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USE OF A ZEBRAFISH MODEL TO IDENTIFY ANTICONVULSANT PROPERTIES OF CANNABINOID AND TERPONOID EXTRACTS AND MIXTURES

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The School of Renewable Natural Resources

by
Courtney Elizabeth Murr
B.S., Louisiana State University, 2018
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ABSTRACT

Epilepsy is a complex group of neurological disorders affecting approximately 65 million people worldwide. Animal models reveal the activation of cannabinoid receptor 1 and cannabinoid receptor 2 reduces the severity of seizures associated with epilepsy. Trials of phytocannabinoids in mammals demonstrate their efficacy of reducing the severity of epileptic seizures. Numerous terpenoid compounds are present in Cannabis plants but most have yet to be clinically investigated. The goal of this research was to use zebrafish (Danio rerio) to identify the potential anticonvulsive properties of phytocannabinoid and terpenoid compounds. During acute cannabinoid exposures, 7-days-post-fertilization (7 dpf) zebrafish larvae were exposed to either cannabidiol (CBD), cannabinol (CBN), tetrahydrocannabinol (Δ^9THC), or tetrahydrocannabivarin (THV) at concentrations of 0.125, 0.25, 0.5, or 1mg/L for 90 minutes. Epileptic-like seizures were tinduced by exposing larvae to a 10mM pentylenetetrazol (PTZ) solution for 10 minutes. After the PTZ incubation, movements were recorded for 10 minutes using a DanioVision™ recording chamber. The total distance moved and average velocity was measured using Ethovision XT™ software. Larvae exposed to phytocannabinoids had significantly reduced seizure behavior ($p < 0.05$) except for the 1mg/L THV treatment. The same methods and dosages were used for acute terpenoid exposures. Larvae exposed to myrcene and limonene had significantly reduced seizure behavior ($p < 0.05$) at all concentrations. For chronic exposures, larvae were exposed CBD or Δ^9THC at 0.0625mg/L or 0.125mg/L at 24-hours-post-fertilization. Solutions were renewed every 24 hours. At 7dpf, larvae were placed into a 48-well plate and exposed to a 10mM PTZ solution for 10 minutes. Larval movement was then recorded for 10 minutes. Zebrafish embryos exposed 0.0625mg/L or 0.125mg/L of CBD or Δ^9-THC for 168 hours did not see a reduction in seizure behavior. Our work has revealed an acute exposure

to selected phytocannabinoids significantly reduces seizure behavior in 7dpf larval zebrafish across a range of concentrations. A chronic exposure to CBD or Δ^9-THC did not yield a reduction in seizure behavior. This study indicates limonene and myrcene reduce seizure behaviors during an acute exposure. It is anticipated this research will support clinical research and use of phytocannabinoids and terpenoids increases in the future.

CHAPTER 1. INTRODUCTION

Epilepsy is a group of neurological disorders affecting approximately 65 million people worldwide characterized by reoccurring seizures due to abnormal electrical activity in the brain (Afrikanova et al. 2013). While the exact mechanisms of the disease are still largely unknown, there are currently over 50 described epileptic syndromes broadly categorized as idiopathic (genetic) or symptomatic (acquired). Idiopathic epilepsies comprise approximately 30% of all epileptic cases and are complex disorders determined by multiple genes. Defects associated with idiopathic epilepsies are most often described in and originate from potassium and sodium voltage-gated channels and ligand-gated channels involving the $GABA_A$ receptor (Berkovic et al. 2006). Symptomatic epilepsy can be caused by a number of environmental factors including head trauma, prenatal injury, stroke, lesions, and tumors. It is widely accepted that multiple factors, both genetic and acquired, interact to result in epileptic phenotypes (Berkovic et al. 2006).

Drugs that reduce the severity of epileptic seizures are available, however, there are currently no known compounds that prevent the development of this disorder. The primary neurotransmitter target of current antiepileptic drugs (AEDs) are the voltage-gated sodium and voltage gated calcium channels of the $GABA_A$ receptor. (Macdonald and Kelly 1993). AEDs are broadly categorized as broad spectrum, narrow spectrum or seizure specific (Sankaranei and Lachhwani 2015). Broad spectrum AEDs are desirable when patients have multiple seizure types or when the seizure-type in unclear (Sankaranei and Lachhwani 2015). While over 24 approved AEDs exist, many have significant adverse side effects (Rosenberg et al. 2015, Sankaranei and Lachhwani 2015). Common adverse side effects include nausea, weight gain, drowsiness, allergic rash, and menstrual cycle irregulates (Sankaranei and Lachhwani 2015). Additionally, in

the United States several AEDs have boxed warnings for rare life-threatening side effects including idiosyncratic fatal hepatic necrosis and Stevens-Johnson syndrome (Sankaranei and Lachhwani 2015). As such, there is increasing interest in discovering new therapeutics for the treatment of this disease (Rosenberg et al. 2015). Interest in the clinical use of phytocannabinoids for the treatment of neurological disorders has increased in recent years as animal models have revealed they have strong anticonvulsive properties (Rosenberg et al. 2015). Animal models show the activation of cannabinoid receptor 1 (CB_1R) and cannabinoid receptor 2 (CB_2R) reduces the severity of seizures associated with epilepsy (Rosenberg et al. 2015). Trials of cannabinoids in mammalian models have demonstrated the efficacy of cannabinoids in reducing the severity of epileptic seizures, preventing seizures, and reducing seizure-related mortality (Marsicano et al. 2003, Wallace et al. 2003, Rosenberg et al. 2015).

The genus Cannabis includes annual plants native to central Asia that have been cultivated and used for medicinal purposes for thousands of years. In recent years, cannabinoid compounds have been increasingly used for medical purposes as Cannabis plants and extracts are increasingly legalized in Europe and North America (Bonini et al. 2018). Cannabis contains over 120 organic compounds derived from the glandular trichomes of the plant known as phytocannabinoids. The two most prevalent phytocannabinoids in Cannabis plants are Δ^9-tetrahydrocannabinol (Δ^9THC) and Cannabidiol (CBD) and are currently of greatest interest for clinical use (Achenbach et al. 2018, Bonini et al. 2018). There is a present debate over whether the genus Cannabis is represented by one species (C. sativa) with two subspecies (C. sativa sativa, C. sativa indica), or whether there is sufficient genetic evidence to classify C. sativa and C. indica as two separate species (Elzinga et al. 2015). There is also debate over whether wild-type Cannabis plants could represent a third species (C. ruderalis) (Elzinga et al. 2015).

The endocannabinoid system (ES) is composed of endogenous cannabinoid compounds that bind to CBRs throughout the central (CNS) and peripheral nervous system (PNS) (Freitas et al. 2017). The primary function of the ES is to maintain homeostasis for bodily functions related sleep, appetite, mood, memory, reward processing, and brain-plasticity (Iseger and Bossong 2015, McPartland et al. 2015, Prud'homme et al 2015). The first endogenous cannabinoid neurotransmitters discovered were anandamide and 2-arachidonoylglycerol, which function as retrograde synaptic messengers. Both of these compounds activate Cannabinoid Receptor 1 (CB_1R) and Cannabinoid Receptor 2 (CB_2R). CB_1R are located primarily in CNS and peripheral neurons but are also expressed by immune cells. The primary function of CB_1R is to regulate the release of neurotransmitters that maintain homeostasis (Pertwee 2008). In the CNS, CB_1R cells are most concentrated at the terminals of neurons within the basal ganglia, cerebellum, hippocampus, and cerebral cortex (Iseger and Bossong 2015). CB_2R cells are located primarily within immune cells including splenocytes, macrophages, monocytes, B-cells and T-cells (McPartland et al. 2015, Pertwee 2008). The activation of CB_2R receptors alters the release of chemical messengers that regulate immune cell migration within the CNS and PNS (Pertwee 2008).

The primary psychoactive component of Cannabis plants is Δ^9THC which has a high affinity for both CB_1 and CB_2Rs in the brain (McPartland et al 2015). Animal models have revealed Δ^9THC has antinociceptive, anti-inflammatory, anti-convulsive, and antioxidant properties, that can relieve or delay progression of certain disorders, and reduce the adverse side effects of certain drug treatments. It has been used to relieve spasticity caused by multiple sclerosis (MS) and neuropathic pain in people with MS and certain cancers (Pertwee 2008). It is

also effective in treating nausea and reduced appetite caused by chemotherapy treatment. However, the therapeutic use of Δ^9THC is potentially limited by its psychoactive side effects. Common adverse effects include dysphoria, depersonalization, anxiety, panic attacks, and paranoia (Pertwee 2008). However, some studies suggest a development of tolerance by CBR agonists may be beneficial, as it could widen the drug's therapeutic potential by increasing tolerance to potential unwanted side effects (Pertwee 2008). Clinical research has revealed that psychological side effects of Δ^9THC tend to occur more frequently when administered in isolate, rather than with whole Cannabis products (McPartland et al 2015).

CBD is a non-psychoactive cannabinoid that constitutes up to 40% of Cannabis extracts (Scuderi et al 2009). Preclinical trials have demonstrated CBD has the potential to provide therapeutic treatment for numerous disorders while avoiding negative side effects and withdrawal symptoms associated with Δ^9THC and other psychotropic cannabinoids (Rosenberg et al 2015). CBD has a low affinity for CB_1 and CB_2Rs, however, CBD interacts with these receptors at low concentrations (Pertwee 2008). Therapeutic properties of CBD include anxiolytic, antidepressant, antipsychotic, anticonvulsant, anti-nausea, antioxidant, anti-inflammatory, antineoplastic, analgesic, and antiallergenic properties, appetite stimulation, neuroprotection, and decreased intraocular pressure (McPartland et al 2015, Scuderi et al 2009). CBD can act as an inverse agonist of CB_2R, which may contribute to its anti-inflammatory properties; CB_2R inverse agonism can inhibit immune cell migration, which reduces the clinical signs of inflammation (Pertwee 2008, Scuderi et al 2009).

CBD also has the potential to agonize and/or antagonize the effects Δ^9THC. CBD exerts a direct blockade of Δ^9THC, thus counteracting some of the actions of Δ^9THC (psychotropic effects), while also potentiating other actions (anti-convulsant activity, anxiolytic effects,

neuroprotection) (McPartland et al 2015, Scuderi et al 2009). Due to a lack of cognitive and psychoactive effects, CBD is a promising candidate for clinical use. Additionally, CBD is tolerated well by humans with low occurrences of adverse side effects and has low toxicity in humans and other species (Scuderi et al 2009). For example, mortality is achieved in Rhesus monkeys at 212 mg/kg when administered intravenously (Scuderi et al 2009). An oral LD_{50} has not been established, but Rosenkrantz et al. 1981 revealed an oral dose of CBD 20-50 times larger than the intravenous dose is required to induce severe intoxication. No significant effects on the CNS, vital signs, or mood have been observed at dosages of up to 1.5 g/day (Devinsky et al. 2014). Presently, there is limited data on the long-term usage of cannabinoids in humans and even less data on the pharmacokinetics and toxicity of cannabinoids in children (Devinsky et al 2014). There are few studies on potential drug interactions of cannabinoids, however laboratory experiments have revealed CBD may have immunosuppressant effects by inhibiting immune cell production and migration (Devinsky et al. 2014, Jensen et al. 2018). Currently, there are no studies that reveal teratogenic or mutagenic effects induced by CBD. In some epilepsy case studies, effects of CBD were considered almost equivalent to those induced by commonly used AEDs. Overall, CBD is considered the most promising candidate for antiepileptic treatments due to its powerful anticonvulsant properties, limited side effects, and low toxicity (Scuderi et al 2009).

Cannabinol (CBN) is a minor, mildly psychoactive constituent of Cannabis extracts (Karniol et al. 1975). It is most frequently found in Cannabis that has aged and/or been exposed to air or ultraviolet light for a prolonged period of time (Andre et al. 2016). CBN is formed as a metabolite of Δ^9THC formed through mono-oxygenation at allylic positions and acts as a partial agonist of CB_1 and CB_2R, with a higher affinity to CB_2R (Andre et al. 2016, McCallum et al.

1975). There currently has been little research into the potential therapeutic properties of CBN, however Chousidis et al. (2020) reported that CBN has both a stimulant and sedative effect on zebrafish larvae.

Discovered in 1970, Tetrahydrocannabivarin (Δ^9THCV or THV) is a C19 propyl-tailed analogue of Δ^9THC found mainly in the hashish of C. sativa cultivated in central Asia (Bonini et al. 2018, Pertwee 2008). Preclinical research in mice has indicated it produces psychotropic effects, but with a potency four to five times less than Δ^9THC. It is likely that THV activates CB_1Rs, but with less potency than Δ^9THC. THV binds to both CB_1 and CB_2R equally, and functions as partial agonist of CB_2R (Pertwee 2008). Studies have revealed a potential use for THV in the treatment of obesity, drug dependence, and drug withdrawal symptoms as it acts as an antagonist of CB_1R (McParland et al. 2015). However much like CBN, there has been little research into the therapeutic properties of THV.

Terpenes are naturally occurring organic compounds found in plants, animals, and microorganisms. Most organisms produce complex mixtures of terpenes which may act synergistically to produce a greater action than the equivalent amount of a single compound. There have been approximately 25,000 terpenoid structures identified but few have been intensively studied for clinical usage. Terpenes in plants function as defense mechanisms or attractants to other organisms. Drimane sesquiterpenes have strong antibacterial and antifungal properties. Many monoterpenes are toxic to insects, fungi, and bacteria and serve as feeding deterrents to insects and mammals (Gershenzon and Dudareva 2007). Terpenes may also have a role in facilitating chemical communication between plant species (Gershenzon and Dudareva 2007). Many monoterpenes and sesquiterpenes have a low molecular weight with high vapor pressures at room temperature and thus serve as good conveyors of information across long

distances (Gershenzon and Dudareva 2007). Additionally, terpenes are one of the major volatile compounds released from flowers and fruits which serve as attractants for pollinators (Gershenzon and Dudareva 2007). In Cannabis plants, most terpenes are found in resin produced within the flowers of the female plant (Booth et al. 2017). Cannabis resin contains a variety of monoterpenes and sesquiterpenes that produce the scent and flavor qualities associated with Cannabis products (Booth et al. 2017). The most abundant terpenoid compounds found in Cannabis plants are α-pinene, limonene, myrcene, β-pinene, terpinolene, β-ocimene, β-caryophyllene, α-humulene, bergamotene, and farnesene (Booth et al. 2017). Presently, there has been limited research on the effects terpenes may have on the medical qualities of Cannabis strains, and limited research on potential therapeutic properties of terpenoids in general.

Within the past two decades, zebrafish (Danio rerio) have become widely used as an experimental organism for drug screening, gene expression, vertebrate development, and human diseases (Howe et al. 2013, Zon and Peterson 2005). The zebrafish genome was fully sequenced by the National Institutes of Health Zebrafish Genome Initiative and was determined that 71.4% of human genes have a zebrafish orthologue; among these, 47% have a one-to-one relationship (Howe et al. 2013). High fecundity and small larval size make zebrafish models cost-effective alternatives to some mammalian models (i.e., rodents, dogs, pigs) (Howe et al. 2013). Female zebrafish can lay up to 200 eggs per clutch and the small transparent larvae are useful for high-throughput, whole-animal screens (Zon and Peterson 2005). Whole-animal screening is particularly advantageous for toxicity screening and disease phenotypes associated with pain, sedation, tumor development, and musculoskeletal and neurological disorders as compared to cultured-cell screens (MacRae and Peterson 2015). Additionally, transparent larvae are useful for visual analysis of early development of whole organ systems sacrificing the organism (Dooley

and Zon 2000, MacRae and Peterson 2015). Thousands of mutant zebrafish phenotypes that mimic human diseases have been developed since the sequencing of the zebrafish genome (Dooley and Zon 2000). Mutations that affect development, physiology, and behavior have provided researchers with information on gene function and the development of complex diseases (Zon and Peterson 2005). Thus, we have determined zebrafish were an appropriate model organism for this research.

The goal of this thesis was to use zebrafish as a model organism to identify the anticonvulsive properties of phytocannabinoids and terpenoids to provide insight for their potential use as anti-epileptic drugs. Specific objectives include using a zebrafish seizure model to test for changes in seizure behavior in the presence of 1) Acute cannabinoids 2) Acute terpenoids and 3) Chronic cannabinoids. The following hypotheses for this work include: 1) An acute exposure to phytocannabinod extracts will significantly reduce seizure activity in 7-day-old zebrafish larvae; 2) Acute exposure to selected terpenoid extracts will reduce seizure behavior in 7-day-old zebrafish larvae; and 3) CBD or Δ^9THC administered every 24 hours for a 168-hour period will significantly reduce seizure behavior in 7-day-old zebrafish larvae.

CHAPTER 2. ACUTE EXPOSURE OF PHYTOCANNABINOD EXTRACTS IN 7-DAY-OLD ZEBRAFISH LARVAE TO INVESTIGATE POTENTIAL ANTI-CONVULSANT ACTIVITY

2.1. Introduction

In recent decades there has been increasing interest in the use of Cannabis extracts for the treatment of epilepsy, particularly for pediatric use (Elliot et al. 2018). Many parents of children with treatment-resistant epilepsy have expressed interest in the use of Cannabis products. (Elliot et al. 2018). Epilepsy is considered treatment-resistant if seizures cannot be controlled after receiving two adequate trials of two or more anti-epileptic drugs (Kwan et al. 2010, Zaheer et al. 2018). Currently, one-third of people with epilepsy receiving treatment with conventional anti-epileptic drugs (AEDs) do not experience a reduction in seizure activity (Thomas and Cunningham 2018, Zaheer 2018). Anecdotal cases of children with treatment-resistant epilepsy reveal Cannabis-derived products improve seizure-control and provide additional sleep and behavioral benefits (Elliot et al. 2018, Perucca 2017). One survey of 75 children and adolescents with seizure disorders conducted in Colorado, United States, where medical and recreational use of Cannabis products is legal, revealed one-third of patients had a 50% reduction in seizure reduction after starting oral Cannabis therapy (Perucca 2017). Another report from the United States on the use of artisanal Cannabis products in 272 children and adults found a 50% reduction in seizure frequency in 55% of cases, with 10% experiencing a complete cessation of seizure activity (Perucca 2017). However, non-clinical data from these types of studies is often difficult to interpret because results are primarily based in uncontrolled observations (Elliott et al. 2018, Perucca 2017). Artisanal Cannabis-derived products are not subject to FDA regulation and thus often lack comprehensive quality-verification. Because of this, many of these products may have cannabinoid concentrations that vary from those stated on their labels (Perucca 2017).

Currently, there has been limited clinical research of the benefits and harms of Cannabis use for people with epilepsy (Elliot et al. 2018). This is likely due to the legal status of Cannabis products in many countries. During three Class I placebo-controlled trials of CBD products in patients with Lennox-Gastaut Syndrome (LGS) and Dravet Syndrome (DS), rare and severe forms of childhood-onset epilepsy, CBD was found to be more effective in reducing the frequency of seizures patients than the placebo (Perucca 2017). A double-blind phase 3 trial consisting of 120 children and adolescents with DS received either a 20mg/kg CBD oral solution or a placebo over a 4-week baseline period and a 14-week treatment period (Devinsky et al. 2017, Perucca et al. 2017). The average frequency of seizures decreased from an average of 12.4 to 5.9 in the CBD treatment group compared to 14.9 to 14.1 with placebo (Devinsky et al. 2017, Perucca et al. 2017). Patients treated with CBD (43%) had a 50% reduction in seizure frequency as compared to 27% of those with placebo. Only 5% of patients became seizure-free with CBD compared to 0% with placebo (Devinsky et al. 2017, Perucca et al. 2017). However, for these trials it was unclear whether seizure control was improved due to direct action from CBD or was the result of drug-interactions with other medications the patients were taking (Perucca 2017). Another double-blind phase 3 trial of CBD consisting of 171 children with LGS found 43.9% of participants in the CBD group experienced a reduction in seizure frequency as compared to 21.8% of the placebo group (Thiele et al. 2018). In June 2018, The United States Food and Drug Administration (FDA) approved Epidiolex®, a CBD-derived oral therapeutic for use in LGS and DS for patients above two years of age (Thomas and Cunningham 2018). Epidiolex® was the first FDA approved drug containing substances derived from Cannabis plants (Thomas and Cunningham 2018). This drug is markedly different from artisanal Cannabis products as it is subject to extensive quality reviews of content and purity (Thomas and Cunningham 2018).

Epidiolex® is 99% CBD in comparison to the nasal spray Sativex®, which contains 50% CBD and 50% Δ^9THC. Sativex® was developed and approved for use in people with Multiple Sclerosis (MS) in the United Kingdom in 2010 but not yet approved for usage in the United States (Lakhan and Rowland 2009). The FDA has since approved two other Cannabis-derived drugs to treat anorexia in patients with AIDS (dronabinol) and nausea associated with chemotherapy (nabilone). Dronabinol contains Δ^9THC, whereas nabilone contains a synthetic cannabinoid that acts like Δ^9THC (Thomas and Cunningham 2018).

Animal models have been used to study epilepsy for several decades and have revealed similarities between experimental seizures and phenotypes observed in patients (Stewart et al. 2012). Seizures can be induced chemically through the use of convulsant drugs (pentylenetetrazol, kainic acid, convulsant barbiturates) or by the use of genetically modified model organisms (Stewart et al. 2012). Rodents are the most widely used model species for understanding the mechanisms of epileptic seizures and actions of AEDs (Baxendale et al. 2012). In recent decades however, zebrafish have emerged as a robust organism for modeling human disease phenotypes, particularly for chemically induced models of epilepsy (Afrikanova et al. 2013 Baxendale et al. 2012). Zebrafish have several advantages over rodent and other mammalian models. They tend to be less costly and less labor intensive than rodent models, and their high fecundity allows for high-throughput analysis (Kalueff et al. 2014). A single pair of zebrafish can produce 200 embryos per week, potentially generating hundreds of thousands of larvae per-year (Afrikanova et al. 2013, Berghmans et al. 2007). Because of their small size, larvae can be housed in volumes as small as 50µl and require only microgram dosages of compounds per experiment (Afrikanova et al. 2013, Berghmans et al. 2007). Larvae develop

rapidly ex-utero making them an advantageous organism for modeling early development and gene expression (Kalueff et al. 2014).

The zebrafish PTZ-induced seizure model has been previously established for larvae at 7 days post-fertilization (dpf) to investigate potential anti-convulsant properties of select agents (Afrikanova et al. 2013, Baxendale et al. 2012, Gawel et al. 2020). Larvae exposed to PTZ exhibit hyperlocomotive seizure phenotypes and epileptic-like electrographic readings (Afrikanova et al. 2013, Gawel et al. 2020). Afrikanova et al. 2013 reported that 7dpf zebrafish larvae exposed to a 20mM solution of PTZ exhibited behavioral responses that correlated to changes in electrographic activity in the brain. Thus, the goal of this work seeks to utilize a zebrafish PTZ-induced seizure model to identify the potential anticonvulsant properties of selected Cannabis extracts.

2.2. Materials and methods

2.2.1. Fish Husbandry

Zebrafish were maintained according to Institutional Animal Care and Use Committee (IACUC) guidelines at Louisiana State University Agricultural Center under protocol numbers A2015-22 and A2018-25. Experimental procedures were reviewed and approved by the Louisiana State University Agricultural Center's IACUC under protocol number A2017-04. Sexually mature adult wild type (WT) zebrafish were obtained from Aquatic Research Organisms (Hampton, NH). Zebrafish, segregated by sex, were housed in a Pentair Aquatic Ecosystems laboratory animal housing aquatic habitat in a room maintained on a 13:11 light-dark cycle. A digitally controlled water heater maintained the aquarium temperature between 27-29°C. Fish were fed Otohime B2 (51% protein, 11% lipid; Marubeni Nisshin Feed Co., Tokyo, Japan) to apparent satiation once daily.

Newly fertilized zebrafish embryos were obtained by pairing adult zebrafish in a 1-L tank within 4 hours of the dark light cycle. Spawning occurred within the first hour of the next light cycle and newly fertilized embryos were collected via siphoning when embryos were approximately 6-hours post-fertilization (hpf).

2.2.2 Embryo Collection

Embryos (6 hpf) were housed in a petri dish containing 150mL of water obtained from the adult zebrafish system at a density of 150 per 150 mL. Petri dishes were kept in an incubation chamber at 27ºC until they were 7-days post-fertilization (dpf). One drop (~0.5 mg) of methylthioninium chloride was added to each dish immediately following collection to prevent fungal growth on the embryos. The final concertation of methylthioninium chloride was concentration of 3 mg/L. Methylthionium chloride was only utilized during the first 24 hours following embryo collection. Water was changed every 24 hours and any dead larvae were removed from the dishes.

2.2.3 Water Quality

Water quality assessments were performed weekly throughout the duration of this study. Alkalinity was measured using the Hach Company phenolphthalein and total method. Total hardness was measured using the EDTA method. Ammonia levels were recorded as Total Ammonia Nitrogen using the high range acid titration method. Nitrite was calculated using the ceric standard solution method. pH was measured using the Oakton pH 700 probe. Water quality assessements were performed between 5 June 2019 and 26 June 2019.

Table 2.1. Mean and Standard Error of the Mean (SEM) values for water quality parameters during the acute exposure to phytocannabinoids. Ammonia (total ammonia nitrogen), alkalinity, and nitrite are reported as mg/L.

	AMMONIA N = 4	ALKALINITY N = 4	NITRITE N = 4	pH N = 4
MEAN	0.015	256.25	0.1825	8.185
SEM	0.006	50.0	0.003	0.04113

2.2.4 Chemical Preparation

Pentylenetetrazol was obtained from Sigma-Aldrich (St. Louis, MO) and stored at -15°C. All phytocannabinoid extracts were obtained from Cerilliant Corporation (Round Rock, TX) and stored at room temperature. Extracts were intended to serve as analytical standard and consisted of 1.0 mg/L of the specific cannabinoid in 1 mL of methanol. Ultra-high purity nitrogen gas was used to evaporate and reconstitute the extracts in 1 mL dimethyl sulfoxide (DMSO). Additionally, DMSO was added to the 10mM PTZ solution achieve a final nominal concentration of 1%. DMSO was selected as the carrier for these compounds due to its low toxicity to aquatic organisms, its effectiveness as an organic solvent, and its facilitation of absorption across the gill membranes (Kais 2013).

2.2.5 Acute Exposure to Phytocannabinoid Extracts

Samples sizes for treatment cannabinoid, PTZ control, and negative control groups were represented by 16 individuals for each treatment concentration. Larvae in the negative control treatment group were not exposed to cannabinoids, PTZ, or DMSO. Seven dpf zebrafish were exposed to cannabidiol (CBD), cannabinol (CBN), tetrahydrocannabinol (Δ^9-THC), or tetrahydrocannabivarin (THV) at nominal concentrations of 0.125, 0.25, 0.5, or 1 mg/L for 90

minutes. Cannabinoid concentrations were based on previous range finding experiments and represent the lowest potentially effective concentrations. Epileptic-like seizures were then induced by exposing larvae to a 10mM pentylenetetrazol (PTZ) solution for 10 min. After the 90-min cannabinoid incubation and 10 min PTZ exposure, larvae were individually placed into wells of a 48-well plate, and movements were recorded for 10 minutes using a DanioVision™ (Noldus Information Technology, Leesburg, VA) recording chamber. All incubations and the 10-minute recording period were done in the dark. During the 10-minute observation period the total distance moved (mm) and average velocity (mm/s) was recorded at a frame rate Of 30 samples per-second using Ethovision XT™ software (Version 14.0, Noldus Information Technology, Leesburg, VA).

2.2.6 Statistical Analyses

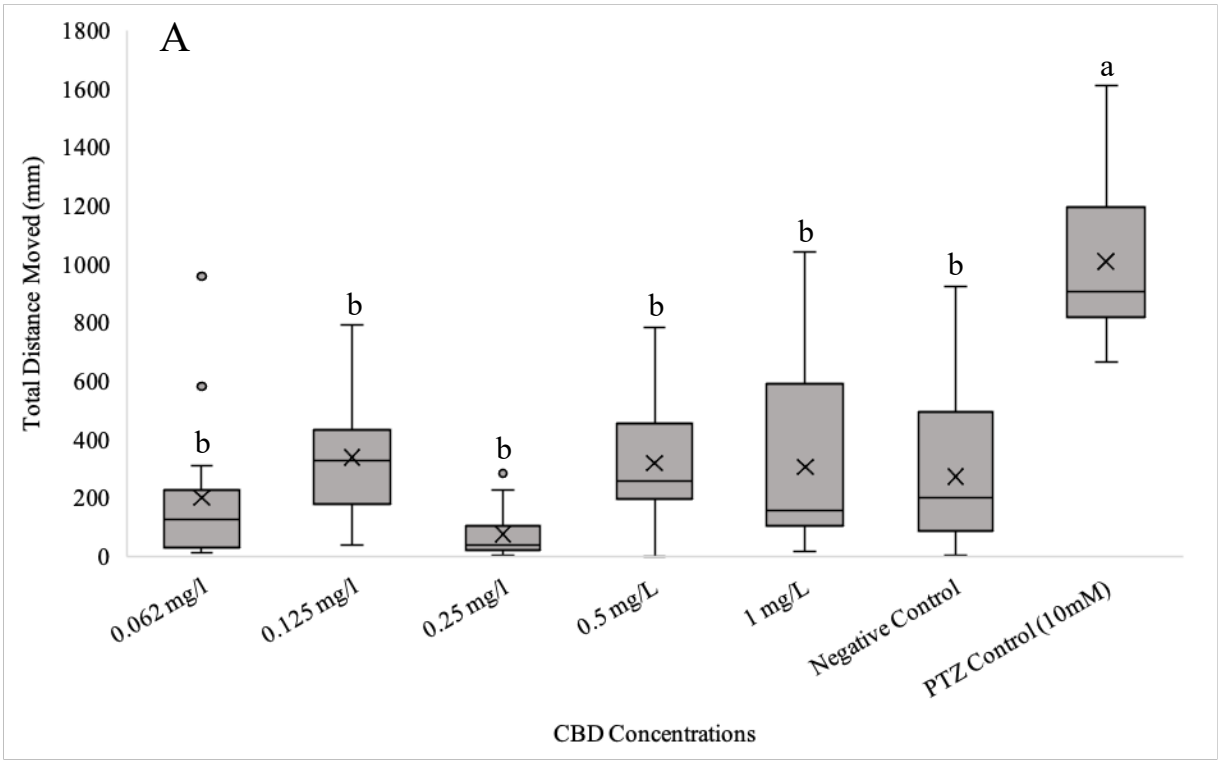
After the 10-min recording period, tracks obtained by the Ethovision XT software were reviewed to ensure there were no errors in the tracking progress. The most common tracking error occurred when the larvae did not move for an extended period of time. When this occured, the software attempts to locate movement and thus has the potential to track movement where there is none. This is particularly an issue when the larva is not moving, and the software detects movements from the reflection of a larva in an adjacent well. Tracks in each individual well were manually reviewed to ensure any false movements were deleted.

Distance moved and velocity recorded during the observation period were compared among concentrations and control treatment for each cannabinoid using one-way analysis of variance (ANOVA). The Ryan-Einot-Gabriel-Welsch (REGWQ) post-hoc test was used for pairwise comparisons of concentrations and controls within each cannabinoid exposure. All

hypotheses were tested at a significance level of α=0.05 and all tests were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC).

2.3. Results

For this model, a significant reduction in total distance moved and velocity indicates a reduction in PTZ-induced seizure behavior. Significant differences in total distance moved were detected for larvae exposed to PTZ after CBD incubations ($F_{6,102} = 13.72$, $p < 0.01$). PTZ control larvae demonstrated greater distance moved over the 10-minute observation period. Total distance moved was not significantly different between CBD treated and the negative control larvae (Figure 2.1A). Negative control larvae were not treated with cannabinoids, PTZ, or DMSO. Mean velocity of CBD incubated larvae was significantly reduced compared to PTZ control larvae ($F_{6,103} = 13.68$, $p < 0.01$). Mean velocity was not significantly different among CBD treated and control larvae (Figure 2.1B)



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(Fig. Cont'd)

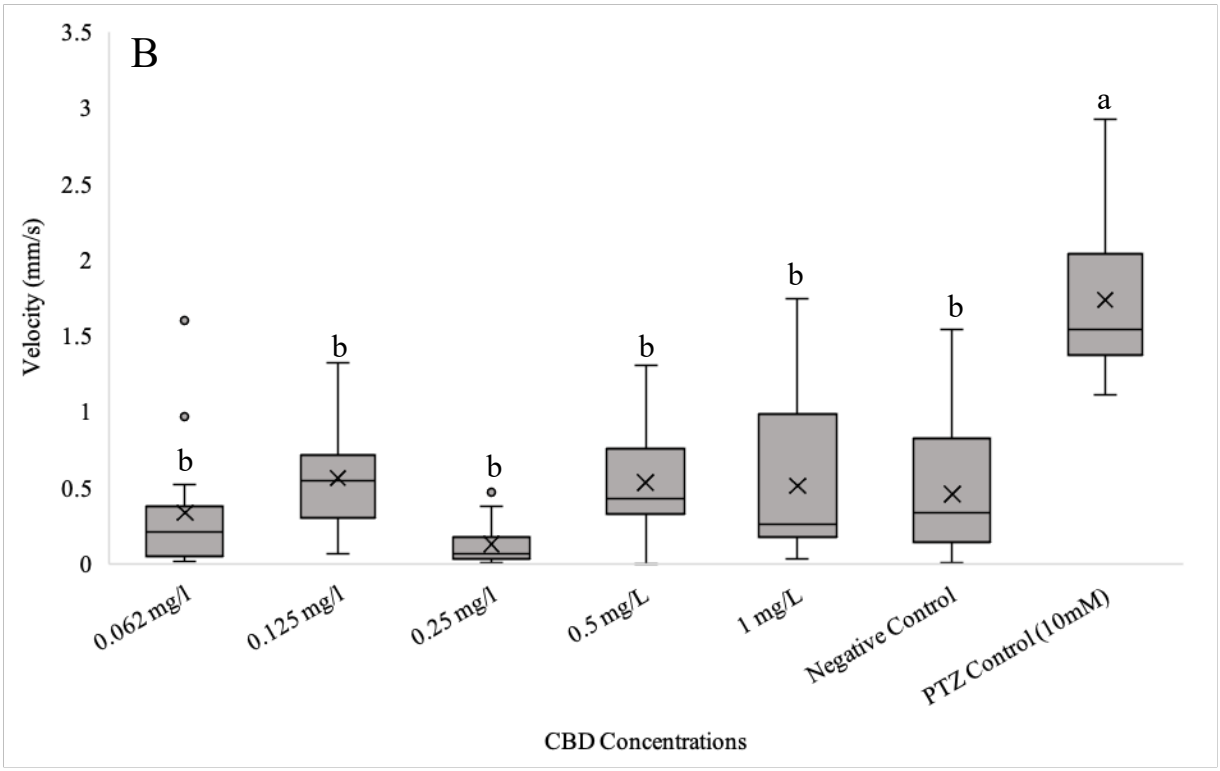
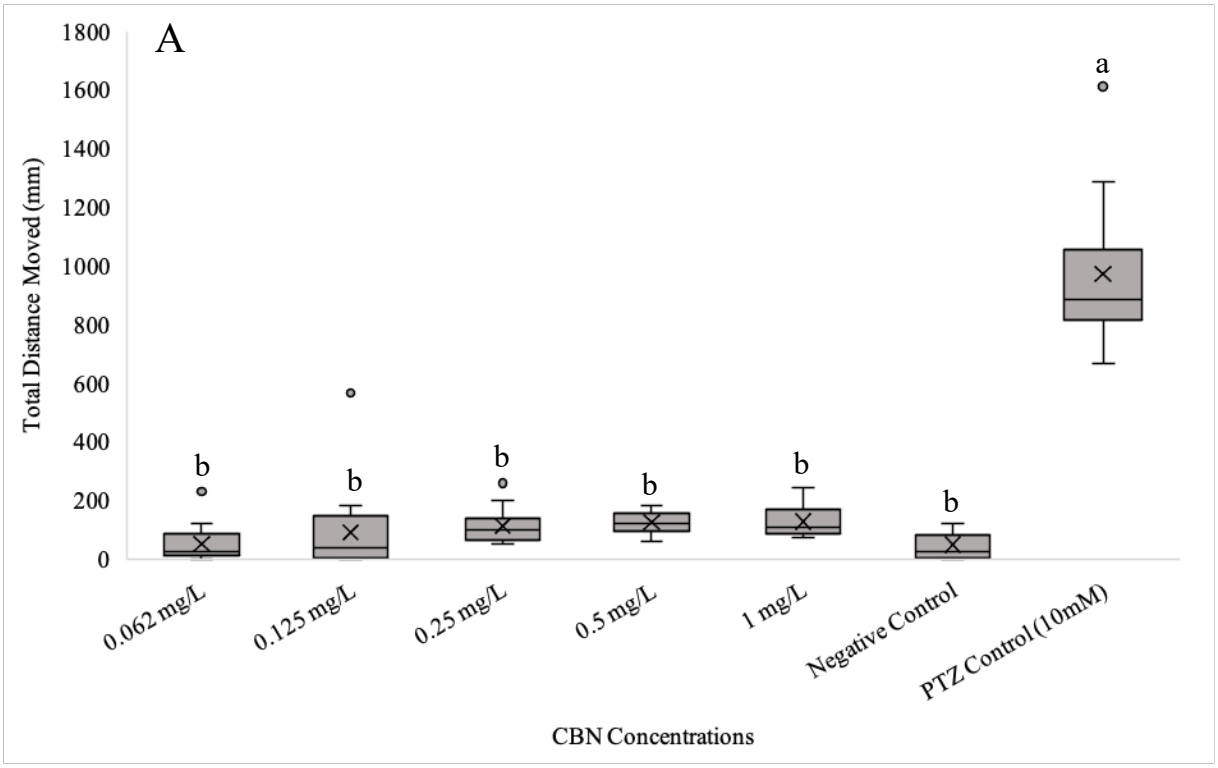


Figure 2.1. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and CBD. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p < 0.05$). N = 16.

Larvae exposed to CBN exhibited significantly reduced distance moved ($F_{6,109} = 53.67$, $p < 0.01$) than larvae exposed to PTZ after the 10-minute observation period. Total distance moved was not significantly different between CBN treated and negative control larvae (Figure 2.2A). Mean velocity of larvae exposed to CBN was significantly less than PTZ-exposed larvae ($F_{6,109} = 53.68$, $p < 0.01$). Mean velocity was not significantly different among CBN treated and control larvae (Figure 2.2B).



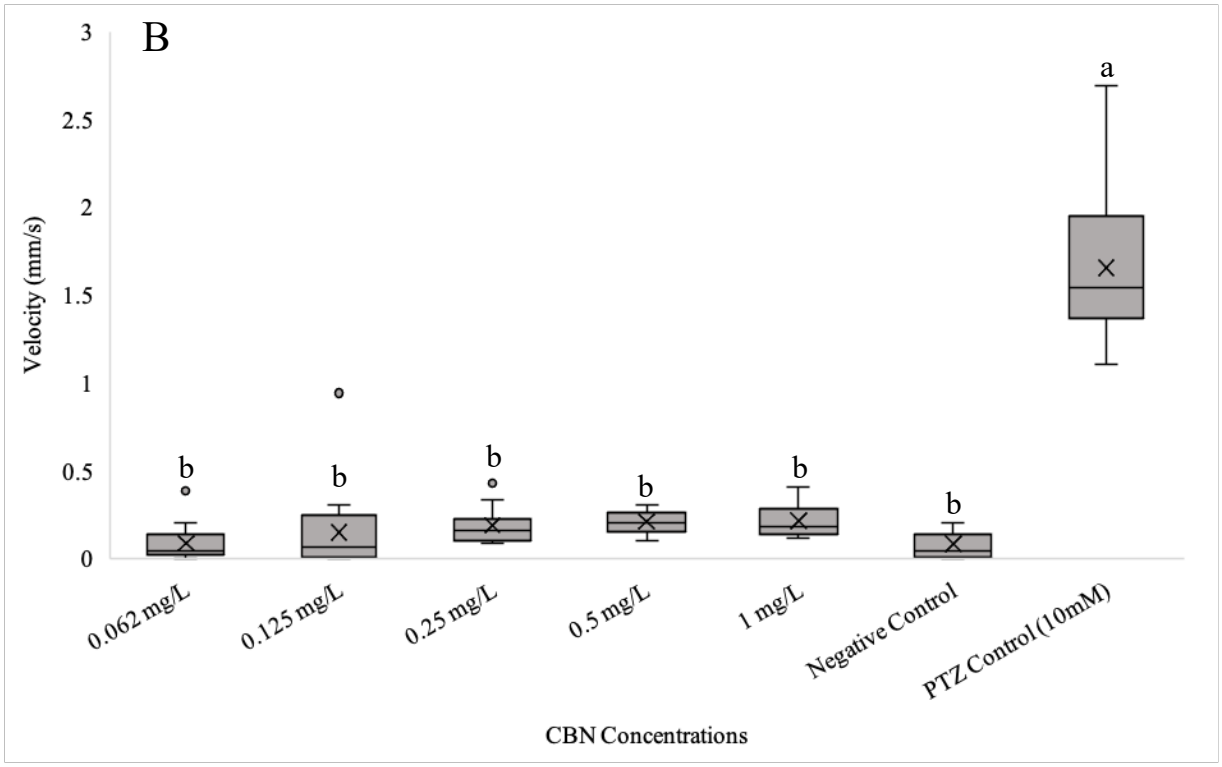
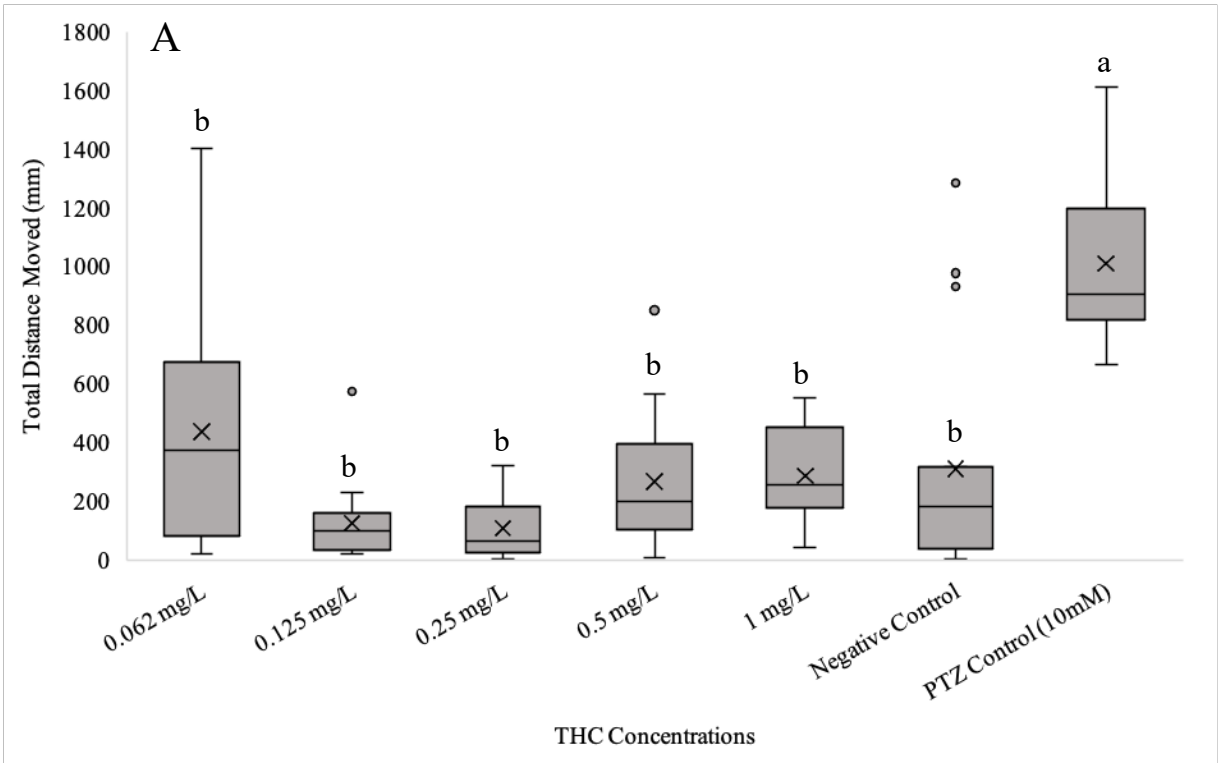


Figure 2.2. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and CBN. Box plots demonstrate median (line within box), 25,

and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$). N = 16.

Larvae exposed to Δ^9THC exhibited significant differences in total distance moved (2.3A) ($F_{6,110} = 2.02$, $p = < 0.01$) velocity (2.3B) ($F_{6,110} = 7.20$, $p < 0.01$) when compared to the PTZ control group across all concentrations. Larvae exposed to Δ^9THC were not significantly different from the negative control group across all concentrations.



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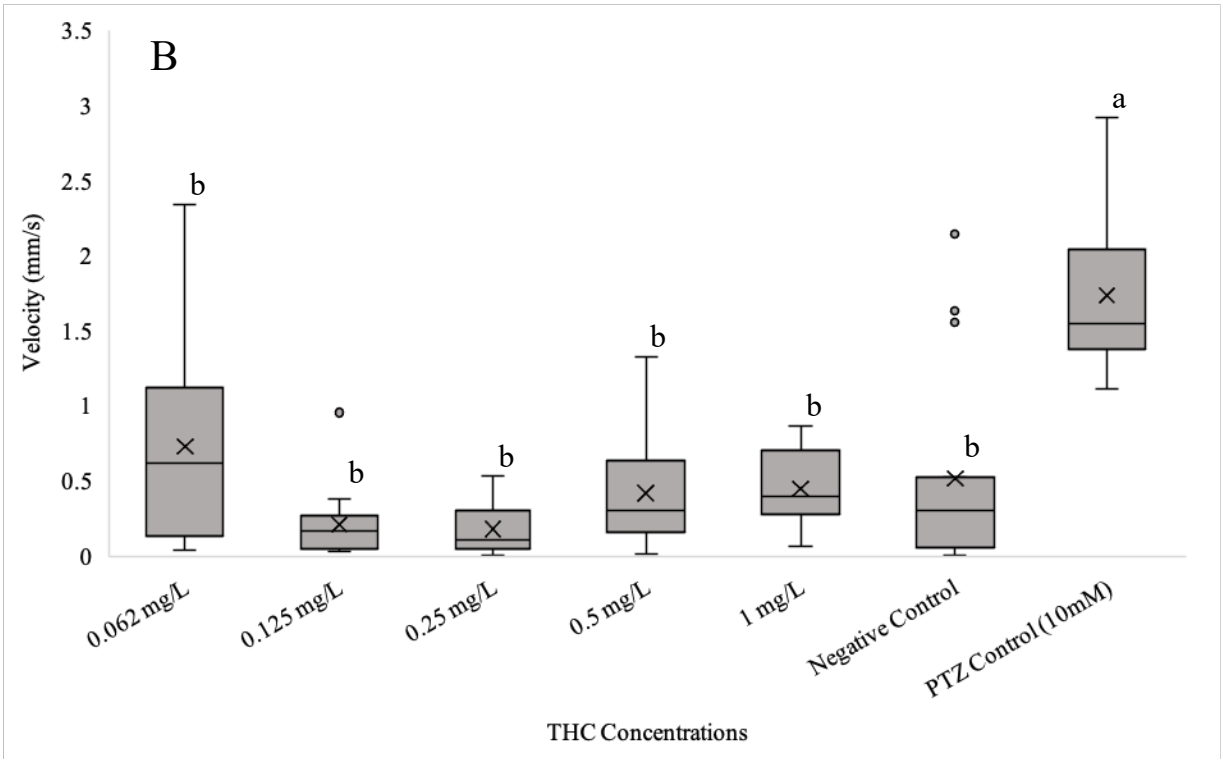
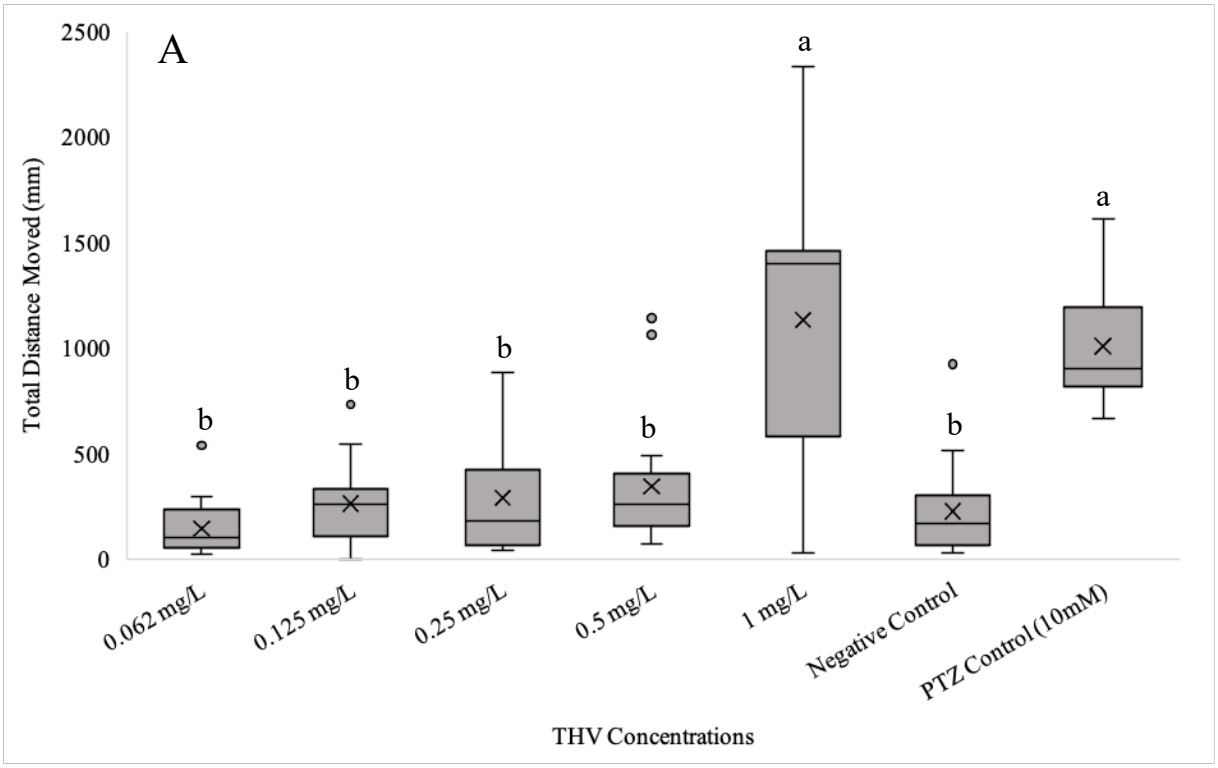


Figure 2.3. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and Δ^9THC. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$). N = 16.

Larvae exposed to THV at 0.125, 0.25, and 0.5 mg/L exhibited significantly reduced total distance moved ($F_{6,110} = 18.57$, $p < 0.01$) and velocity ($F_{6,110} = 18.57$, $p < 0.01$). Larvae at these concentrations were not significantly different from the negative control group. The 1 mg/L group of THV exhibited behavior that was not significantly different from the PTZ control group and total distance moved and average velocity were significantly higher than the negative control group.



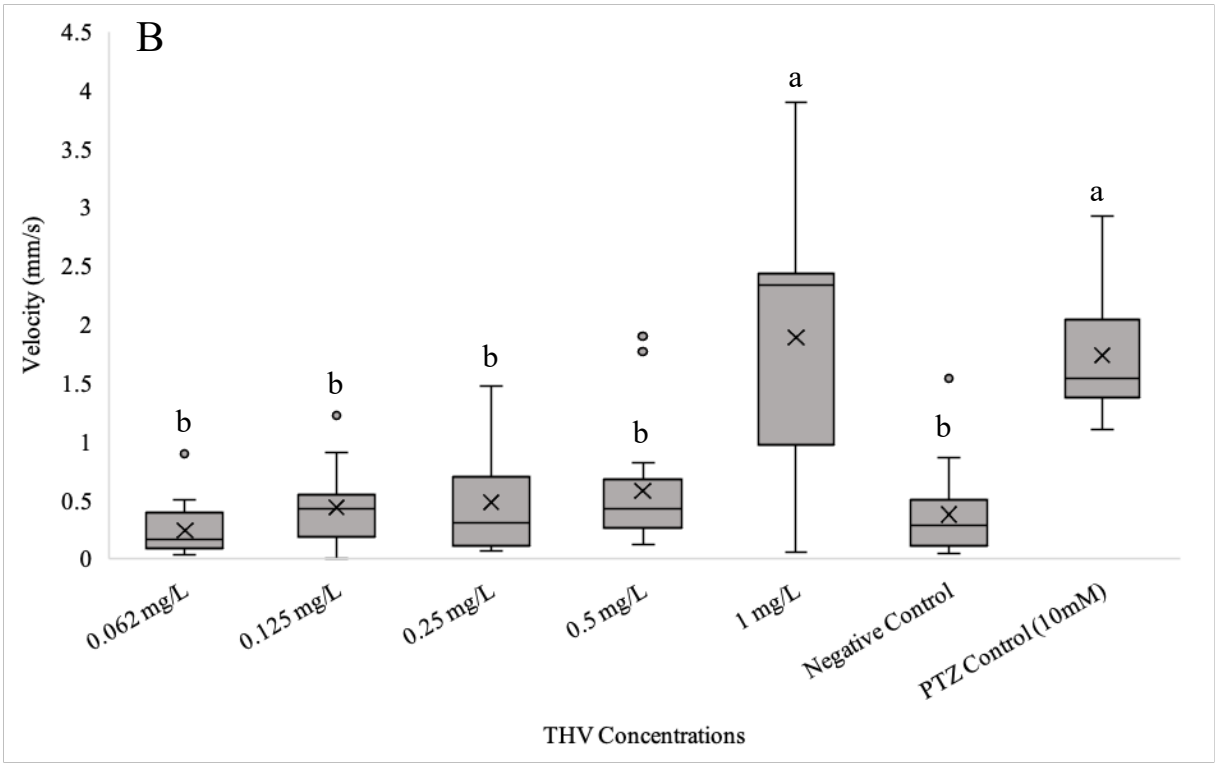
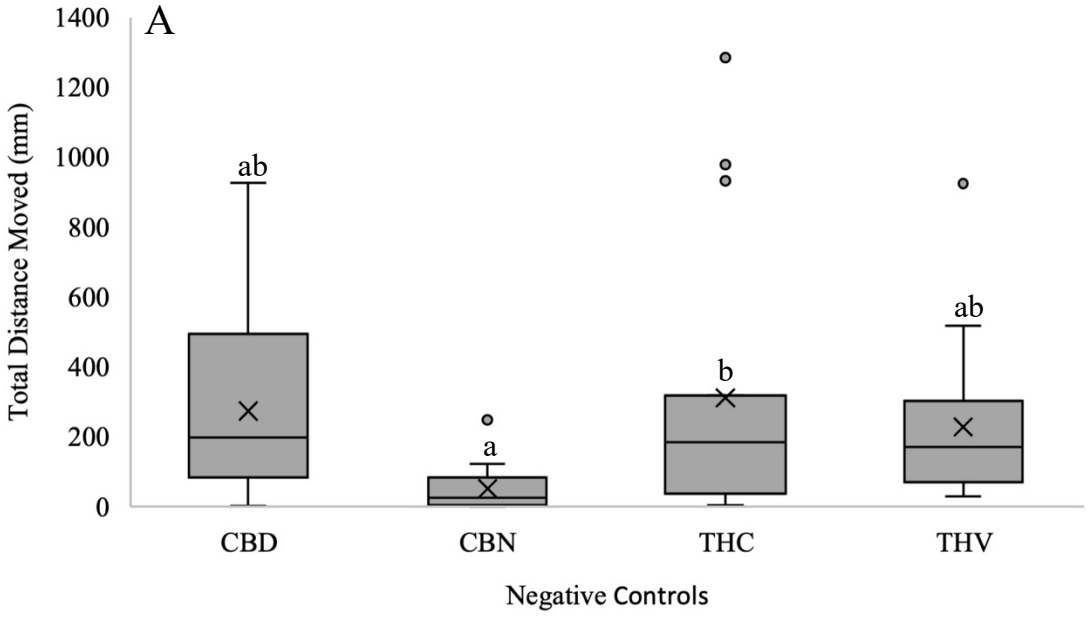


Figure 2.4. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and THV. Box plots demonstrate median (line within box), 25,

and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$). N = 16.

Comparison of negative control treatments among cannabinoid experiments detected marginally significant differences ($F_{3,51} = 2.74$, $p = 0.0517$). Specifically, post hoc analysis among negative controls indicated negative control treatments performed for THC and CBN were different (Figure 2.5A). When comparing movements of each cannabinoid from the lowest concentration treatment, significant differences in total distance moved were detected for THC incubations (Figure 2.5B; $F_{3,59} = 7.15$, $p < 0.01$)



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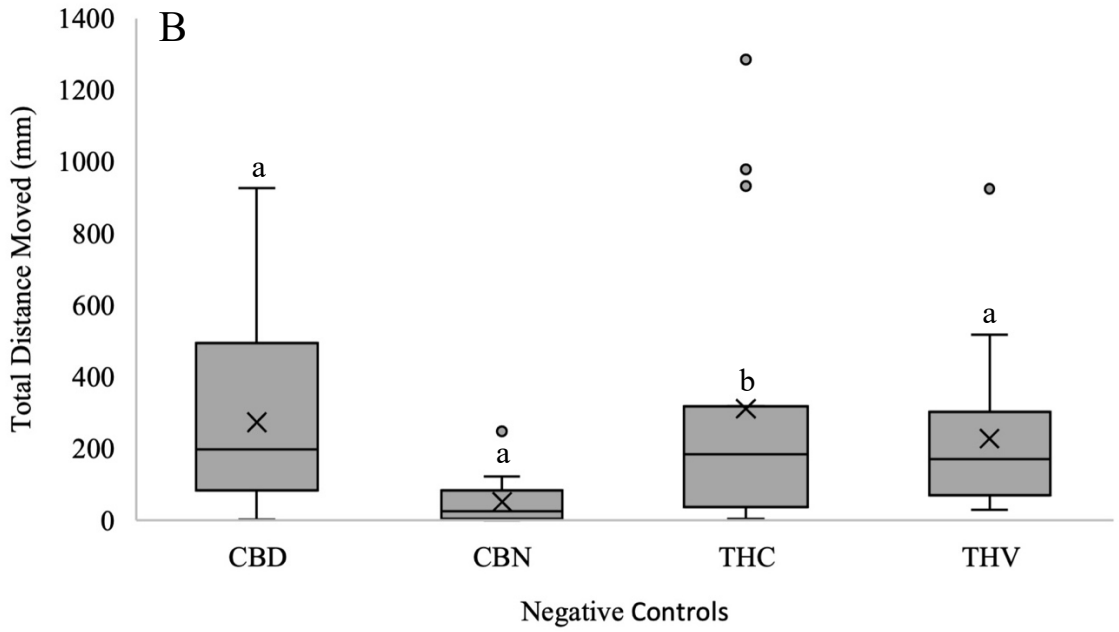


Figure 2.5 Total distance moved among negative control (A) and lowest cannabinoid treatments (B) 7dpf zebrafish larvae. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p \leq 0.05$).

2.4. Discussion

Zebrafish larvae exposed to phytocannabinoids prior to a chemically induced seizure had significantly reduced seizure behavior except for the highest concentration (1 mg/L) of the THV treatment group. A majority of our findings were consistent with those found in other chemically induced zebrafish seizure models of THC, CBD, and CBN (Ellis et al. 2018, Thornton et al. 2020). Furthermore, the cannabinoid treatments and concentrations in the current study did not appear to reduce movement metrics from control conditions. However, Thornton et al. (2020) reported zebrafish larvae exposed to 4 μM (1.25 mg/L) THC exhibited a significant reduction in seizure behavior but was likely because of a sedative effect rather than antiseizure activity. Ellis et al. (2018) reported similar findings; Δ^9-THC had little to no effect other than sedation. Our

results do not indicate a sedative effect, as none of our concentrations were significantly different from the negative control.

CBD reduces seizure behavior in rodents exposed acutely to maximal electroshock, PTZ, 3-mercaptopropionic acid, picrotoxin, cobalt, cocaine, penicillin, and through the induction of audiogenic seizures (Devinsky et al. 2014 Gobria et al .2015, Jones et al. 2012 Perucca 2017). Similar results are observed in zebrafish models of epilepsy. Griffin et al. (2020) observed reduced epileptiform behavior in 5dpf scn1lab homozygous mutant zebrafish larvae exposed to five different synthetic cannabinoids for 10 minutes at 1µM (0.31 mg/L), 10µM (3.1 mg/L) and 100µM (31.4 mg/L). Thorton et al. (2020) found CBD and Δ^9THC significantly reduced total distance moved in both 5-6 dpf wild-type PTZ treated and 5-6 dpf scn1lab homozygous mutant zebrafish larvae when exposed for 15 minutes with 100% light.

The results demonstrate phytocannabinoids are effective at reducing seizure behavior at relatively low dosages across a gradient of 5 concentrations. As a result, this model could be useful as a high-throughput screening tool for higher vertebrate models providing potential recommendations for future research of isolated cannabinoids or complex extracts. The recommended standard dosage of Epidiolex® (99% CBD) for the treatment of LGS and DS in patients with mild hepatic impairment is 10 mg/kg/day and up to a maximum of 20 mg/kg/day administered orally. Recommended dosage for those with moderate hepatic impairment is 5 mg/kg/day to a maximum of 10 mg/kg/day. For those with severe hepatic impairment, the recommended dosage is 2 mg/kg/day with a maximum dose of 4 mg/kg/day (Greenwich Biosciences Inc., Carlsbad, CA). The recommended dosage for Sativex® is between 4-8 nasal sprays per day. Each 100 µl spray Sativex®contains 2.7 mg Δ^9THC and 2.5 mg CBD (GW Biosciences, Cambridge, UK). The current study revealed Δ^9-THC and CBD decreased PTZ-

induced seizure activity. could provide further refinement for potential dosages in higher vertebrate models.

There is currently little information on the uptake, kinetics, and metabolism of cannabinoids by zebrafish larvae (Achenbach et al. 2018). Park et al. (2020) indicates the route of administration has an effect on the number and types of metabolites found in zebrafish larvae. In this study, larvae administered a synthetic cannabinoid through microinjection revealed metabolites found in zebrafish larvae had a higher similarity to metabolites found in human urine samples when compared to those larvae exposed to the compound in the water (Park et al. 2020). Therefore, route of administration is something to consider for a zebrafish cannabinoid model.

Investigation into the anticonvulsant properties of THV revealed that larvae in the 1 mg/L treatment group exhibited a hyperlocomotive phenotype and this treatment group was not statistically different than larvae exposed to 10mM PTZ. Some studies reveal that Δ^9THC can induce hyperlocomotive effects when administered at high concentrations (Achenbach et al. 2018, Devinsky et al. 2014). In a dog model, acute exposure to a sub-convulsant dosage of penicillin and smoked Cannabis produced muscular jerks and chronic exposures produced colonic-type seizures in the occipital and frontal cortices (Devinsky et al. 2014). The authors determined Δ^9THC had either reduced the seizure threshold or increased the permeability of the blood-brain barrier (Devinsky et al. 2014). Studies in seizure-prone rabbits and rodents found Δ^9THC produced epileptiform phenotypes (Devinsky et al. 2014). These studies suggest that in some cases Δ^9THC when administered in isolation may not produce antiseizure effects, but rather potentiate them (Devinksy et al. 2014). Since the THV molecule is structurally similar to Δ^9THC, this may explain the hyperlocomotive phenotype exhibited by the 1 mg/L treatment group in the current study. Future testing of THV will be necessary to verify this

hyperlocomotive behavior. To our knowledge, there are currently no studies investigating the anticonvulsant properties of THV.

Improvements to the model in the current study could include the elimination of the velocity analysis. Total distance moved and velocity are colinear as velocity is calculated from total distance divided by the total observation period (CBD: $R^2 = 0.99$; $F_{7,101} = 12601.9$, $p < 0.01$). Although there does not appear to be a clear dose-response relationship across the gradient of cannabinoid concentrations for our results, a dose response analysis could be useful for future work utilizing this model. The lowest concentration of THC in the current study (0.062 mg/L) appears to demonstrate a value with diminished anti-seizure properties, however the other cannabinoids did not reach lower limits of effectiveness. Further work with lower concentrations will be needed to elucidate these lower limits and were employed in following chapter. The inclusion of a negative control allowed for distinguishing reduced seizure behavior from any potential hypolocomotive behavior induced by the phytocannabinoids. It has been documented that phytocannabinoids (most notably Δ^9-THC) can induce hypolocomotive behavior in zebrafish larvae, mammalian models, and humans (Stewart and Kalueff 2014). The use of movement metrics for screening potential anti-convulsant compounds within this larval zebrafish model holds promise for future development of anti-seizure drugs.

CHAPTER 3. ACUTE EXPOSURE TO MAJOR TERPENOID COMPOUNDS PRESENT IN CANNABIS SPP. TO INVESTIGATE POTENTIAL ANTI-CONVULSANT ACTIVITY

3.1. Introduction

Currently, Δ^9THC, CBD, and other phytocannabinoids have been the primary focus of Cannabis research for clinical use (Russo 2011). Within the past decade there has been increasing interest in terpenoid compounds present in Cannabis plants (Erickson 2019, Russo 2011). Over 200 terpenoids have been identified in Cannabis plants but relatively little clinical or peer-reviewed research has been conducted on their pharmacology and potential use as therapeutants (Russo 2011). Interest in the medicinal properties of terpenoids in Cannabis resin has led many artisanal Cannabis product manufacturers to experiment with terpene profiles in their products (Erickson 2019). Terpenoid composition in Cannabis resin varies, often substantially so, based on genetic, environmental, and developmental factors (Booth 2017). While phytocannabinoid concentrations are generally predictable in Cannabis strains, terpenoid concentrations are often unknown or inconsistent (Booth 2017). Studies have indicated Cannabis plants with the same genetic makeup may have differing terpenoid composition based on variation in soil nutrient composition, temperature, growing medium, and exposure to ultraviolet light (Erickson 2019, Russo 2011). Thus, Cannabis strains sold under the same name may have differing terpenoid profiles based on the environment in which they are grown (Erickson 2019). Currently there is a need to identify genes responsible for terpene synthesis to produce Cannabis strains with consistent and desirable terpenoid profiles (Booth 2017).

There is evidence that terpenoid profiles can vary not only between Cannabis species and strains, but also within species and strains (Casano et al. 2010, Elzinga et al. 2015, Hillig 2004). Casano et al. (2010) examined inflorescence samples from 16 Cannabis plants derived from

"mostly sativa" or "mostly indica" strains. The authors designated strains as "mostly sativa" or "mostly indica" based on genetic, morphological, and chemotaxonomic information (Casano et al. 2010). They revealed "mostly indica" strains had high concentrations of β-myrcene. This is also supported by Elzinga et al. (2015) who describe "mostly indica" strains as dominated by mostly β-myrcene with limonene or α-pinene as the second most abundant terpenoids. Elzinga et al. (2015) and Casano et al. (2010) both describe "mostly sativa" strains having more complex terpenoid profiles than "mostly indica." Some "mostly sativa" plants consisted mostly of α-pinene and/or α-terpinolene while other "mostly sativa" strains were predominantly β-myrcene with α-terpinolene or trans-β-ocimene second most abundant terpenoids (Casano et al. 2010). Elzinga et al. (2015) investigated 53 different Cannabis strains using gas chromatography and flame ionization detection and determined the strain name alone cannot accurately convey the terpenoid profiles as several strains of the same name had variations in chemical composition. Hillig (2004) investigated ways to characterize terpenoid profiles Cannabis plants to determine whether differences in terpenoid profiles could be useful for taxonomic differentiation. However, they determined differences in terpenoid profiles are not useful for chemotaxonomic differentiation due to the variability of terpenoid profiles of Cannabis plants belonging to the same strain and/or originating in the same geographical location (Hillig 2004). Additionally, the terpenoid composition of many Cannabis strains are incomplete as terpenoids can be difficult to identify (Booth and Bohlmann 2019, Hillig 2004). This is due mostly due to the lack of sufficient genetic characterization of Cannabis plants and lack of reference materials for many terpenoid compounds. (Booth and Bohlmann 2019, Hillig 2004).

Preclinical research has indicated several terpenoids have therapeutic properties when administered in isolation and may act synergistically with phytocannabinoids to potentiate their

therapeutic actions (Russo 2011). Research has indicated select terpenoids have anxiolytic (linalool, limonene), antifungal (caryophyllene oxide), anti-inflammatory (α-pinene, β-myrcene), and sedative (nerolidol, linalool, β-myrcene) effects (Booth 2017, Erickson 2019, Russo 2011). Terpenoids are potent and highly bioavailable; they have been shown to affect human and animal behavior at ng/mL concentrations (Russo 2011). Thus, there is increasing interest in the pharmacodynamics of terpenoids for the treatment of pain, inflammation, depression, anxiety, addiction, epilepsy, and cancer (Russo 2011). Presently, four terpenoid compounds (limonene, linalool, β-myrcene, citral) have demonstrated anticonvulsant activity in animal trials (Bahr et al. 2019, de Barros Viana et al. 2000, Russo 2011). The current study was designed to use a zebrafish seizure model to identify the potential anticonvulsant properties of terpenoid compounds most commonly found in Cannabis plants.

3.2. Materials and methods

3.2.1. Fish Husbandry

Zebrafish were maintained according to Institutional Animal Care and Use Committee (IACUC) guidelines at Louisiana State University Agricultural Center under protocol numbers A2015-22 and A2018-25. Experimental procedures were reviewed and approved by the Louisiana State University Agricultural Center's IACUC under protocol number A2017-04. Sexually mature adult wild type (WT) zebrafish were obtained from Aquatic Research Organisms (Hampton, NH). Zebrafish, segregated by sex, were housed in a Pentair Aquatic Ecosystems laboratory animal housing aquatic habitat within a room maintained on a 13:11 light-dark cycle. A digitally controlled water heater was used to maintain aquarium temperature between 27-29°C. Fish were fed Otohime B2 (51% protein, 11% lipid; Marubeni Nisshin Feed Co., Tokyo, Japan) to apparent satiation once daily.

Newly fertilized zebrafish embryos were obtained by pairing adult zebrafish in a 1-L tank within 4 hours of the dark light cycle. Spawning occurred within the first hour of the next light cycle and newly fertilized embryos were collected via siphoning when embryos were approximately 6-hours post-fertilization (hpf).

3.2.2 Embryo Collection

Embryos (6 hpf) were housed in petri dishes containing 150mL of water obtained from the adult zebrafish system at a density of 150 per dish. Petri dishes were kept in an incubation chamber at 27ºC until they were 7-days post-fertilization (dpf). One drop (~0.5mg) of methylthioninium chloride was added to each dish immediately following collection to prevent fungal growth embryos at a final concentration of 3 mg/L. Methylthionium chloride was only utilized during the first 24 hours following embryo collection. Water was changed every 24 hours and any dead larvae were removed from the dishes.

3.2.3 Water Quality

Water quality assessments were performed weekly throughout the duration of this study. Alkalinity was measured using the Hach Company phenolphthalein and total method. Total hardness was measured using the EDTA method. Ammonia levels were measured using the high range acid titration method. Nitrite was calculated using the ceric standard solution method. pH was measured using the Oakton pH 700 probe. Water quality assessments were performed between 3 July 2019 and 20 September 2019.

Table 3.1. Mean and Standard Error of the Mean (SEM) values for water quality parameters during the acute exposure to phytocannabinoids. Ammonia (total ammonia nitrogen), alkalinity, and nitrite are reported as mg/L.

	AMMONIA	ALKALINITY	NITRITE	pH
	N = 11	N = 13	N = 11	N = 14
MEAN	0.009	267.69	0.0112	8.23
SEM	0.007	33.52	0.0018	0.090

3.2.4 Chemical Preparation

All terpenoid compounds were obtained from Sigma-Aldrich (St. Louis, MO) and stored at room temperature. DMSO was added to the 10mM PTZ solution achieve a final concentration of 1%. DMSO was selected as a carrier for these terpenoid compounds because of its low toxicity to aquatic organisms, its effectiveness as an organic solvent, and its facilitation of absorption across the gill membranes (Kais 2013).

3.2.5 Acute Exposure to Terpenoid Extracts

Samples sizes for treatment terpenoids, PTZ control, and negative control groups were represented by 16 individuals for each treatment concentration. Negative control larvae were not exposed to terpenoid compounds, PTZ, or DMSO. Seven dpf zebrafish were exposed to linalool, pinene, terpinolene, myrcene or limonene at nominal concentrations of 1, 5, 10, and 25 mg/L, for 90 minutes. These concentrations were based upon volumetric formulation. The solubility for linalool, pinene, terpinolene, myrcene, and limonene in water are 1590, 2.49, 6.812, 5.60, and 7.57 mg/L at 25°C respectively. Epileptic-like seizures were then induced by exposing larvae to a 10mM pentylenetetrazol (PTZ) solution for 10 min. After the 90-min terpenoid incubation and 10 min PTZ exposure, larvae were individually placed into wells of a 48-well plate, and movements were recorded for 10 minutes using a DanioVision™ (Noldus Information

Technology, Leesburg, VA) recording chamber. During the 10-minute observation period the total distance moved (mm) and average velocity (mm/s) was recorded at a frame rate of 30 samples per-second using Ethovision XT software (Version 14.0, Noldus Information Technology, Leesburg, VA). All incubations and the 10-minute recording period were done in the dark.

3.2.4 Statistical Analyses

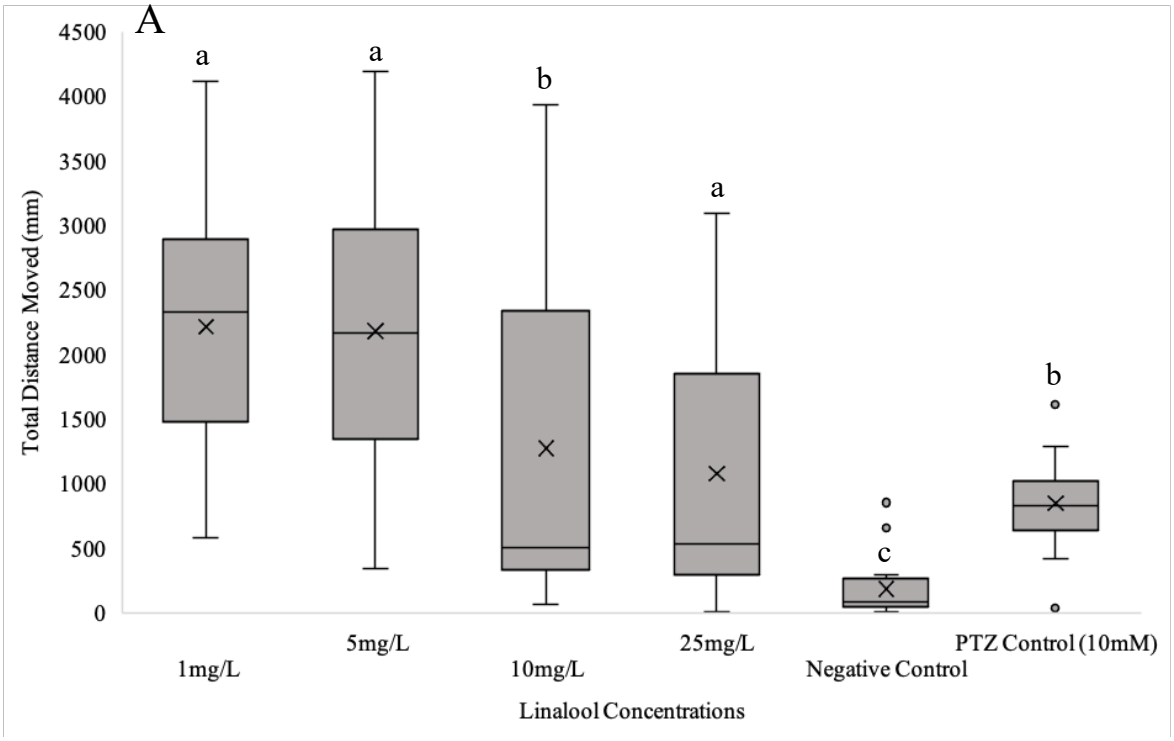
After the 10-min recording period, tracks obtained by the Ethovision XT software were reviewed to ensure there were no errors in the tracking progress. The most common tracking error occurred when the larvae did not move for an extended period of time. When this occurs, the software attempts to locate movement, and thus has the potential to track movement where there is none. This is particularly an issue when the larva is not moving, and the software detects movements from the reflection of a larva in an adjacent well. Tracks in each individual well were manually reviewed to ensure any false movements were deleted.

Distance moved and velocity recorded during the observation period were compared among concentrations and control treatment for each terpenoid using one-way analysis of variance (ANOVA). The Ryan-Einot-Gabriel-Welsch (REGWQ) post-hoc test was used for pairwise comparisons of concentrations and controls within each terpenoid exposure. All hypotheses were tested at a significance level of $\alpha=0.05$ and all tests were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC). A general linearized model two-way ANOVA was performed to test for significant differences in total distance moved (mm) among CBD concentrations and the terpenoid myrcene or limonene.

3.3. Results

3.3.1 Terpenes

For this model, a significant reduction in total distance moved and velocity indicates a reduction in PTZ-induced seizure behavior. Significant differences in total distance moved and velocity were detected among linalool, negative, and PTZ control treatments (total distance moved: $F_{6,94} = 11.42$, $p < 0.01$; velocity: $F_{6,94} = 11.29$, $p < 0.01$). All linalool treatments moved greater distances and at a higher velocity than negative control larvae. Larvae in the 1 mg/L and 5 mg/L treatment group moved significantly greater than the 10mM PTZ control, indicating a hyperlocomotive phenotype. (Figure 3.1A). No mortality was observed during linalool exposures.



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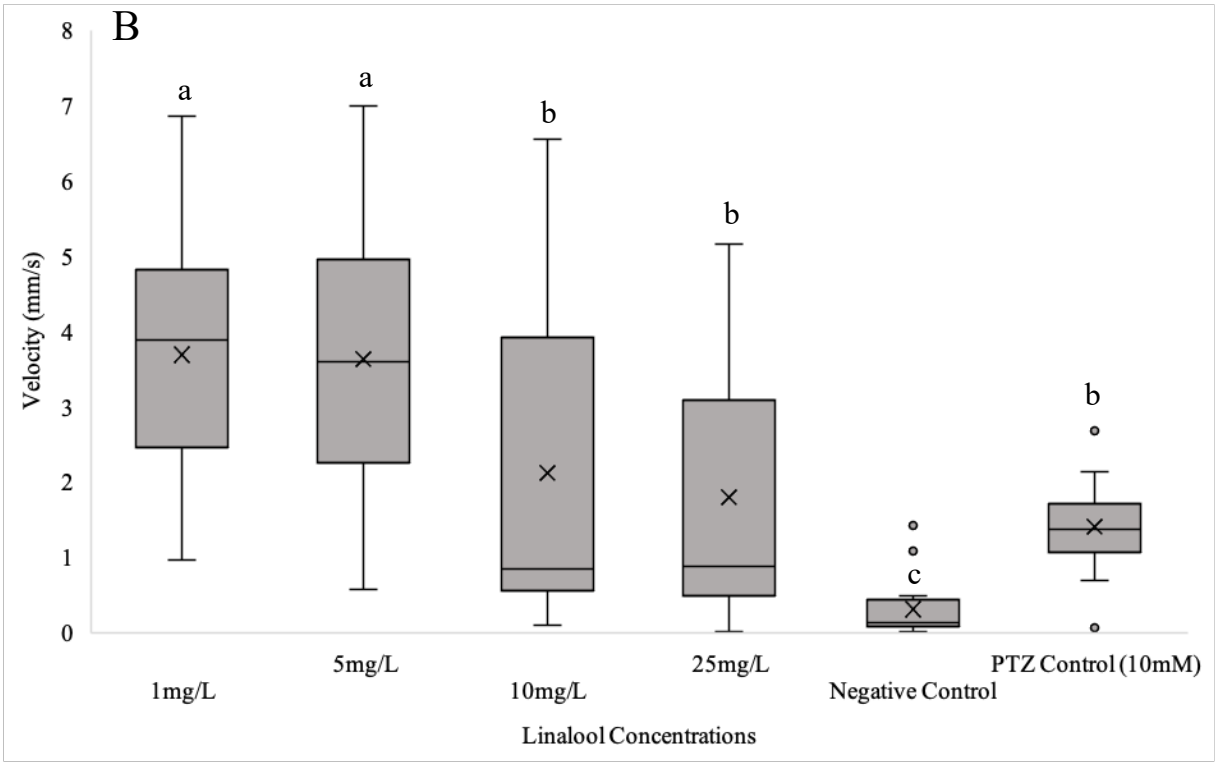


Figure 3.1. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and linalool. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p < 0.05$). N = 16.

Larvae exposed to pinene experienced significant differences in total distance moved among pinene, negative, and PTZ controls (total distance moved: $F_{6,107} = 30.18$, $p < 0.01$; velocity: $F_{6,107} = 30.29$, $p < 0.01$). The 1 mg/L treatment group displayed a hyperlocomotive phenotype, moving a significantly greater distance and significantly faster than the 10mM PTZ control (Figure 3.2A). There were no significant differences between the 5 mg/L treatment group and the PTZ control. Larvae in the 10 mg/L and 25 mg/L treatment group moved significantly less distance and significantly slower than the 10mM PTZ control and were not statistically different from the negative control. However, this is attributed to mortality rather than a reduction in seizure behavior. The 10 mg/L treatment group experienced 6.25% mortality, and the 25mg/L treatment group experienced 12.5% mortality.

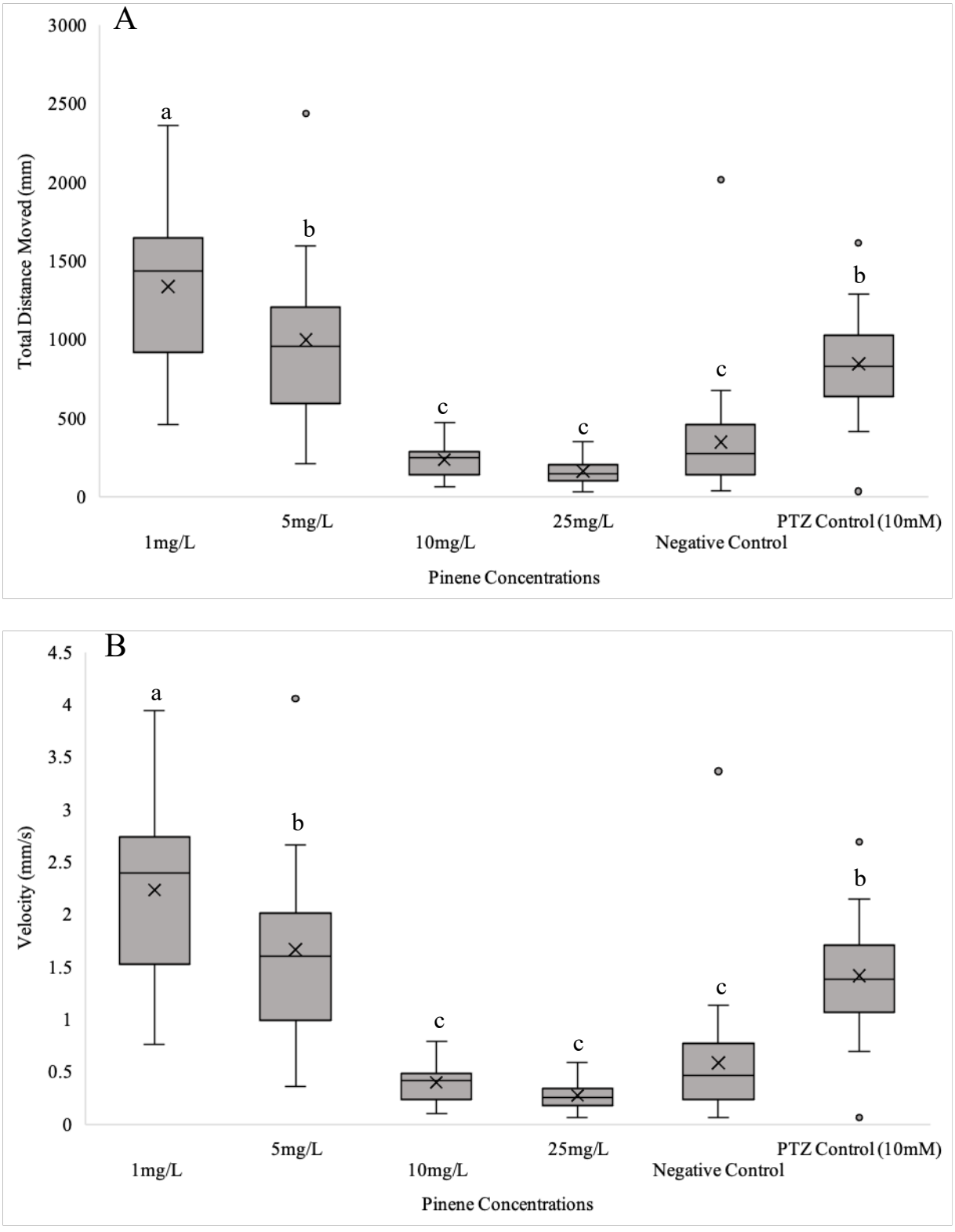
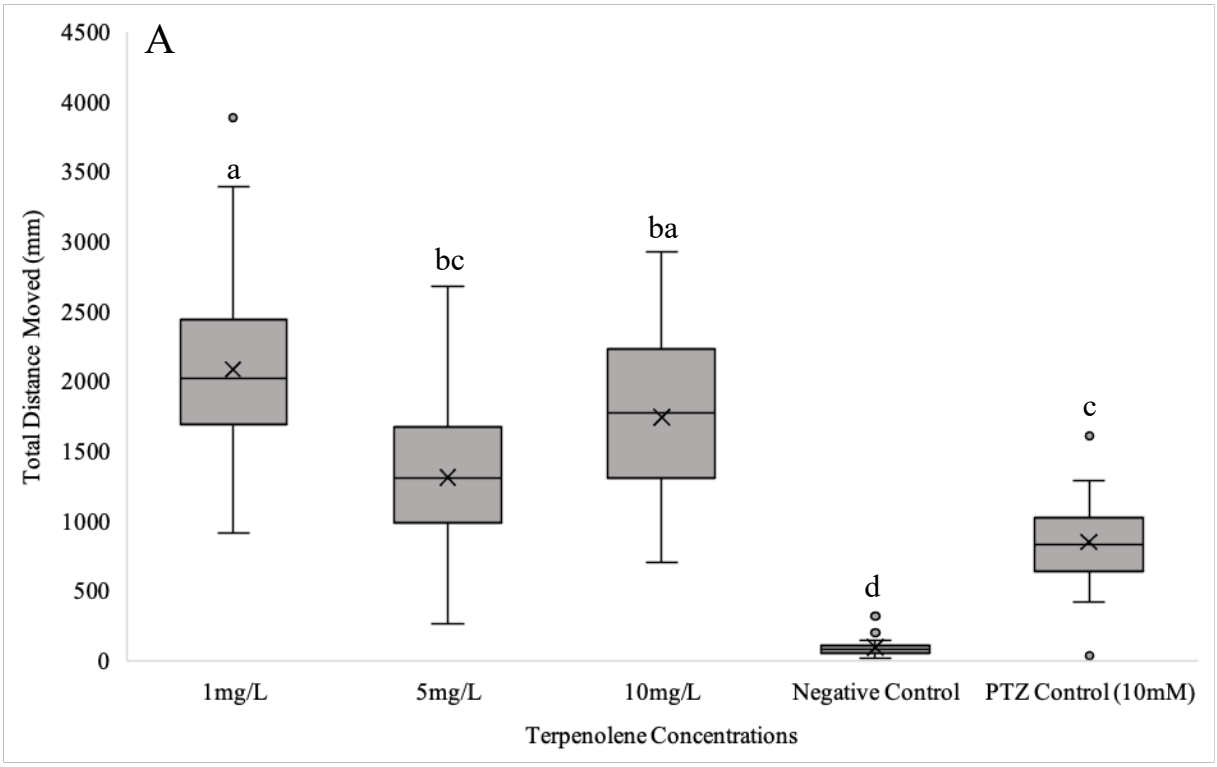


Figure 3.2. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and pinene. Box plots demonstrate median (line within box), 25,

and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p < 0.05$). N = 16.

Larvae exposed to terpinolene experienced no reduction in seizure behavior at any concentration (total distance moved: $F_{5,79} = 30.82$, $p < 0.01$; velocity: $F_{5,79} = 30.36$, $p < 0.01$). The 1mg/L and 10mg/L treatment group moved significantly more and significantly faster than the negative control and 10mM PTZ control, indicating a hyperlocomotive phenotype (Figure 3.3A). Larvae in the 5mg/L group were not statistically different from the PTZ control. The 25 mg/L treatment group experienced 100% mortality, and thus was eliminated from this trial.



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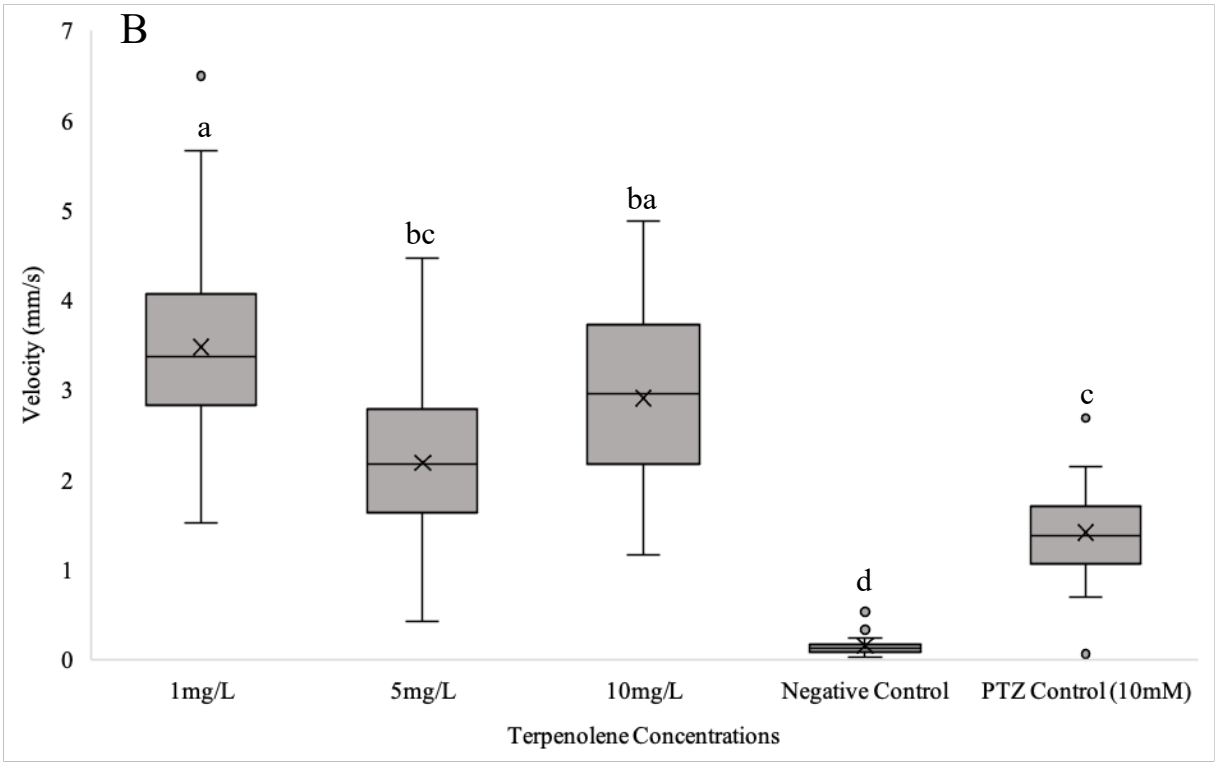


Figure 3.3. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and terpinolene. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p < 0.05$). N = 16.

Larvae exposed to myrcene exhibited a significant reduction in seizure behavior at all concentrations (total distance moved: $F_{6,111} = 7.04$, $p < 0.01$; velocity: $F_{6,111} = 7.55$, $p < 0.01$). The 1, 5, 10, and 15mg/L treatment groups were not significantly different from the negative control, indicating potential anticonvulsant properties of myrcene (Figure 3.4A).

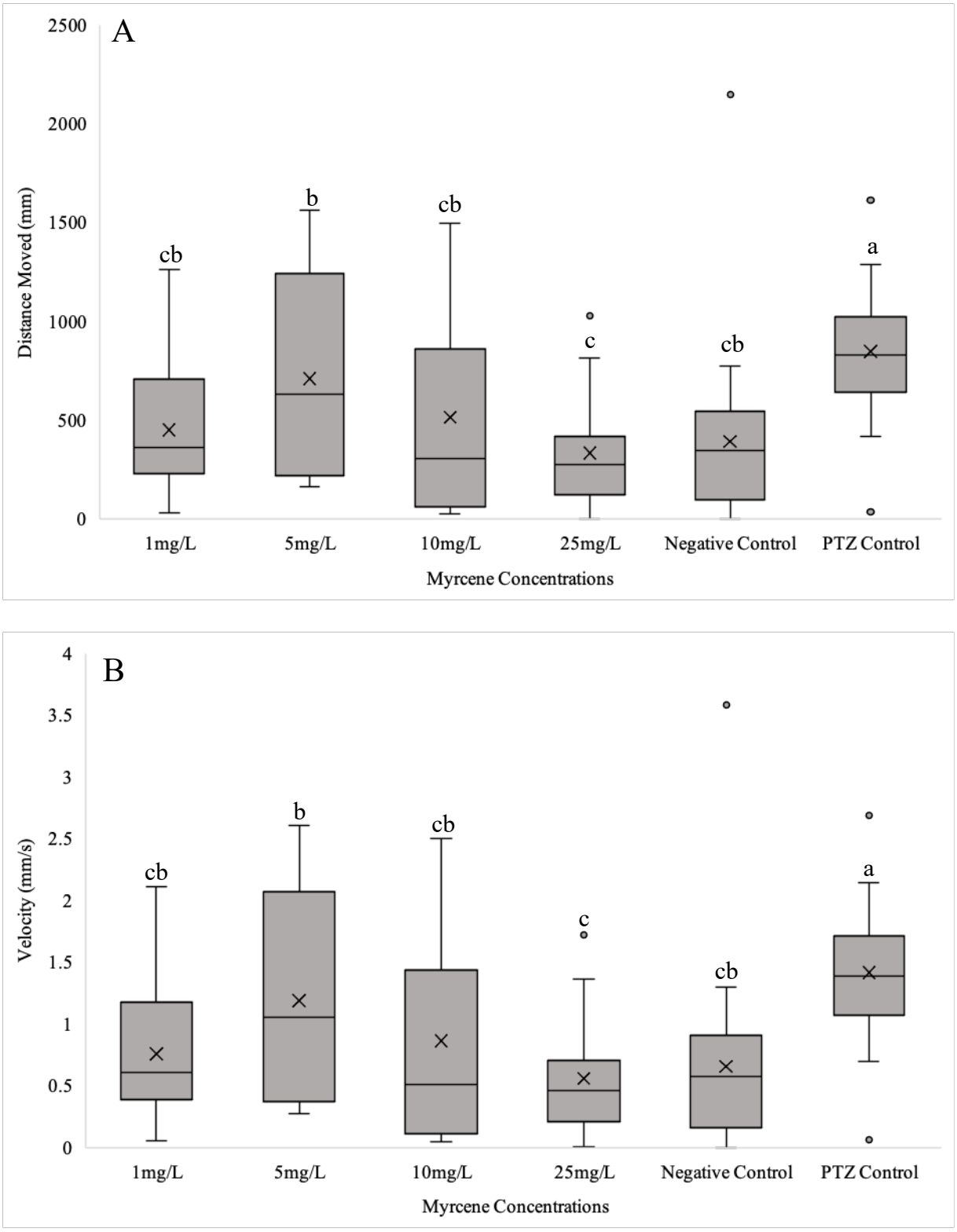
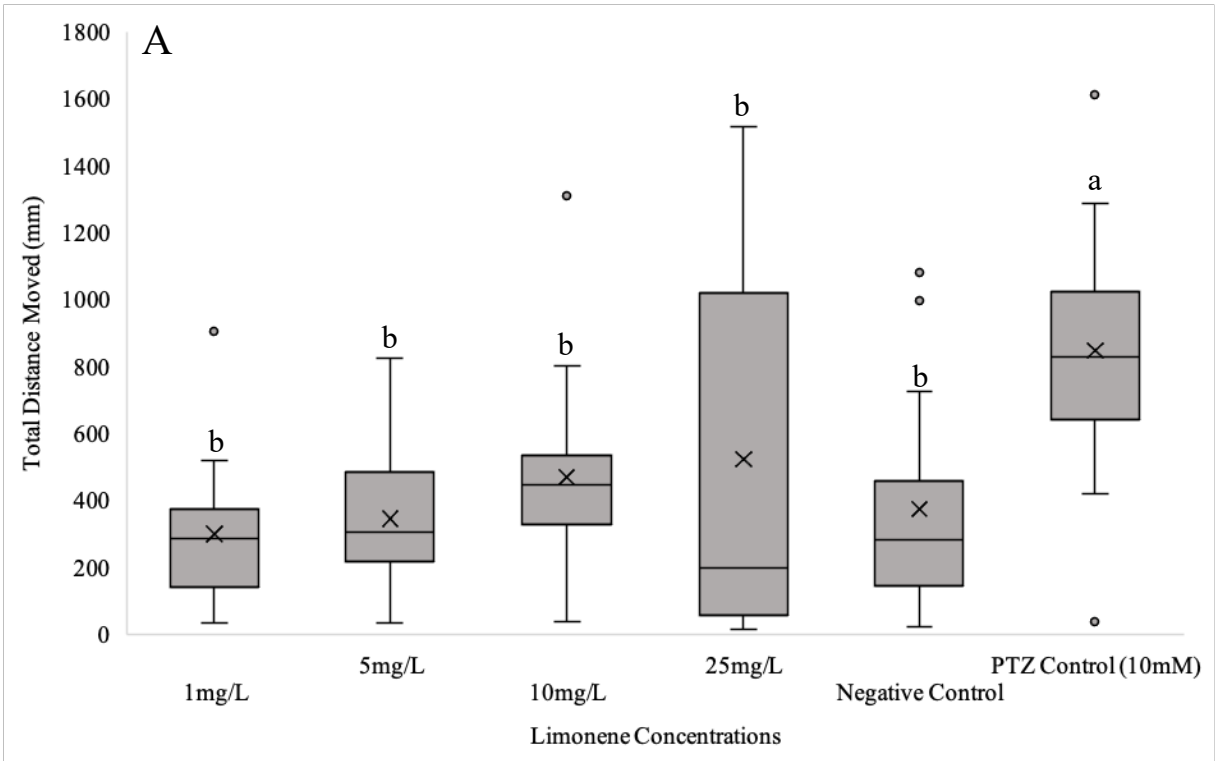


Figure 3.4. Total distance moved (A) and mean velocity (B) for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and myrcene. Box plots demonstrate median (line within box),

25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p < 0.05$). N = 16.

Larvae exposed a limonene gradient moved significantly less and significantly slower than the 10mM PTZ control, but not significantly less or significantly slower than the negative control across all concentrations (total distance moved: $F_{6,90} = 10.54$, $p < 0.01$; velocity: $F_{6,90} = 11.27$, $p < 0.01$).



(Fig Cont'd)

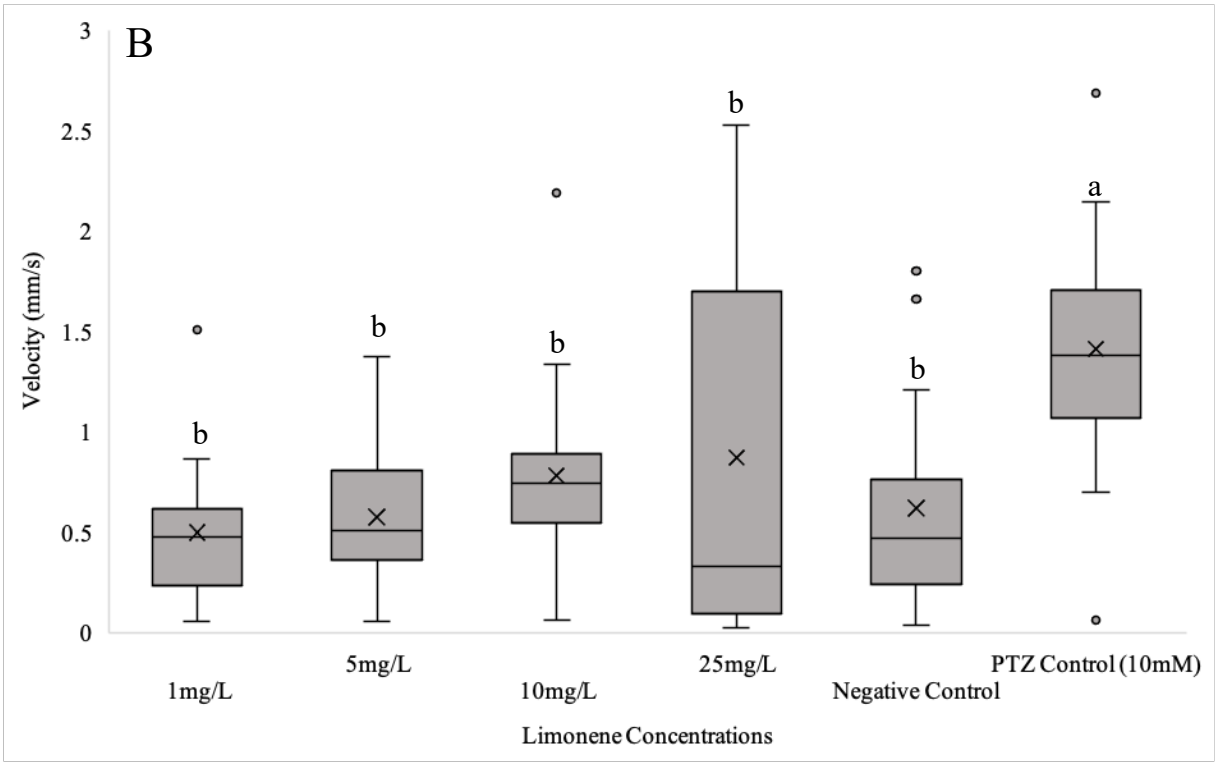


Figure 3.5. Total distance moved (A) and men velocity for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and a limonene gradient. Median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different ($p \geq 0.05$).

A comparison of the lowest terpenoid concentrations revealed significant differences in total distance moved ($F_{4,74} = 33.4$, $p < 0.01$). The two terpenes that indicated a redution in PTZ-induced seizure behavior (limonene and myrecene) were not significantly different in total distance moved (Figure 3.6)

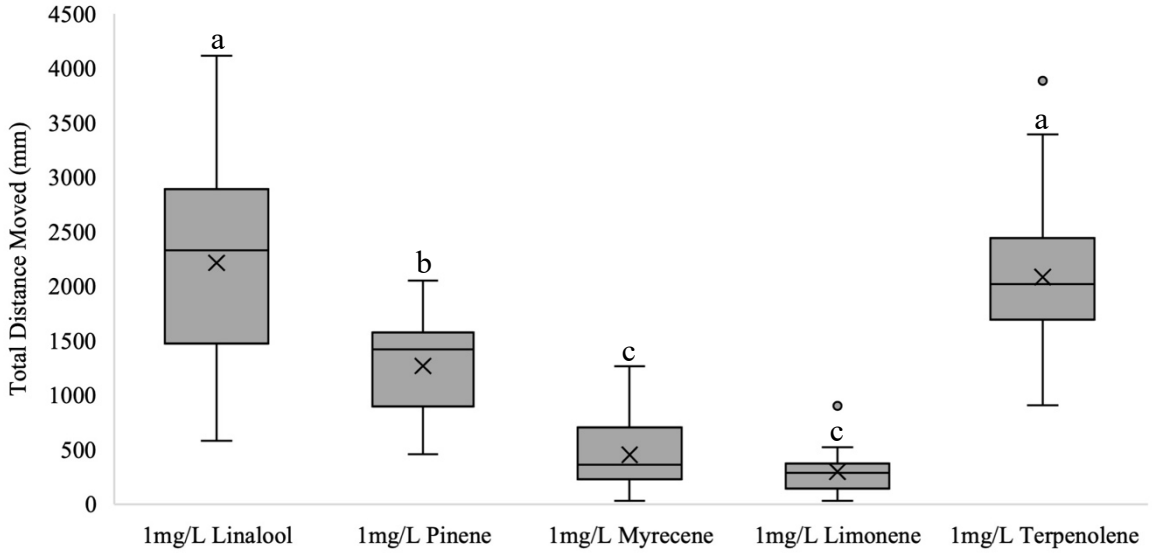


Figure 3.6. Total distance moved among lowest concentration of terpene incubation treatments in 7dpf zebrafish larvae exposed to PTZ. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Letters denote statistical significance among treatment groups (REGWQ; $p \leq 0.05$).

3.3.2 Terpenes and CBD

Significant differences in total distance moved were detected among CBD concentrations, negative and PTZ control treatments (total distance moved: $F_{6,124} = 28.49$, $p < 0.01$) with significantly greater movement among CBD concentration below 0.5 mg/L. Total distance moved for the lowest CBD concentration was not different from the PTZ control (Figure 3.6).

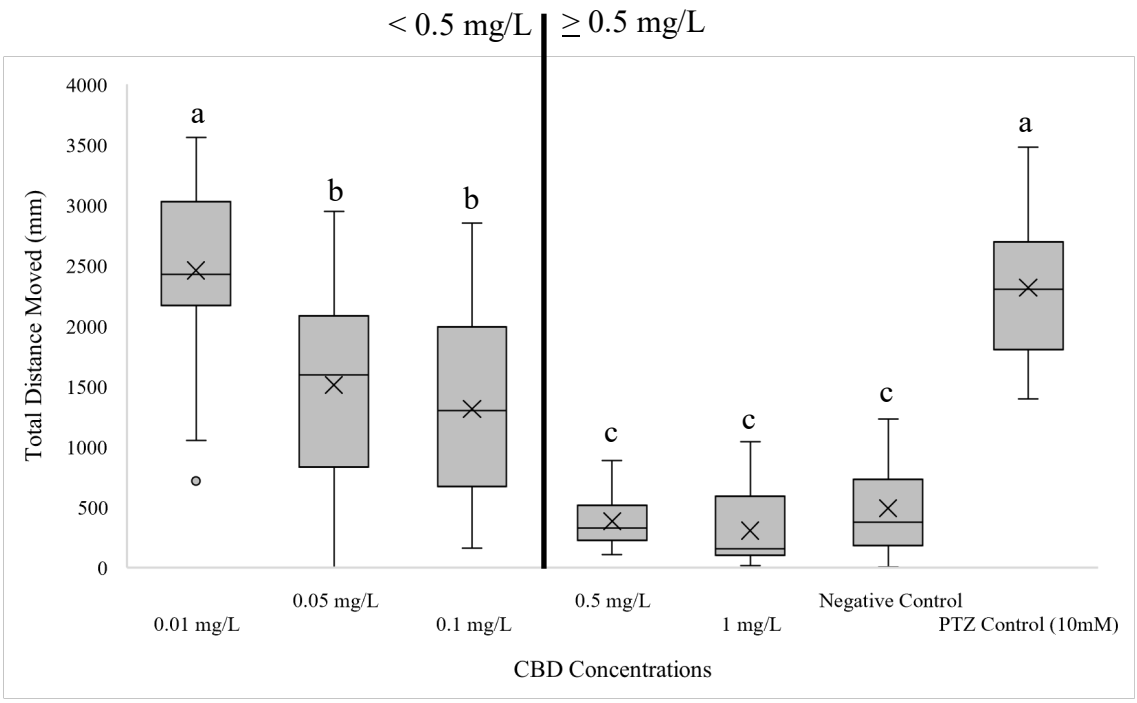


Figure 3.7 Total distance moved for 7dpf zebrafish larvae exposed to a negative control, PTZ control, and CBD. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

Concentrations of CBD below 0.5 mg/L in the presence of 1 mg/L myrcene did not result in anti-convulsant properties (Figure 3.7). Myrcene did not significantly influence total distance moved ($F = 0.58$, df =1,4, $p = 0.44$) and there was no significant myrcene*CBD concentration interaction ($F = 2.21$, df = 1,4, $p = 0.07$), indicating that overall main effect significant differences were attributed to CBD concentrations ($F = 58.2$, df = 1,4, $p < 0.01$).

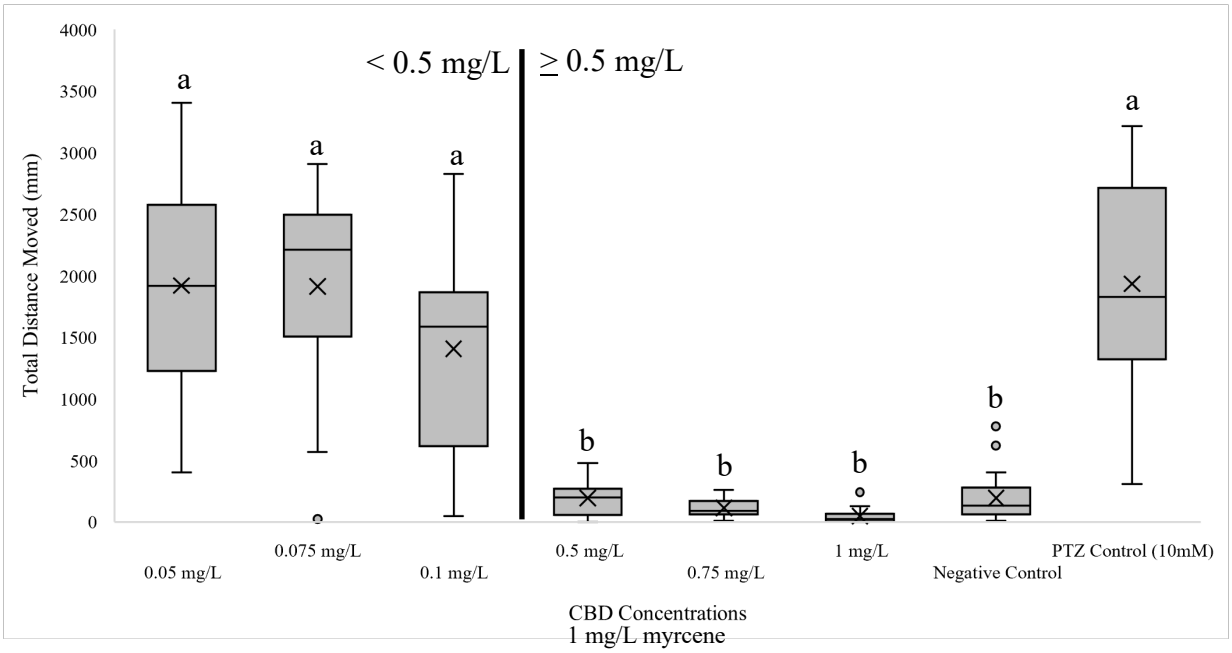


Figure 3.8 Total distance moved for 7dpf zebrafish larvae incubated with 1 mg/L myrcene and different CBD concentrations prior to a PTZ (10 mM) challenge. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

The CBD concentrations of 0.1 mg/L and 0.75 mg/L in the presence of 2 mg/L limonene were significantly different from control (Figure 3.8). Limonene did not significantly influence total distance moved ($F = 1.35$, df =1,6, $p = 0.24$) and there was no significant limonene*CBD concentration interaction ($F = 1.76$, df = 1,4, $p = 0.13$), indicating that overall main effect significant differences were attributed to CBD concentrations ($F = 58.13$, df = 1,6, $p < 0.01$).

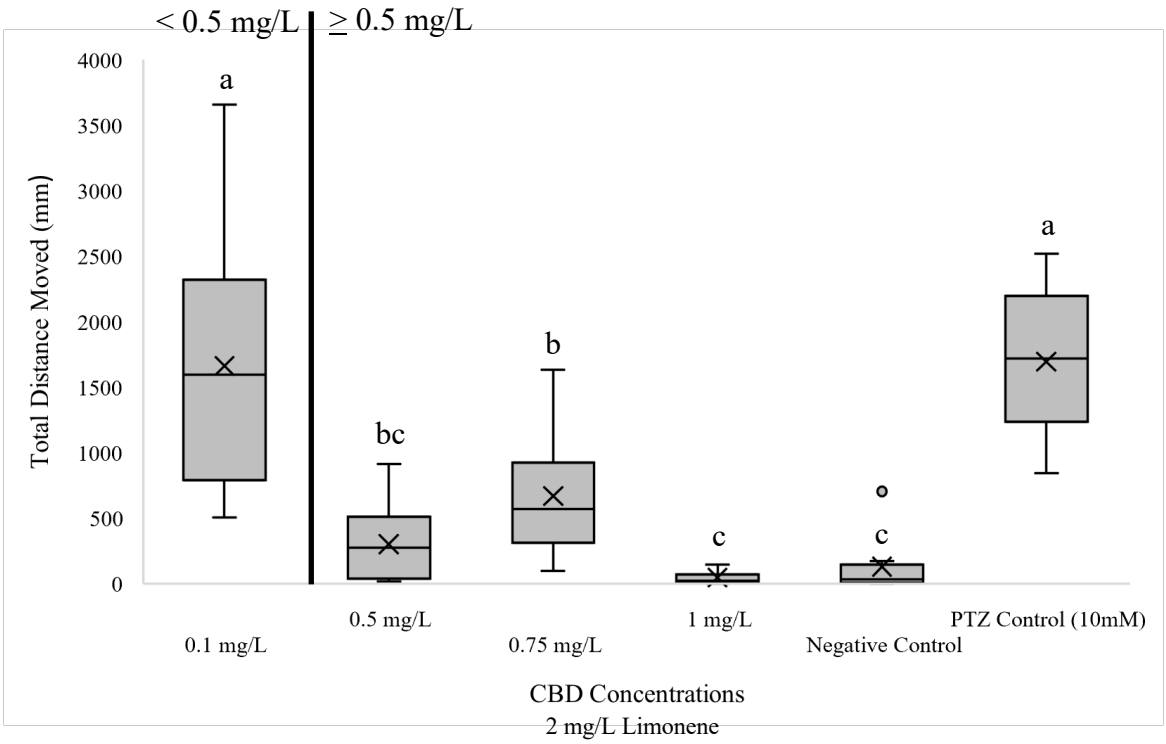


Figure 3.9 Total distance moved for 7dpf zebrafish larvae incubated with 2 mg/L limonene and different CBD concentrations prior to a PTZ (10 mM) challenge. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

Concentrations below 0.5 mg/L in the presence of 1 mg/L limonene were significantly different from control (Figure 3.9). Limonene significantly influenced total distance moved ($F = 3.85$, $df = 1,6$, $p = 0.05$) and there was a significant limonene*CBD concentration interaction ($F = 4.37$, $df = 1,4$, $p < 0.01$). Concentration of CBD was significant; however, 1 mg/L limonene decreased the anti-seizure properties of CBD at the 0.5 mg/L treatment group ($F = 55.82$, $df = 1,6$, $p < 0.01$).

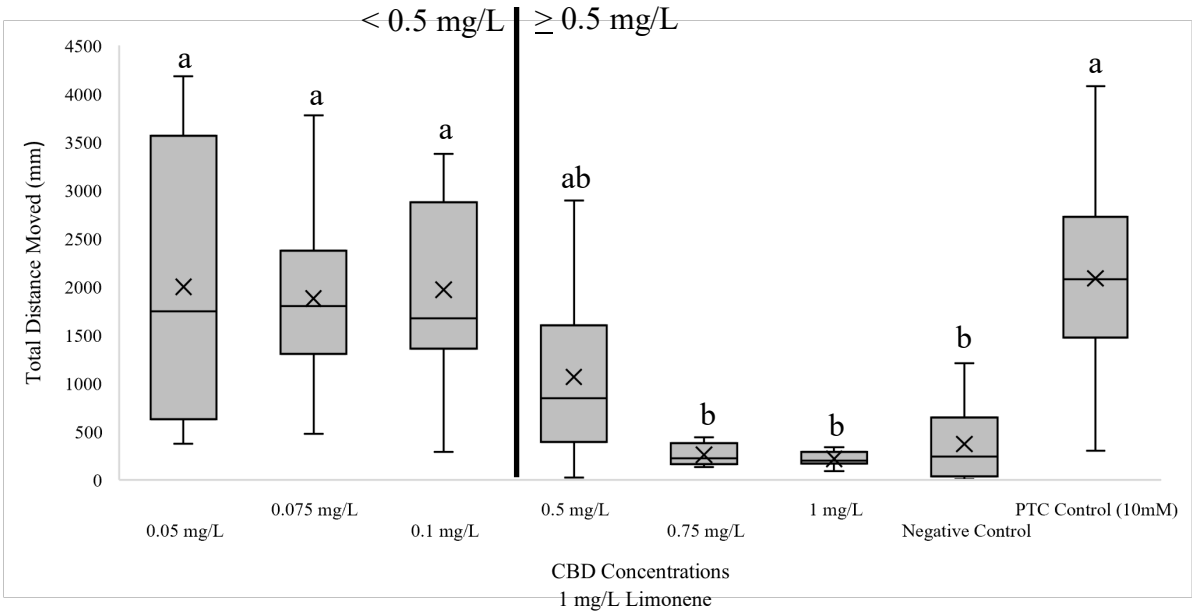


Figure 3.10 Total distance moved for 7dpf zebrafish larvae incubated with 1 mg/L limonene and different CBD concentrations prior to a PTZ (10 mM) challenge. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

The lowest CBD concentrations of 0.1 mg/L in the presence of 0.5 mg/L limonene was significantly different from control (Figure 3.10). Limonene did not significantly influence total distance moved ($F = 0.82$, $df = 1,6$, $p = 0.36$) and there was no significant limonene*CBD concentration interaction ($F = 0.89$, $df = 1,4$, $p = 0.47$), indicating that overall main effect significant differences were attributed to CBD concentrations ($F = 56.55$, $df = 1,6$, $p < 0.01$).

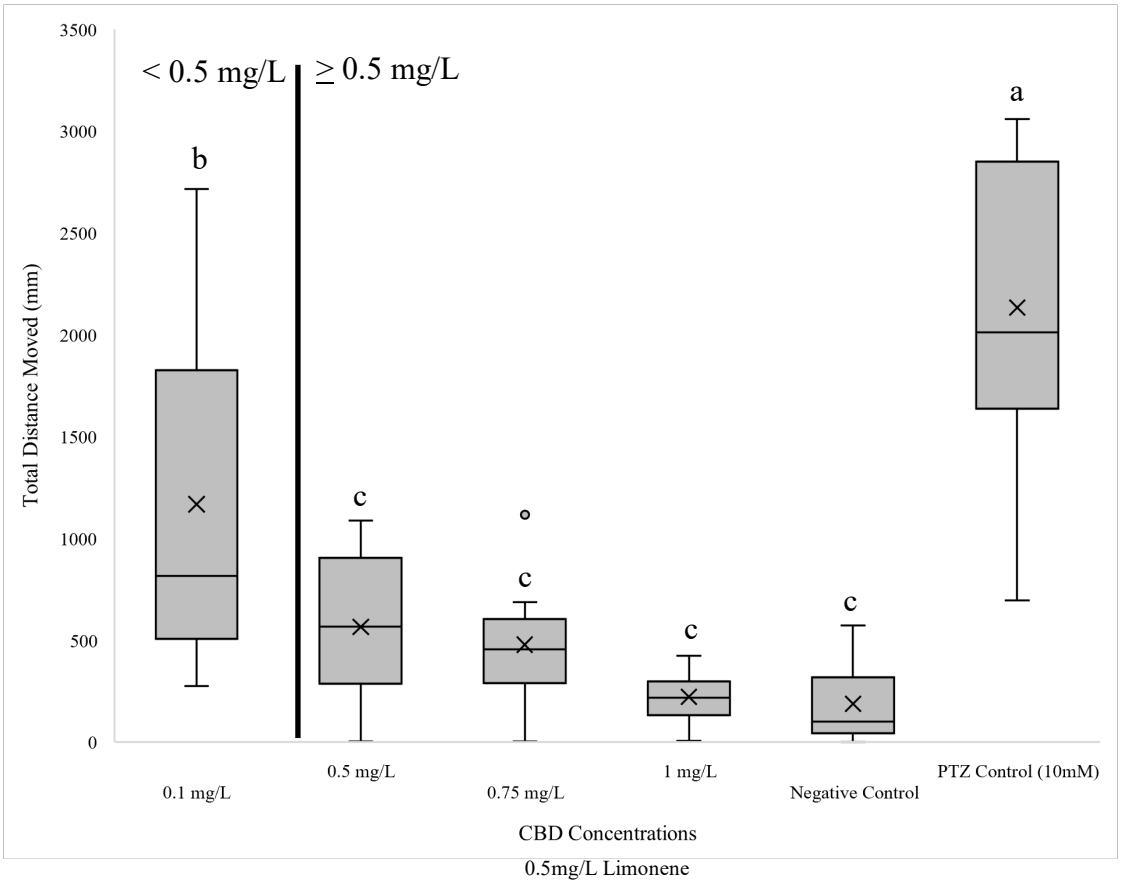


Figure 3.11 Total distance moved for 7dpf zebrafish larvae incubated with 0.5 mg/L limonene and different CBD concentrations prior to a PTZ (10 mM) challenge. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

3.4. Discussion

This experiment examined five of the most predominant terpenes present in Cannabis plants. Of the compounds examined, two (myrcene and limonene) reduced seizure behavior in 7 dpf zebrafish larvae. Review of current literature indicates these results are consistent with other investigations into the anticonvulsant properties of limonene and myrcene (Pathak et al. 2010, Seo et al. 2020, Sorocco de Barros Viana et al. 2000). The remaining three terpenoid compounds (linalool, pinene, terpinolene) did not reduce seizure behavior in 7dpf zebrafish. Additionally, pinene and terpinolene produced mortality at 10 mg/L and 25 mg/L over the course of the

treatment administration and observation period (110 minutes total). However, several studies utilizing terrestrial model organisms have revealed anticonvulsant activity of pinene, linalool, and linalool oxide at varying concentrations for each compound (Milanos et al. 2017, Negromonte Souto-Maior 2016). In the current study, linalool, pinene, and terpenolene all induced hyperactivity in 7 dpf zebrafish larvae. Vatanparast et al. (2017) reported that 0.4mM for an exposure duration of 4-6 minutes of linalool induced epileptiform activity in the snail Caucasotachea atrolabiata. The zebrafish model may not be appropriate for studies of terpenoid anticonvulsant properties as terpenoid compounds may be more toxic to aquatic organisms and invertebrates than in higher vertebrate model species.

There is currently speculation over whether terpenes potentiate the therapeutic actions of phytocannabinoids compounds (Ferber et al. 2020, Finlay et al. 2020). In a randomized double-blind, double-dummy trial, 539 adults with Generalized Anxiety Disorder received either 160 or 80 mg of Silexan (an essential oil found in lavender), or 20 mg of paroxetine (a selective serotonin reuptake inhibitor anti-depressant), or a placebo for 10 weeks (Ferber et al. 2020). The study found that Silexan was more effective at producing anxiolytic properties than paroxetine (Ferber et al. 2020). Studies on the anxiolytic properties of essential oils similar to those described in Ferber et al. 2020 have partially contributed to the idea that whole-cannabis products may be more effective in eliciting desirable effects, than with cannabinoids in isolation (Russo 2011). However, a different study utilizing an animal model found that linalool, one of the major terpenoids present in lavender plants, did not show any anxiolytic effects (Ferber et al. 2020). Finley et al. (2020) investigated whether select terpenes present in Cannabis (myrcene, α- and ß-pinene, ß-caryophyllene, and limonene) elicited any receptor-mediated activity, altered the activity of Δ^9-THC, CBD, or elicited an effect at the CBRs. They determined there was no

evidence to support any of the terpenes tested had any direct interactions with CB_1 or CB_2R, Δ9-THC, or CBD. Additionally, there is limited experimental data that supports terpenoid compounds have any synergistic effects in relation to anticonvulsant properties of cannabinoids (Russo 2011).

Effects of CBD on seizure behavior was not changed by the addition of myrcene or limonene. Neither compound decreased the concentration at which convulsant behavior was significantly reduced. Whole-Cannabis extracts are complex mixtures of cannabinoids and terpenoids and thus research into potential interactions is important. Our research indicated that terpenes do not appear to alter the antiseizure properties of CBD or the severity of movement from PTZ based seizures. The current study indicates they are not needed as an additional therapeutic for reducing seizures. There are reports of allergic reactions to essential oils in humans, which should be considered when developing Cannabis products that include terpenoid compounds (Bleasel et al. 2002, de Groot 2016). Using isolates or mixtures of only phytocannabinoids could reduce potential adverse side effects of terpenoid compounds.

CHAPTER 4. INVESTIGATING POTENTIAL DRUG RESISTANCE; CHRONIC DEVELOPMENATAL EXPOSURE TO CBD AND Δ^9THC IN ZEBRAFISH LARVAE.

4.1. Introduction

Despite approximately 3,000 years of recreational and medicinal Cannabis use there have been few comprehensive studies on the long-term effects of Cannabis use in humans (Devinsky et al. 2014, Nutt et al. 2013, Ware et al. 2015). This, in part, is due to its legal status in many countries. The United Kingdom Misuse of Drugs Act (1971) classifies Cannabis and Cannabis resin, Δ^9-THC, and THV as a Class B substance (Nutt et al. 2013). United States Controlled Substances Act (1971) classifies Cannabis and Cannabis resin as a Schedule-I drug and Δ^9THC as a Schedule-III drug (Nutt et al. 2013). Since these laws were enacted however, Cannabis products have been deemed less harmful than other recreational drugs including alcohol (Nutt et al. 2013). In a 2009 report, the United Kingdom's Advisory Council on the Misuse of Drugs (ACMD) evaluated the short- and long-term health risks of Cannabis use. The ACMD report deemed Cannabis to be less harmful than other Class B drugs including amphetamines, ketamine, methoxetamine, and codeine (Nutt et al. 2013., Weissenborn and Nutt 2012, United Kingdom Government 2014). Lung diseases associated with pulmonary exposure to Cannabis partially contribute to its legal status in many countries (Nutt et al 2013). Smoked Cannabis is often used in self-management of chronic pain, anxiety, and sleep disorders (Nutt et al. 2013, Ware et al. 2015). Long-term effects of pulmonary exposure to Cannabis products include damage to the respiratory tract, increased risk of chronic bronchitis, and lung cancer (Nutt and Weissborn 2012). Smoke from herbal Cannabis contains similar chemical constituents (with the exception of nicotine) as tobacco smoke, and tar from Cannabis resin contains higher concentrations of certain carcinogenic compounds than tobacco smoke (Ashton 2008). However,

there is still debate over whether Cannabis usage is more or less likely to cause damage to the respiratory system as compared to tobacco smoking (Nutt and Weissborn 2012). There are additional concerns about the short-term mental health effects of Cannabis usage (Ashton 2008, Nutt and Weissborn 2012). For light users, short-term effects (including impaired motor performance and cognition) are considered easily reversable with cessation of use and are currently thought to have no significant lasting effects (Nutt and Weissborn 2012). However heavy, chronic cannabis use could experience cognitive impairments for longer periods of time (weeks to months to years) after cessation of use (Ashton 2008). It is not currently known if heavy, long-term cannabis use can permanently impair cognitive function (Ashton 2008).

There is in increased need for studies on the effects of long-term Cannabis usage as interest in Δ^9-THC for management of chronic pain, especially for patients with cancer-related chronic pain has increased in recent decades (Johnson et al. 2013, Ware et al. 2015). Currently, opioids are the primary means of managing cancer-related chronic pain; long-term adverse effects of opioid usage often offset their therapeutic benefits (Johnson et al. 2013). There is increasing interest in the usage of Cannabis products and extracts as an alternative to opioids for the management of chronic pain (Johnson et al. 2013, Stewart and Kalueff 2014). Despite its psychoactive effects, Class B status in the United Kingdom and Schedule-1 status in the United States, Cannabis has relatively low addiction potential and is significantly less addictive than recreational opioids such as diacetylmorphine (heroin) (Nutt et al. 2013, Stewart and Kalueff 2014). In the United States, Cannabis is currently legal in 18 states and the District of Columbia and potentially safe for clinical usage under medical supervision (Nutt et al. 2013).

Ware et al. (2015) performed a study in which 431 individuals with chronic pain participated in a 1-year study to assess potential long-term negative impacts of Cannabis use

when used for the treatment of pain. The control group consisted of 216 individuals with chronic pain but no current or previous Cannabis use. The Cannabis group consisted of 215 individuals 141 of which were current users, and 58 former users; participants were administered standardized herbal cannabis product with 12.5% THC at a median daily dose of 2.5 g/day (Ware et al. 2015). Results of this study determined under medical supervision; Cannabis use for the treatment of chronic pain was reasonably safe over a one-year period (Ware et al. 2015). Additionally, the study found that members of the control group were more likely to use opioids for the management of pain than members of the Cannabis group pain (55% in the Cannabis group, 66% in the control group) (Ware et al. 2015). The authors noted that there is a need for data on the long-term pulmonary and cognitive functioning beyond one year (Ware et al. 2015). Johnson et al. (2013) conducted study on the long-term safety of a THC/CBD oromucosal spay in patients with cancer-related pain. Of the 43 patients in the original two-week, trial, 13 patients received a THC/CBD spray, 11 received a THC spray, and 19 received a placebo (Johnson et al. 2013). Patients were allowed to use continue usage of other analgesic drugs throughout the duration of the study. The most commonly used non-opioid medications used were paracetamol and diclofenac; the most common opioids used during the trial were diamorphine and morphine sulfate (Johnson et al. 2013). This study found overall, the proportion of patients reporting analgesic effects was greater for the THC/CBD group than the placebo group (Johnson et al. 2013).

The pharmacokinetics, toxicity, and potential adverse health effects of Cannabis are even less understood in children, as most studies on the effects of long-term Cannabis usage are performed with adults (Devinsky et al. 2014). Information on the pharmacokinetics of cannabinoids in children is especially relevant as there is an increased interest in the use of

Cannabis products and extracts for treatment-resistant epilepsy in children (Reference section 2.1). Toddlers and adolescents may be more sensitive adverse effects of cannabinoids on the nervous system as they are undergoing important periods of neuronal development (Carty 2019). Overall, there is a need for more clinical investigations into the effects of long-term Cannabis use, especially over a time period greater than one year.

Drug tolerance is the reduction of effects of a drug resulting from repeated exposure (Mount Sinai Ichan School of Medicine, New York, NY). Tolerance is observed in humans who engage in frequent Cannabis use, whether medicinally or recreationally (Ashton 2008). Laboratory experiments in mammals reveal that a tolerance to primary (analgesia, motor inhibition, anti-convulsant activity, hypothermia, etc.) and peripheral (immunosuppression, hypotension, etc.) effects develop when administered cannabinoids over a prolonged period of time (Gonzalez et al. 2005). In laboratory animals (dogs, rats, mice), the degree of tolerance depends on the species, dosage, and duration of treatment (Gonzalez et al. 2005, Martin et al. 2004). In general, the pharmacodynamics of cannabinoids have the most significant impact in the development and extent of tolerance, however, there is evidence pharmacokinetics can also influence degrees of tolerance (Gonzalez 2005). In addition to laboratory animals, tolerance of cannabinoids has also been observed in in vitro cell culture experiments (Gonzalez et al. 2005, Pertwee 1991). Presently there is debate over whether chronic exposure to cannabinoids creates tolerance through down regulation of receptors, changes in bioavailability, and/or neuronal adaption (Gonzalez et al. 2005). Martin et al. (2004) states that the downregulation of CB_1R accompanies cannabinoid tolerance development, however, the processes responsible for this downregulation are unknown. McKinney et al. (2008) determined, however, there is a dose-dependent relationship in the development of Δ^9-THC tolerance and changes in CB_1R in mice

treated with escalating doses (10, 20, 30, 60 mg/kg) of Δ^9-THC over a 6.5-day period versus mice treated with only 10 mg/kg over the same period of time.

The development of tolerance may lead Cannabis users to escalate dosage. This has generated debate over whether Cannabis tolerance might have a "gateway" effect by increasing the likelihood that users will consume drugs with a higher addiction potential when they no longer experience the desired effects of Cannabis use (Gonzalez et al. 2005, Ashton 2008). Additionally, there is debate over whether the development of tolerance encourages continued Cannabis use in order to avoid withdrawal symptoms associated with cessation of Cannabis use (Ashton 2008). Withdrawal symptoms have been observed in laboratory animals and humans and are most frequently associated with heavy, chronic cannabis use (Ashton 2008, Lichtman and Martin 2005). The most common withdrawal symptoms include restlessness, insomnia, anxiety, anorexia, and muscle tremors (Ashton 2008). Jones (1983) determined a dose of 180 mg of THC for a period of 11-21 days will produce withdrawal symptoms in humans (Ashton 2008). Thus, withdrawal symptoms must be considered for patients using Cannabis for an extended period of time. As previously mentioned, there is a need for robust studies on the effects of long-term Cannabis use in humans as phytocannabinoid compounds for medical use becomes more common.

This study seeks to determine the potential anti-convulsant activity of CBD and Δ9-THC when administered to 7dpf larval zebrafish over a 168-hour period. We predict a 168-hour exposure to CBD or Δ9-THC will reduce seizure behavior in 7dpf zebrafish larvae.

4.2. Materials and methods

4.2.1. Fish Husbandry

Zebrafish were maintained according to Institutional Animal Care and Use Committee (IACUC) guidelines at Louisiana State University Agricultural Center under protocol numbers A2018-25. Experimental procedures were reviewed and approved by the Louisiana State University Agricultural Center's IACUC under protocol number A2017-04. Sexually mature adult wild type (WT) zebrafish were obtained from Aquatic Research Organisms (Hampton, NH). Zebrafish, segregated by sex, were housed in a Pentair Aquatic Ecosystems laboratory animal housing aquatic habitat within a room maintained on a 13:11 light-dark cycle. A digitally controlled water heater was used to maintain aquarium temperature between 27-29°C. Fish were fed Otohime B2 (51% protein, 11% lipid; Marubeni Nisshin Feed Co., Tokyo, Japan) to apparent satiation once daily.

Newly fertilized zebrafish embryos were obtained by pairing adult zebrafish in a 1-L tank within 4 hours of the dark light cycle. Spawning occurred within the first hour of the next light cycle and newly fertilized embryos were collected via siphoning when embryos were approximately 6-hours post-fertilization (hpf).

4.2.2 Embryo Collection

150 Embryos (6 hpf) were housed in a petri dish containing 150mL of 30% Danieau's embryo media solution (pH of 7.6). One drop (~0.5mg) of methylthioninium chloride was added to each dish immediately following collection to prevent fungal growth embryos at a final concentration of 3 mg/L. Methylthionium chloride was only utilized during the first 24 hours following embryo collection. Water was changed every 24 hours and any dead larvae were

removed from the dishes. Petri dishes were kept in an incubation chamber at 27ºC until they were 7-days post-fertilization (dpf).

4.2.3 Chemical Preparation

PTZ was obtained from Sigma-Aldrich (St. Louis, MO) and stored at -15°C. All Cannabinoid extracts were obtained from Cerilliant Corporation (Round Rock, TX) and stored at room temperature. Extracts were intended to serve as analytical standard and consisted of 1.0 mg/L of the specific cannabinoid within 1 mL of methanol. Ultra-high purity nitrogen gas was used to evaporate and reconstitute the extracts in 1mL dimethyl sulfoxide (DMSO). Additionally, DMSO was added to the 10mM PTZ solution achieve a final concentration of 1%. DMSO was selected as a carrier for these compounds because of its low toxicity to aquatic organisms, its effectiveness as an organic solvent, and its facilitation of absorption across the gill membranes (Kais 2013).

3.2.5 Chronic exposure to Cannabinoid Extracts

Seven dpf zebrafish were exposed to CBD or Δ9-THC at concentrations of 0.0625, 0.125mg/L, for 168 hours. Samples sizes for CBD and Δ^9-THC treatment groups, PTZ control, and negative control groups were represented by 24 individuals for each treatment concentration except for the 0.0625mg/L + 10mM treatment group, which was represented by 48 individuals. Negative control larvae were not exposed to cannabinoids, PTZ or DMSO. Epileptic-like seizures were induced by exposing larvae to a 10mM pentylenetetrazol (PTZ) solution for 10 min on day 7 of the incubation period. After the 10-minute PTZ exposure, larvae were individually placed into wells of a 48-well plate, and movements were recorded for 10 minutes using a DanioVision™ (Noldus Information Technology, Leesburg, VA) recording chamber. During the 10-minute observation period the total distance moved (mm) was recorded was recorded at a frame rate of 30 samples per-second using Ethovision XT software (Version 14.0,

Noldus Information Technology, Leesburg, VA). All incubations and the 10-minute recording period were done in the dark.

3.2.4 Statistical Analyses

After the 10-min recording period, tracks obtained by the Ethovision XT™ software were reviewed to ensure there were no errors in the tracking progress. The most common tracking error occurred when the larvae did not move for an extended period of time. When this occurs, the software attempts to locate movement, and thus has the potential to track movement where there is none. This is particularly an issue when the larva is not moving, and the software detects movements from the reflection of a larva in an adjacent well. Tracks in each individual well were manually reviewed to ensure any false movements were deleted.

Distance moved and velocity recorded during the observation period were compared among CBD and Δ^9-THC concentrations and control treatments using one-way analysis of variance (ANOVA). The Ryan-Einot-Gabriel-Welsch (REGWQ) post-hoc test was used for pairwise comparisons of concentrations and controls within each exposure. All hypotheses were tested at a significance level of α=0.05 and all tests were performed using Statistical Analysis Software (SAS Institute Inc., Cary, NC).

4.3. Results

For this model, a significant reduction in total distance moved indicates a reduction in PTZ-induced seizure behavior. Total distance moved for 7dpf zebrafish larvae incubated in CBD throughout larval development and challenged with PTZ were significantly greater than negative control and PTZ treatments ($F_{5,164} = 11.44$, $p < 0.01$). Post-hoc analysis indicated that both CBD concentrations (0.125 and 0.0625 mg/L) moved significantly greater distances than negative control treatments when challenged with PTZ. Neither the 0.0625 mg/L or 0.125 mg/L treatment

groups not challenged with PTZ were statistically different from the negative control. However, the negative control and 0.125 mg/L treatment groups were not significantly different from the PTZ control (Figure 4.1).

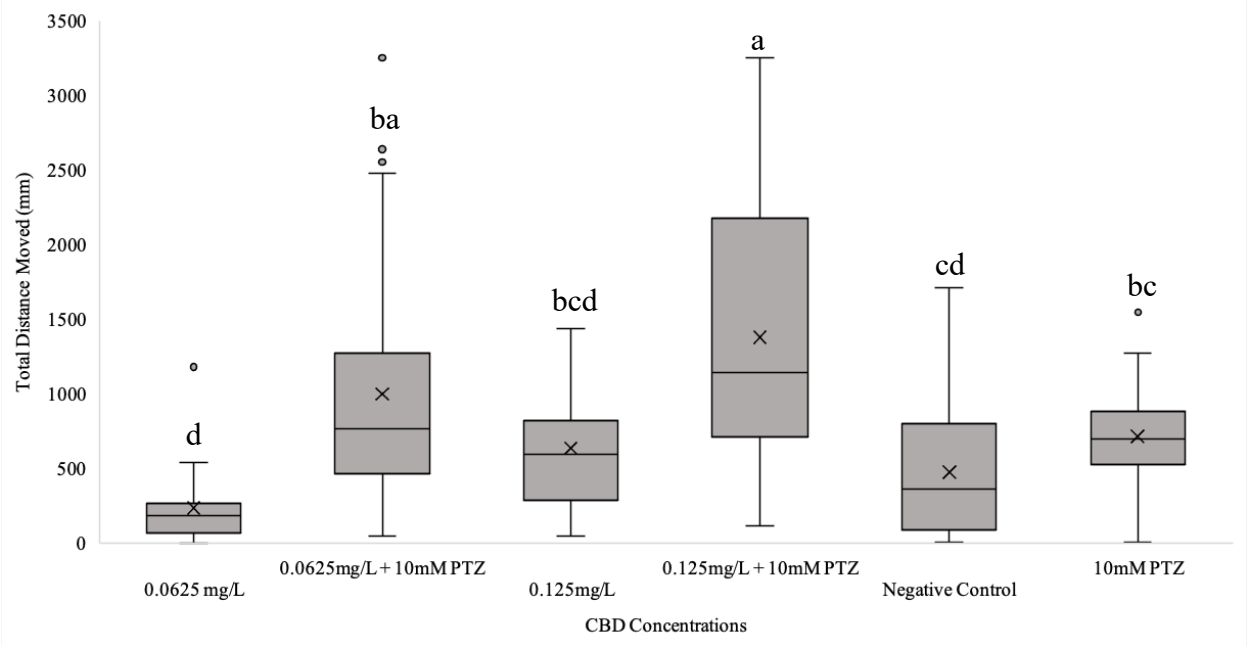


Figure 4.1. Total distance moved for 7dpf zebrafish larvae exposed to a negative control, PTZ control, CBD, and CBD with PTZ. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

Total distance moved for 7dpf zebrafish larvae incubated in Δ^9-THC throughout larval development and challenged with PTZ were significantly different than treatments not challenged with PTZ ($F_{5,138} = 8.57$, $p < 0.01$). Post-hoc analysis indicated the 0.0625 mg/L and 0.126 mg/L Δ^9-THC-only treatment groups moved less than the PTZ control. The negative control was not significantly different from the 10mM PTZ control group or Δ^9-THC treatments challenged with PTZ (Figure 4.2).

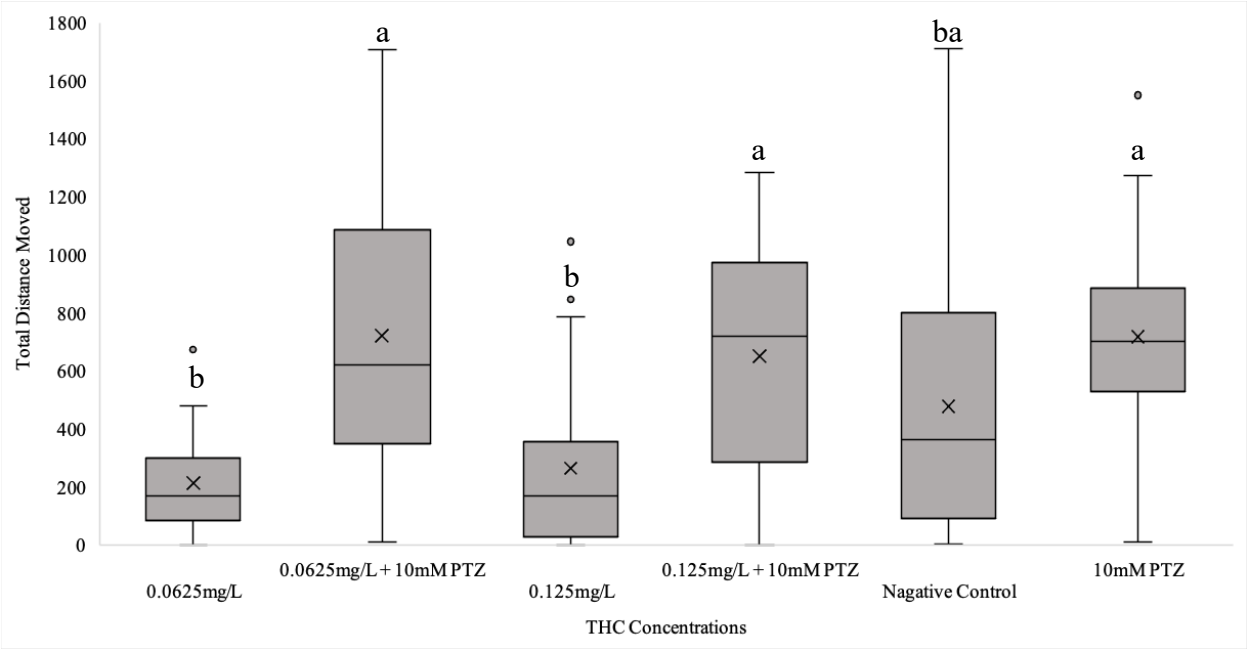


Figure 4.2. Total distance moved for 7 dpf zebrafish larvae exposed to a negative control, PTZ control, Δ^9-THC, and Δ^9-THC with 10mM PTZ. Box plots demonstrate median (line within box), 25, and 75% confidence intervals (CI; boundary of box), 10 and 90% CI (error bars), and outliers (◦). Boxes with the same letters are not statistically different (REGWQ; $p \geq 0.05$).

4.4. Discussion

Results indicate 7dpf zebrafish larvae experienced no protective anti-convulsant effects from PTZ when exposed to CBD or Δ9-THC over a 7-day period. Though not significantly different, larvae exposed to 0.0625 mg/L and 0.125 mg/L Δ^9-THC moved less than the negative control, indicating a possible sedative effect. There is a need for more investigation into the efficacy of this chronic model, as the negative controls for both cannabinoid trials were not statistically different from the PTZ control. Several PTZ challenged treatment groups within both CBD and Δ^9-THC exposed larvae experienced greater variance than has been previously displayed with this seizure model (see Chapter 2).

Though there are numerous studies supporting CBD's anticonvulsant effects in acute models, there is less evidence for CBD's effects in chronic models (Devinsky et al. 2014). Rats given a cobalt implant showed no reduction in epileptiform activity when exposed to CBD over a

36-hour period (Devinsky et al 2014). The same study found Δ^9-THC only had anticonvulsant effects for the first 24 hours of the exposure period (Devinsky et al. 2014). An experiment in which mice were injected with 10mg/kg of Δ^9-THC over a six-day period became 5-10 times less sensitive to Δ^9-THC than control mice (Fan et al. 1994, Martin et al. 2004). Sim-Selly and Martin (2002) exposed mice to escalating doses (10mg/kg starting dose to a final dose of 160mg/kg) of Δ^9-THC over a two-week period and reported mice became 50-100 times less sensitive to the effects of Δ^9-THC than control mice.

Exposure to CBD and Δ^9-THC during the first seven days of embryo and larval development may have interfered with normal developmental trajectories and thus inhibited the main effect of investigating potential drug resistance. Akhtar et al. (2013) described malformations of zebrafish embryos after exposure to 0.6 mg/L and 2.4 mg/L Δ^9-THC over a five-day period. Observed malformations included yolk sac and pericardial edema and a bent tail and body axis (Ahktar et al. 2013). The current study did not observe developmental deformities, potentially due to the lower chronic concentrations of CBD and Δ^9-THC employed (0.625 and 0.125 mg/L). Carty et al. (2018) exposed zebrafish embryos at the blastula stage through the larval stage (96 hpf) to Δ^9-THC at concentrations of 0.3, 0.6, 1.25, 2.5, and 5 mg/L or CBD at 0.07, 0.3, 0.6, 1.25 mg/L. They observed similar deformations for both Δ^9-THC and CBD including edemas, curved body axis, deformations of the eyes, snout, jaw, and fins, distention of the swim bladder, and abnormal behavior (Carty et al. 2018). Laval deformations were observed at lower CBD concentrations (0.3 mg/L) than in Δ^9-THC (5 mg/L). Thus, this experiment could potentially be improved utilizing adult zebrafish to mitigate any potential deleterious developmental effects associated with chronic phytocannabinoid exposure. A possible explanation for the high variation, lack of reduced seizure behavior, and non-significant

differences between PTZ and negative control groups could be due to the Daneau's embryo media solution. Further investigation will be necessary to determine if this embryo media solution would be appropriate for this seizure model. Finally, concentrations administered may have been too low to exert an anti-convulsant effect. We examined the lower end of phytocannabinoid concentrations shown to produce anticonvulsant effects when administered acutely.

This work investigates chronic exposures of cannabinoids to an established PTZ model. Recommendations for future chronic models include exposure to phytocannabinoids at higher concentrations and investigation into whether an adult zebrafish model would be more appropriate for this type of experiment. This model might not be the best approach due to potential developmental effects. Requires greater investigation into development to further this work or validate this model.

CHAPTER 5. SUMMARY AND CONCLUSIONS

Interest in the clinical use of phytocannabinoids for the treatment of neurological disorders has increased in recent years as animal models have revealed the activation of CB_1 and CB_2R reduces the severity of seizures associated with epilepsy (Rosenberg et al. 2015). Mammalian models have demonstrated the efficacy of cannabinoids in reducing the severity of epileptic seizures, preventing seizures, and reducing seizure-related mortality (Marsicano et al. 2003, Wallace et al. 2003, Rosenberg et al 2015).

In recent decades, there has been particular interest in the use of Cannabis extracts for treatment-resistant epilepsy in children (Elliot et al. 2018). Many parents of children with treatment-resistant epilepsy have expressed interest in the use of Cannabis products as anecdotal cases of children with treatment-resistant epilepsy reveal Cannabis-derived products improve seizure-control. Currently, there has been limited clinical research of the benefits and harms of Cannabis use for people with epilepsy, especially in children and adolescents (Elliot et al. 2018). This is often attributed to the legal status of Cannabis products in many countries.

This body of work reveals zebrafish larvae exposed to phytocannabinoids prior to a chemically induced seizure had significantly reduced seizure behavior except for the highest concentration (1 mg/L) of the THV treatment group. A majority of our findings were consistent with those found in other chemically induced zebrafish seizure models of Δ^9-THC, CBD, and CBN (Ellis et al. 2018, Thornton et al. 2020). Furthermore, the cannabinoid treatments and concentration in the current study did not appear to reduce movement metrics from control conditions, indicating the absence of a sedative effect often associated with Δ^9-THC.

Currently, Δ^9THC, CBD, and other phytocannabinoids have been the primary focus of Cannabis research for clinical use however, there is increasing interest in terpenoid compounds

present in Cannabis plants (Erickson 2019, Russo 2011). Over 200 terpenoids have been identified in Cannabis plants, but relatively little clinical or peer-reviewed research has been conducted on their pharmacology and potential use as therapeutants (Russo 2011). Terpenoid composition in Cannabis resin varies, often substantially so, based on genetic, environmental, and developmental factors and while phytocannabinoid concentrations are generally predictable in Cannabis strains, terpenoid concentrations are often unknown or inconsistent (Booth 2017).

This experiment examined five of the most predominant terpenes present in Cannabis plants and revealed that two terpenoids (myrcene and limonene) reduced seizure behavior in 7 dpf zebrafish larvae. Review of current literature indicates that these results are consistent with other investigations into the anticonvulsant properties of limonene and myrcene (Pathak et al. 2010, Seo et al. 2020, Sorocco de Barros Viana et al. 2000). The remaining three terpenoid compounds (linalool, pinene, terpinolene) did not reduce seizure behavior in 7dpf zebrafish. Additionally, pinene and terpinolene produced mortality at 10 mg/L and 25 mg/L over the course of the treatment administration and observation period (110 minutes total). However, several studies utilizing terrestrial model organisms have revealed anticonvulsant activity of pinene, linalool, and linalool oxide at varying concentrations for each compound (Milanos et al. 2017, Negromonte Souto-Maior 2016). The zebrafish model may not be appropriate for studies of terpenoid anticonvulsant properties as terpenoid compounds may be more toxic to aquatic organisms and invertebrates than in higher vertebrate model species.

There is in increased need for studies on the effects of long-term Cannabis usage, as there is interest in CBD and Δ^9-THC for management of chronic pain as an alternative to opioid compounds. (Johnson et al. 2013, Ware et al. 2015). The pharmacokinetics, toxicity, and potential adverse health effects of Cannabis are still being revealed through laboratory

experiments and clinical trials and are even less understood in children. To date, most studies on the effects of long-term Cannabis usage are performed with adults (Devinsky et al. 2014). Overall, there is a need for more clinical investigations into the effects of long-term Cannabis use, especially over a time period greater than one year.

Results indicate 7dpf zebrafish larvae experienced no protective anti-convulsant effects from PTZ when exposed to CBD or Δ9-THC over a 7-day period. Though not significantly different, larvae exposed to 0.0625 mg/L and 0.125 mg/L Δ^9-THC moved less than the negative control, indicating a possible sedative effect. There is a need for more investigation into the efficacy of this chronic model, as the negative controls for both cannabinoid trials were not statistically different from the PTZ control. Several PTZ challenged treatment groups within both CBD and THC exposed larvae experienced greater variance than has been previously displayed with this seizure model (see Chapter 2). Our findings are consistent with other investigations into the anticonvulsant properties of CBD and Δ^9-THC as there are numerous studies supporting CBD and Δ9-THC's anticonvulsant effects in acute models, but less evidence in chronic models (Devinsky et al. 2014).

The use of movement metrics for screening potential anti-convulsant compounds within this larval zebrafish model holds promise for investigating the neuroprotective properties of phytocannabinoids, select terpenoids, and the future development of anti-seizure drugs.

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