Vaugeois, J. M. (2000). The stimulant effects of caffeine on locomotor behaviour in mice are mediated through its blockade of adenosine A2A receptors. *British Journal of Pharmacology, 129*(7), 1465-1473.

Yang, S., Kim, W., Choi, B. H., Koh, H. Y., & Lee, C. J. (2003). Alcohol impairs learning of T-maze task but not active avoidance task in zebrafish. *Korean Journal of Biological Sciences, 7*(4), 303-307.

Yeh, C.-M., Glöck, M., & Ryu, S. (2013). An optimized whole-body cortisol quantification method for assessing stress levels in larval zebrafish.

Young, D., Lawlor, P. A., Leone, P., Dragunow, M., & During, M. J. (1999). Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. *Nature Medicine, 5*(4), 448-453.

Zakhary, S. M., Ayubcha, D., Ansari, F., Kamran, K., Karim, M., Leheste, J. R., . . . Torres, G. (2011). A behavioral and molecular analysis of ketamine in zebrafish. *Synapse (New York, N.Y.), 65*, 160-167. doi:10.1002/syn.20830

Zhang, P., Hirsch, E. C., Damier, P., Duyckaerts, C., & Javoy-Agid, F. (1992). c-fos protein-like immunoreactivity: distribution in the human brain and over-expression in the hippocampus of patients with Alzheimer's disease. *Neuroscience, 46*, 9-21.