"I tried to replicate what looked simple from the literature, and wasn't able to. Then I had a crisis of confidence, and then I learned that my experience wasn't uncommon."

- Baker M. 2016**.** 1,500 scientists lift the lid on reproducibility. Nature.

Characterizing the effects of poorly studied cannabinoids on larval zebrafish behaviour

by

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Bachelor of Science, University of New Brunswick 2019

A Thesis Submitted in Partial Fulfillment
of the Requirements for the Degree of

Master of Science in Biology

in the Graduate Academic Unit of Biology

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This thesis is accepted by the
Dean of Graduate Studies

THE UNIVERSITY OF NEW BRUNSWICK

August 2023

ABSTRACT

Cannabis sativa has been used medicinally for thousands of years. However, research on its health effects has been hindered due to prohibition and social stigma. Legalization provides Canadian researchers with the opportunity to apply modern research methods to better understand the benefits and dangers of pharmaceutical cannabinoids. The two main phytocannabinoids are tetrahydrocannabinol (THC) and cannabidiol (CBD). In addition, there are many lesser-known phytocannabinoids such as cannabinol (CBN), and cannabigerol (CBG) each with isomeric variants, which are poorly studied. In this thesis I document the behavioural effects of THC isomers as well as unexpected deleterious effects of CBD and CBN. I also bring attention to the importance of cannabinoid solubility in the context of aquatic research models. This research provides refinements to the use of zebrafish as model organisms for cannabinoid research, improves our fundamental understanding of how cannabinoids affect vertebrate behaviour, and highlights some of their potential as therapeutics.

DEDICATION

To my life partner Matt,

I couldn't have accomplished this without you, thank you.

ACKNOWLEDGEMENTS

As with any big accomplishment, this thesis could not have been completed on my own, and I have many people to thank.

Thank you to Dr. Bryan Crawford for taking me on as a student in a field that was new to both of us. Thank you for allowing me to use your lab space, and for supporting me throughout this long and tumultuous process. I wasn't sure if I'd ever make it to the end, but you never lost hope. And just as importantly, thank you to Ethicann Pharmaceuticals and Dr. Bruce Mackler for funding the project. I'd also like to thank you for your unwavering support during the many setbacks from both COVID and the numerous technical issues that we encountered. Thank you for your patience and for providing me with your extensive knowledge of the field. This project could not have been completed without you.

I also want to thank my lab mates, Rachael Wyatt, Nhu Trieu, Chris Small, Kate Gallant, Shahrzad Rahmanian, Clare Kilgour, Jill Hickey and Kate MacGregor. Thank you for always lending a helping hand, great conversation, and friendship. I truly wish we could have spent more time working together in-person. I'd also like to thank Marybelle Pack-Cameron for continuing this work.

I'd also like to thank several professors, Dr. Myriam Barbeau for taking the time out of her busy schedule to explain the statistics to me, and Dr. René Malenfant for helping me write my R code. I'd also like to thank Dr. Steve Heard for allowing me to use his controlled environment chamber, and Dr. Mike Duffy for allowing me to use his aquarium equipment. I also want to thank my supervisory committee, Dr. Yang Qu for letting me use your lab space and for your help conducting experiments, and Dr. Tillmann Benfey, thank you for all your support.

I have many people to thank for helping me with animal care. Krista Latimer and Heather Burke, you work so hard to take care of our fish. The UNB Aquatic Facility would not be able to do research without you, thank you. I also want to thank all the animal care workers over the past few years, Becky Jensen, Alex Dickey, and so many more. You saved our fish during the COVID shutdown.

Nicole Daigle and Taylor Burke, thank you for all that you do, and for supporting me when I wasn't sure where to turn. You are so knowledgeable and great assets in the department, as well as wonderful friends. You fight for the students who can't currently fight for themselves.

I'd like to thank my best friend and partner Matt Mabey for helping me with my calculations, experiments, and so much more. Thank you for your unwavering support throughout this entire process.

Lennox and Lexi, thank you for always being by my side and bringing me joy during the challenging days.

I also want to thank all my friends and family for their support and kindness over the past few years.

Thank you, thank you, thank you!

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List of Symbols, Nomenclature or Abbreviations

2-AG	2-Arachidonoylglycerol
AD	Alzheimer's Disease
ANA	Anandamide
CB_1	Cannabinoid receptor 1
CB_2	Cannabinoid receptor 2
CB receptor	Endocannabinoid receptor
CBC	Cannabichromene
CBD	Cannabidiol
CBDA	Cannabidiolic acid
CBDV	Cannabidivarin
CBG	Cannabigerol
CBN	Cannabinol
Cnr1	Cannabinoid receptor 1 gene
Cnr2	Cannabinoid receptor 2 gene
CNS	Central nervous system
dpf	Days post-fertilization
eCBs	Endogenous cannabinoid
ECS	Endocannabinoid system
fMRI	Functional magnetic resonance imaging
HD	Huntington's Disease

HIV/ AIDS	Human Immunodeficiency Viruses/ Acquired Immune Deficiency Syndrome
hpf	Hours post-fertilization
HU-210	(-)-1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol
HU-211	(+)-1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol
LC-MS	Liquid chromatography-mass spectrometry
MeOH	Methanol
MMAR	Marihuana Medical Access Regulations
MS	Multiple Sclerosis
PD	Parkinson's Disease
PTZ	Pentylenetetrazole
RGC	Retinal Ganglion Cells
THC	Tetrahydrocannabinol
THCA	Tetrahydrocannabinolic acid
THCV	Tetrahydrocannabivarin

1. Introduction

1.1 Cannabinoid Biology

The *Cannabis sativa* plant has been used for medicinal purposes for thousands of years, and in western medicine since 1839 (O'Shaughnessy & Calcutta, 1840). It is also the most widely used illicit drug in the world (Krug & Clark, 2015). Raphael Mechoulam described it as a "pharmacological treasure trove" when he and his colleagues first isolated and synthesized tetrahydrocannabinol (THC) and cannabidiol (CBD) in the 1960's (Mechoulam & Burstein, 1973). The two main phyto-cannabinoids found in *Cannabis sativa* are CBD and THC (Figures 1.1 and 1.2). They are approved for pharmaceutical use in many countries, as are synthetic cannabinoids and cannabinoid extracts.

Since the 1960's many other cannabinoids have been characterized, but we are only beginning to understand their pharmaceutical effects and the underlying molecular mechanisms by which these compounds function, and the details of the endocannabinoid system (ECS) with which they interact. There are thousands of research articles and hundreds of clinical studies on the pharmacodynamics and pharmacology of cannabinoids, focusing variously on how they affect learning, appetite, sleeping, healing, and many other physiological and pathological processes. Because there are so many compounds in *Cannabis* plants it is important to consider both how these compounds may interact (i.e. synergistic and 'entourage effects', which is the suggested positive contribution from the addition of terpenes to cannabinoids (Ferber *et al.* 2020)) and the pharmacological potential of minor compounds and subtle chemical variations that may be present (Russo & Marcu, 2017).

In addition to THC and CBD, other less well-studied cannabinoids include cannabinol (CBN), cannabichromene (CBC), cannabigerol (CBG), tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), tetrahydrocannabinolic acid (THCA), and cannabidiolic acid (CBDA) (Figure 1.1). Adding further complexity to the chemical cocktail found in extracts of *Cannabis* plants are complex mixtures of terpenes and other alkaloids, all of which may have effects on the biological activities of the primary cannabinoids.

1.1.1 The Endocannabinoid System

The endocannabinoid system (ECS) is very important in the developing nervous system and modulates neuronal activity and network function in the mature nervous system (Lu & Mackie, 2021). It is made up of the endocannabinoid receptors (CB receptors), their ligands the endogenous cannabinoids (eCBs), and proteins that synthesize, transport, and degrade eCBs (Lu & Mackie, 2021). The ECS is multifunctional and plays a role in many signaling pathways, which is important to note when discussing drugs that target the ECS (Lu & Mackie, 2021). The two main receptors in the endocannabinoid system are the cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2). Both are G-protein-coupled receptors, primarily interacting with inhibitory G-proteins. They inhibit certain voltage-sensitive calcium channels, stimulate kinases and mitogen-activated proteins, and also recruit beta-arrestin, which provides feed-back inhibition for the signaling pathway, and have other actions (Howlett *et al.* 2002).

CB_1 receptors are most abundant in the central nervous system (CNS), but are also present in the liver, adipose tissue, and skin (Lu & Mackie, 2021). In the neurons CB_1 is found at synaptic terminals, reflecting its role in modulating synaptic transmission (Nyiri *et al.* 2005). CB_2 is expressed by microglia in the CNS, and by other immune effector cell types (Cabral *et al.* 2015), consistent with its reported role in modulating the inflammatory response. Tanaka *et al.* (2020) speculate that CB_2 receptor signaling changes the balance of metabolic and gene regulatory states of the microglial cells away from neuroinflammatory M1 genes and towards neuroprotective M2 genes and homeostatic M0 genes.

1.1.2 Isomers

Multiple isomers of cannabinoids exist and are present in extracts of various strains of *Cannabis* (Figures 1.2 and 1.3). The two most common isomers of THC are **Δ**9-THC, and **Δ**8-THC (Hollister & Gillespie, 1973; Hollister, 1974). In addition to structural isomers, there are also cis and trans stereo isomers (i.e. enantiomers) of each of these THC isomers (Dos Santos *et al.* 2018). Another important compound is 11-hydroxy-**Δ**9-THC (Figure 1.4), a metabolite of **Δ**9-THC, which has different properties and differing behavioural effects than **Δ**9-THC. CBD has (+) and (–) enantiomers as well, of which only (+) CBD has been found to oppose seizures (Leite *et al.* 1982).

In addition to phytocannabinoids, there are also synthetic cannabinoids with differing effects. (-)-1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol (HU-210; Figure 1.5) is a synthetic cannabinoid derived from Δ8-THC that is 80-800 times more psychoactive than Δ9-THC, while the (+) enantiomer of the same compound (HU-211; Figure 1.5) has no detectable psychoactive effect. Interestingly, HU-211 is not effectively bound by the CB receptors but does retain potentially useful pharmacological effects (Shoami & Mechoulam, 2000).

1.1.2.1 THC

THC is the most studied phytocannabinoid with hundreds of published clinical studies on its properties and effects (Russo & Marcu, 2017). THC interacts with both CB_1 and CB_2 receptors (Russo, 2011). As an example of the pharmacological potential of cannabinoids, THC has 20x the anti-inflammatory power of aspirin as well as anti-nociceptive properties in rats, based on paw edema formation (Duane Sofia *et al.* 1974). When bound to the CB_2 receptor THC is associated with pain relief and anti-inflammatory activities but is not associated with behavioural effects (Russo & Marcu, 2017).

Δ8-THC is a minor constituent of *Cannabis sativa.* Very little research has been done on this or other isomers of THC, but a few studies have revealed similar effects with similar metabolites. In 1973, Hollister and colleagues concluded that the placement of the double-bond, changing the length of the side chain, or producing hydroxy metabolites at the 11- or 8- position, does not alter the actions of THC, but can drastically change the potency (Hollister & Gillespie, 1973).

1.1.2.2 CBD

CBD is the main non-intoxicating cannabinoid found in *Cannabis sativa*. CBD has a low affinity for the CB receptors, and unlike THC, does not appear to function as an agonist of the CB_1 receptor. However, CBD can compete with and thereby antagonize THC binding to CB_1 if the concentration of CBD is high relative to that of THC. In 2010 Bhattacharyya and colleagues administered **Δ**9-THC after either placebo or CBD and used functional magnetic resonance imaging (fMRI) to determine that CBD decreases the psychogenic effect of **Δ**9-THC in humans (Bhattacharyya *et al.* 2010). Similarly, Achenbach *et al.* (2018) exposed zebrafish larvae to **Δ**9-THC with and without CBD and observed that larvae exposed to THC in the presence of CBD exhibit reduced behavioural effects.

In addition to antagonizing the psychoactive effects of THC, CBD has many pharmacological properties, and is used as an antioxidant, anti-psychotic, anti-convulsive, and anti-inflammatory (Russo & Marcu, 2017). It has neuroprotective properties and can help treat neurological disorders, such as multiple sclerosis (MS), Parkinson's disease (PD), and epilepsy (De Lago & Fernandez-Ruiz, 2007). CBD can also counteract undesirable effects of THC, such as anxiety, hunger, sedation, as well as the intoxicating effects (Russo & Marcu, 2017). CBD is used in a specific ratio of 1:1 with THC in medical *Cannabis* preparations such as Sativex®, and, despite the limited amount of research to support it, is becoming widely viewed as a panacea for a plethora of pathologies (Barnes, 2006).

1.1.2.3 CBG

CBG is structurally very similar to CBD (Figure 1.1), and like CBD it is non-psychoactive. It is of commercial and pharmaceutical interest, but little is known about its anti-convulsant properties (Hill *et al.* 2014). Hill and colleagues concluded that CBG has no effect on the severity of seizures in a pentylenetetrazole (PTZ) model of generalized seizures in mice (Hill *et al.* 2014). In a PTZ model of seizures in zebrafish CBG reduced seizure index at a concentration of 4 μM but increased seizure index at 2 μM (Kollipara *et al.* 2023). Interestingly, when CBG was combined with CBD Kollipara and colleagues found a 95% reduction in seizure index (Kollipara *et al.* 2023). CBG's binding affinity for the CB receptors has been reported to change based on concentration (Navarro *et al.* 2018), but it is reported to be in the micromolar range. Some speculate that it may be functioning as a sedative as opposed to antagonizing seizures (Kollipara *et al.* 2023), and it also binds several other receptors expressed by a variety of neural and non-neural cell types (Cascio *et al.*2010), so more research is needed to elucidate the mechanism(s) by which CBG functions in the vertebrate nervous system.

1.1.2.4 CBN

The non-enzymatic oxidation product of THC is CBN (Figure 1.1). It is commonly found after prolonged or improper storage of THC and was the first cannabinoid to be isolated and identified (Wood & Spivey, 1899). Using COS-7 cells expressing the rat CB_1 receptor as well as rat brain synaptosomal membrane preparations, Rhee *et al.* (1997) found that CBN maintains ¼ of the potency at the CB_1 receptor compared to THC. Like THC, CBN has demonstrated many therapeutic effects, such as anti-inflammation, anti-biotic, anti-convulsant, as well as sedative effects (Rhee *et al.* 1997).

Chousidis *et al.* (2020) found that the LD_{50} of CBN for zebrafish larvae at 5 days post-fertilization (dpf) is 1.12 mg/l (3.6 μM), and embryos exposed to concentrations of 0.75 mg/l (2.4 μM) or higher after 72 hours post-fertilization (hpf) showed abnormalities such as tail bending, pericardial edema, and yolk-sac abnormalities. Embryos exposed to CBN also showed a significantly higher heart rate at 96 hpf when given 0.75 mg/l (2.4 μM) compared to vehicle controls, which is indicative of a stimulant effect (Chousidis *et al.* 2020). Larvae exposed to CBN also showed a significant decrease in their distance traveled in both light and dark environments, compared to vehicle controls, which is indicative of a sedative effect (Chousidis *et al.* 2020).

1.1.3 Other Secondary Metabolites in *Cannabis*

Secondary metabolites are organic compounds that are not critical for the growth, development, or reproduction of the plant. They may have a variety of survival functions such as functioning as odorants to attract pollinators, and they often have unusual and complex chemical structures produced by complex biosynthetic pathways (Nazir Tahir *et al.* 2021). Plant secondary metabolites are important defense mechanisms against herbivory and plant pathogens, and function in inter- and intra-species competition. Humans use plant secondary metabolites for many things such as flavourings (e.g., herbs and spices), essential oils, pigments, food additives, pharmaceuticals, and industrial feed stocks, and as recreational drugs (Tiwari & Rana, 2015).

In addition to THC and CBD and other phyto-cannabinoids present in *Cannabis sativa* there are many terpenoids and other compounds. There may be synergistic relationships and complimentary roles of these chemicals with respect to the therapeutic efficacy in the treatment of pain, epilepsy, multiple sclerosis, and other diseases (Russo & Marcu, 2017).

1.1.3.1 Terpenoids

Terpenoids can be found in many plants and the *Cannabis* plant is no exception with as many as 200 described. Terpenoids are aromatic compounds that fulfill many ecological roles for the plant, such as attraction of pollinators and protection from predation (Russo & Marcu, 2017). Some of the most common terpenoids are myrcene, terpinolene, limonene, caryophyllene, and bisabolol (Fischedick, 2017) (Figure 1.6). Like other secondary metabolites, terpenoids play many roles for the plant such as protection from herbivores and plant pathogens, and attraction of pollinators. They are aromatic compounds that are often produced most abundantly in dedicated structures called the trichomes, which is also where the cannabinoids are produced. The aromatic mixtures sold as "essential oils" consist primarily of the plants' terpenoids. Unlike cannabinoids, which have been the focus of research for centuries, terpenoids remain poorly studied and represent a promising avenue of investigation with respect to pharmaceutically active phytochemicals (Booth *et al.* 2017). There has been a lot of debate on whether the terpenoids found in *Cannabis* are important. However, even at low concentrations they have behavioural effects, such as an increase or decrease in activity levels of rodents (Buchbauer *et al.* 1993). Historically in wild and naturally cultivated *Cannabis* plants, terpenoid concentrations in the flower have been in the 1% range, and up to the 10% range within the trichomes (Potter, 2009). Recently with selective breeding the concentration within the flower has risen to 3.5% and higher (Fischedick *et al.* 2010).

Observational studies have reported that adding terpenoids to THC can result in different subjective psychogenic effects. When limonene and THC were given together the experience was reported to have a “cerebral and euphoric” effect, while myrcene and THC was reported to have more of a “physical, mellow, and sleepy” effect (Russo & Marcu, 2017). THC given alone was more dysphoric than euphoric (Calhoun *et al*. 1998).

1.2 Historical Research into the Medicinal Use of *Cannabis*

Cannabis was first exploited by humans more than 10 000 years ago. Potsherds found at the Okinoshima archaeological site in Japan (12500 – 2300 BC) have *Cannabis* seed and fruit macrofossils attached (Okazaki *et al.* 2011). The first historical use of *Cannabis* as a medicinal was documented in the Chinese book, “Shen Nung Pen Ts’ao Ching” which was written during the first century B.C., although it is believed they were using hemp, as none of the psychological effects were described (Li, 1974; Jiang *et al.* 2006). Since then, *Cannabis* use has been documented in many countries around the world such as India, Egypt, Greece, and many Latin American countries. The first evidence of *Cannabis sativa* being used medicinally comes from around 400 A.D. in Egypt where it was found in a family tomb site from this period (Zias *et al*. 1993). Cannabis has also been used in various rituals for thousands of years in cultures across the world (Chasteen, 2016). Its first documented use in western medicine was in the 1830’s when O’Shaughnessy realized the *Cannabis* from India was vastly different than the *Cannabis* from Europe. He tested the two types of *Cannabis* on many animal species before using it in the treatment of patients. He found *Cannabis* was a great anti-convulsant (O'Shaughnessy, 1839). The molecular mechanisms by which consumption of *Cannabis* results in psychogenic and physiological effects were first investigated by Raphael Mechoulam and colleagues, who discovered and isolated THC in the 1960's (Mechoulam, 1973). The first cannabinoid receptor (CB_1) as well as the first endocannabinoid (anandamide (ANA)) were discovered by Devane *et al.* (1988) in the late 1980's. Due to prohibition, research on the *Cannabis* plant has been hindered, only recently becoming more productive after legalization in certain countries.

1.3 Effects of Prohibition on Research

Research on the *Cannabis sativa* plant has been hindered since the early 1900's due to legal prohibition and social stigma. *Cannabis sativa* was made illegal in Western Countries in the 1920's, with Canada adding it the Confidential Restricted List under the *Narcotics Drug Act Bill* in 1923 (House of Commons debates, 14 parliament, 2nd session: Vol 3). In 2001 Canada legalized *Cannabis sativa* for medicinal purposes and legalized medical and recreational use of the *Cannabis sativa* plant in 2018 (Government of Canada, Medical uses of *Cannabis*, 2021). Beyond criminalization, prohibition created many restrictions and limitations on research by restricting access of *Cannabis* for academic purposes (Bridgeman, 2017). Now that *Cannabis* is legal in Canada this provides Canadian researchers with an unprecedented opportunity to apply modern research methods to better understand the potential benefits and dangers of pharmaceutical cannabinoids.

1.4 Cannabinoids as Pharmaceuticals

As discussed previously, cannabinoids have various analgesic, anti-inflammatory, immunomodulatory, anti-anxiolytic, and anti-spasmodic properties, and they have become increasingly popular in the treatment of many disorders over the past twenty years (Russo & Marcu, 2017). Canada was the first nation to establish a national medical *Cannabis* program (Lucas & Walsh, 2017). In 2001 the Marihuana Medical Access Regulations (MMAR) allowed patients with incurable or chronic illnesses to apply to Health Canada for permission to possess a small amount of medical *Cannabis*. A few of the illnesses included in the list were HIV/AIDS, severe chronic pain, cancer, and multiple sclerosis (Walsh *et al.* 2013). Lucas *et al.* (2019) conducted a survey in Canada and found that most patients use medical *Cannabis* for pain and mental health conditions. These conditions accounted for 83.7% of the respondents (Lucas *et al.* 2019). The main side effects and drawbacks of using *Cannabis* as a pharmaceutical are the psychoactive effects. These psychoactive effects are associated with activation of the CB_1 receptor in the CNS. Consequently, drugs that solely target the CB_2 receptor should not have these side effects and should therefore have improved pharmaceutical potential (Ogawa *et al.* 2018). For example, the CB_2 receptor agonist ABK5 is a good anti-inflammatory and analgesic agent, which does not have the psychoactive side effects (Tang *et al.* 2021).

1.4.1 Anti-Inflammatory Effects

CB_1 is expressed primarily in the CNS, especially in the hippocampus, cerebellum basal ganglia, and olfactory bulb (Zou & Kumar, 2018), whereas CB_2 is primarily expressed by immune and glial cells, suggesting molecules modulating activity at CB_2

are more likely to be relevant to inflammatory disorders (Ogawa *et al.* 2018). The specific role of endocannabinoids in the immune system is still unclear; however, cannabinoids have potent effects on cytokine production in immune cells (Croxford & Yamamura, 2005). The endocannabinoid 2-Arachidonoylglycerol (2-AG) binds and activates the CB_2 receptor, whereas plant-derived cannabinoids such as THC act as partial agonists at the receptor level (Croxford & Yamaura, 2005). As a partial agonist, THC will occupy the receptor and only partially activate it, and therefore will reduce signaling from the endogenous ligand. The use of different concentrations and types of cannabinoid ligands has made comparing across studies extremely difficult, and more research must be done. Cannabinoids have been successfully used in the treatment of neuropathic pain mediated through the CB_2 receptor in auto-immune disorders such as diabetes and rheumatoid arthritis (Ibrahim *et al*. 2003), demonstrating some of their potential in this context.

1.4.2 Anti-Anxiety Effects

264 million people worldwide have been diagnosed with an anxiety disorder, making this one of the most prevalent mental health disorders (Smith-Apeldoorn *et al.* 2019). Even though there are many treatments available for those who suffer with an anxiety disorder, such as behavioural cognitive therapy and medication, only 40-60% of adults are responsive to this kind of intervention (Bystritsky, 2006). This leaves many patients looking for alternative remedies. This has prompted research into how cannabinoids affect emotional regulation and anxiety management (Papagianni & Stevenson, 2019). Some studies have shown promising results using CBD as an

anxiolytic, but effects vary with treatment duration as well as dose. Another study demonstrated that THC has an anxiogenic effect on mice (Schramm-Sapyta *et al*. 2007). *Cannabis* that has a high concentration of THC appears to induce anxiety behaviour, with the effects being dose-dependent (Rubino *et al.* 2015). The evidence to support *Cannabis* as a beneficial treatment for anxiety disorders is weak, with some of the side effects exacerbating the problems; this is likely an issue arising from poorly-characterized differences in the chemical composition of treatments based on extracts of plants producing different suites of cannabinoids and/or other secondary metabolites that alter the activity of the cannabinoids, as well as potential differences in the composition (including isomeric variations) of the cannabinoids themselves. Clinical trials must be conducted, and more research must be done to elucidate the underlying molecular mechanisms of these effects, and the pharmacodynamics in humans as well as the risks of *Cannabis* use in anxiety related disorders (Graczyk *et al.* 2021).

1.4.3 Anti-Spasmodic / Seizure-Antagonizing Effects

Epilepsy is a common disease that affects 1% of adults and 2% of children, and cannabinoids are potentially valuable therapeutics in this context as well (Hauser & Hesdorffer, 1990). Schmidt & Sillanpaa (2012) estimate that between 25% - 40% of newly diagnosed epilepsy patients will have seizures that are resistant to current treatment. One of the goals of the International League Against Epilepsy/American Epilepsy Society is to find new research strategies and model systems to find new methods of seizure control (Wilcox *et al.* 2013). Because the CB_1 receptor is prevalent in the central nervous system it seems plausible that these seizure-antagonizing effects are mediated through CB_1. Previous findings from a multitude of studies using both *in vivo* and *in vitro* models of seizures show that activation of the pre-synaptic CB_1 receptor suppresses epileptic activity (Blair *et al.* 2015). Recently there have been many clinical trials assessing the ability of CBD to oppose epileptic seizures. The response rate ranges between 36% to 50%, which is very high compared to other forms of treatment; however, we do not yet have a good understanding of the mechanism(s) by which CBD antagonizes seizures (Zhang *et al.* 2023). And as with any medication there are still some adverse effects, such as somnolence, fatigue, and decreased appetite, but these were moderate to mild (Morano *et al.* 2020). Further research using biomedical models will help better understand and mitigate these risks (Morano *et al.* 2020).

1.4.4 Other Neurodegenerative Disorders

Other neurodegenerative disorders for which cannabinoids are promising treatments include Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's Disease (HD), and Multiple Sclerosis (MS) (Zhang *et al.* 2023). Aso and colleagues demonstrated that a mixture of CBD and **Δ**9-THC could reduce cognitive impairment in a murine model of AD (Aso *et al.* 2015).

In PD models there is an increase in CB_2 receptor expression, which could indicate dysregulation of the endocannabinoid system. There is also substantial evidence to suggest that the CB_2 receptor plays in important role in the regulation of neuroinflammation that is associated with PD (Duffy *et al.* 2021). By modulating the CB receptors and their ligands, we may be able to provide an effective treatment for this disease (Zhang *et al.* 2023).

In HD there is a downregulation of the CB_1 receptor and an accumulation of mutant Huntington proteins (Fernandez-Ruiz, 2009; Welcome, 2020). This accumulation of aberrant protein can cause inflammation and tissue damage in the CNS, but activation of the CB_2 receptor may decrease this inflammatory response. Using cannabinoids such as CBD and THC may prove to be an effective therapy for treating HD.

Yiangou and colleagues found that the endocannabinoid system could play a role in the development of MS (Yiangou *et al.* 2006). MS patients have reported relief in pain and muscle spasm when using a combination of THC and CBD (Holgado *et al.* 2017). Frustratingly little is known about the underlying molecular mechanisms of MS, or how molecules like CBD and THC mitigate the pathology of this disorder, but cannabinoids are a promising new treatment (Zhang *et al.* 2023).

1.5 Biomedical Research Models for *Cannabis* Research

All vertebrates, as well as many invertebrates such as leeches, mussels, and sea urchins have an endocannabinoid system (McPartland *et al.* 2001). This means that there are many biomedical models to choose from when doing biomedical *Cannabis* research. However, the endocannabinoid system of invertebrates such as *Drosophila* are not very similar to humans as they lack the CB_2 receptor, which makes them less-suitable for biomedical research (McPartland *et al.* 2001). Mammalian models such as rats, rabbits, and mice are all very expensive to house, feed, and care for, and raise ethical issues regarding the use of animals in research. Zebrafish are an excellent vertebrate biomedical model that are especially valuable in the early stages of drug development as they are widely used for toxicological analysis of drugs, and increasingly for pursuit of lead compounds before more costly mammalian models are used (Beliaeva *et al.* 2010).

1.5.1 Zebrafish are a Good Model for Biomedical Research

Zebrafish are emerging as a premier biomedical research model for investigating basic biology and human diseases, and for testing potential drugs (Marrs & Sarmah, 2021). But they are not without disadvantages. The most obvious shortcoming of the zebrafish with respect to their use as a biomedical research model is that they are not mammals. Zebrafish are teleost (bony) fish of the family *Cyprinidae*. The last common ancestor of zebrafish and humans lived over 400 million years ago (Zhu *et al.* 2006), and while many aspects of vertebrate biology have been stable since then, there are obviously many differences that must be considered when trying to generalize results from fish to humans. And even obvious differences can have subtle implications; for example, fish are poikilotherms and thus the way they metabolize compounds is temperature dependent, affecting the pharmacokinetics of a drug differently from mammals (Lardelli, 2008). A great example of this is the use of tricaine for anesthetizing zebrafish embryos. Tricaine methanesulfonate is not effective in mammals as it gets metabolized too quickly due to their higher body temperatures, but it works well and is widely used on fish (Wayson *et al*. 1976).

Nevertheless zebrafish, particularly their larvae, have many advantages over other model organisms. They are a low-cost, high-throughput alternative to costly mammalian models. Captive zebrafish spawn year-round and a tank of 20 zebrafish can yield hundreds to thousands of embryos at each spawning. They develop externally and very quickly, reaching larval stage at 120 hpf. Zebrafish larvae can absorb small molecules across their skin and their genome is fully sequenced, well annotated, and highly amenable to forward and reverse genetic manipulation. The zebrafish research community has developed and shares literally thousands of transgenic and mutant strains, as well as over a dozen 'wildtype' lines.

In 2000 Peterson *et al.* (2000) demonstrated that live zebrafish could be used for screening chemicals in a 96-well plate by simply adding the compounds to their media (Peterson *et al.* 2000). Since then, hundreds of examples of small molecules have been shown to have similar effects in zebrafish and humans (Rennekamp & Peterson, 2015).

1.5.2 Zebrafish as a Model for *Cannabis* Research

The zebrafish genome encodes homologous CB_1 and CB_2 receptors (encoded by the *cnr1* and *cnr2* genes, respectively), that are expressed by 24 hpf, making zebrafish larvae an excellent model system in which to study the effects of cannabinoids in vertebrates (Krug & Clark, 2015).

The zebrafish *cnr1* receptor shares 65.5%–69% similarity in nucleotide sequence and 65.9%–74.9% similarity in amino acid sequence with tetrapod orthologues (Lam *et al.* 2006). Both CB receptors are present in zebrafish at 24 hpf, and by 48 hpf the CB_1 receptor is expressed widely in the CNS (Rodriguez-Martin *et al.* 2007; Oltrabella *et al.* 2017). Recently researchers have begun using zebrafish to investigate the behavioural, toxicological, metabolic, and other biological effects of cannabinoids on zebrafish (Achenbach *et al.* 2018; Samarut *et al.* 2019; Bailone *et al.* 2022; Kollipara *et al.* 2023).

Carty and colleagues demonstrated that both THC and CBD were toxic to embryos at concentrations near their limits of solubility (The LD_{50} for CBD is 0.53 mg/L (1.69 μM). The LD_{50} for THC is 3.65 mg/L (11.6 μM), and the solubility of THC is 8.9 μM (Yalkowsky *et al.* 2010)). They also demonstrated that THC and CBD cause developmental delays in zebrafish and lead to many dysmorphologies (Carty *et al.* 2018). CBD is sparingly soluble in aqueous solutions with an empirically measured solubility of 0.2 μM determined by phase solubility (Mannila *et al.* 2007; Lv *et al.* 2019). This draws into question research reporting its use at concentrations well above this limited solubility (e.g., Achenbach *et al.* 2018; Jensen *et al.* 2018; Carty *et al*. 2018; Chousidis *et al.* 2020; Kollipara *et al.* 2023) and increases the relevance of recent investigations into ways to improve CBD solubility and bioavailability in aqueous media (Lv *et al.* 2019).

1.6 Cannabinoids Affect the Swimming Behaviour of Larval Zebrafish

Solubility limitations notwithstanding, researchers have reported treating adult zebrafish with 40 mg/L (127.19 μM) CBD and saw that they swam significantly less than adults exposed to vehicle controls (Jensen *et al.* 2018). However, this effect is not seen in larval zebrafish. When Achenbach *et al.* (2018) exposed larval zebrafish to a reported concentration of 3.75 μM CBD, they did not see any decrease in total swimming distance. When zebrafish were exposed to as little as 2 μM **Δ**9-THC, Achenbach *et al.* (2018) saw a significant dose-dependent decrease in distance traveled.

1.6.1 Behavioural Effects of Cannabinoids on Zebrafish Larvae

Achenbach *et al.* (2018) quantified the short-term effects of THC and CBD on larval swimming behaviour using a high-throughput behavioural analysis system. This experimental paradigm measures the basal activity in a continuously lit environment as well as the response to light/dark transitions, which produce both a startle-response and anxiety-based hyperactivity (Ellis *et al.* 2012). The startle response is triggered by a light to dark transition. Retinal ganglion cells (RGC) integrate signals from photoreceptors and other neurons in the retina and transmit signals via their axons projecting into the optic tectum and contralateral thalamus (Robles *et al.* 2014). The thalamus relays this information to the deep layers of the tectal neuropil (Heap *et al.* 2018). This information is further processed through poorly understood pathways, resulting in activation of pathways to premotor structures that shape the motor response (Sato *et al.* 2007). The initial response to 2 μM **Δ**9-THC is a decrease in distance traveled for a few minutes, followed by approximately half an hour of hyperactivity. After 30 minutes swimming distance decreases to below control levels, and this hypoactivity persists for at least 150 minutes. Light/dark transitions trigger startle responses in larvae, and THC-treated larvae exhibited startle responses comparable to controls. However, there does appear to be reduced activity during the (anxiogenic) ten-minute dark periods in larvae exposed to the highest dosages (2 μM) of THC.

Inconsistent with widely held views regarding CBD's lack of psychogenic effects associated with THC, zebrafish larvae exposed to CBD also exhibit initial hyperactivity in a dose-dependent manner between 1 μM and 3.75 μM CBD, after which larvae return to basal activity levels comparable to controls. Achenbach and colleagues report a strongly repressed startle response and complete absence of anxiety behaviour during the light/dark transitions in larvae treated with 3.75 μM CBD. Finally, these authors report co-administration of 0.5 μM THC with 0.5 μM CBD yielded larval activity patterns indistinguishable from controls, and the administration of 1.5 μM THC with 0.5 μM CBD restored normal activity after the half hour of hyperactivity characteristic of the initial effects of THC.

Achenbach and colleagues also determined the concentration of cannabinoids, as well as some of their metabolites, in the larval tissues using mass spectrometry. The amount of THC detected increases very quickly in the first hour, and then more gradually until it eventually begins to drop after about 6 hours. The highest concentration of THC recorded in a larva was 70 mg/kg a little more than five hours after the addition of the compound to the medium. In larvae exposed to CBD, they saw a similar rapid increase during the first hour of exposure. After the first hour the concentration rose at a slower pace and leveled off at 200 minutes. The concentration then dropped at a steady pace until the end of the experiment (500 minutes). The highest concentration of CBD measured in a larva was 35 mg/kg at around 200 minutes (Achenbach *et al.* 2018).

1.6.2 Antagonizing Seizures with Cannabinoids in a Zebrafish Larval Model of Epilepsy

THC and CBD synergistically antagonize chemical and genetic models of epileptic seizures in zebrafish larvae (Samarut *et al.* 2019). Using both a chemically induced seizure model using PTZ, and spontaneous seizures in GABA receptor subunit alpha 1 mutant larvae, Samarut and colleagues showed that both THC and CBD alone reduce the fast-twitch swimming representative of seizure, and combining both cannabinoids increase their effectiveness in this model (Samarut *et al.* 2019). PTZ causes changes to zebrafish behaviour such as an increase in swimming behaviour followed by fast-darting activity, and a loss of posture. It also causes electrophysiological changes of small amplitude discharge reminiscent of interictal bursts and is accompanied by regionalized elevation in neuronal activity (Ellis *et al.* 2012). *GABRA1* knockouts are fish which produce sporadic seizures that are triggered when they are exposed to light. Targeted knockouts of either GABA receptors or sodium channels also results in spontaneous seizures (Samarut *et al.* 2018).

Thus, cannabinoids represent extremely promising compounds for the treatment of seizure disorders, as well as a plethora of other disorders as discussed above. Furthermore, the high-throughput behavioural analysis of larval zebrafish activity is an excellent experimental paradigm for these types of investigations.

1.6.3 For Optimal Seizure Reduction, CBD can be Combined with Additional Cannabinoids

THC, CBD, CBN, CBC, and CBG can be used independently or in combination to antagonize chemical models of epilepsy in zebrafish larvae (Kollipara *et al.* 2023). Using PTZ to chemically induce seizures, Kollipara and colleagues showed that CBD, **Δ**9-THC, **Δ**8-THC, CBN, CBC, and CBG independently lower seizure index. They also showed that combinations of CBD and **Δ**9-THC, CBD and CBG, as well as CBD and **Δ**8-THC, further reduce seizure index more than the independent cannabinoids (i.e. they act synergistically). A combination of CBD and CBN, as well as CBD and CBC, were not significant in reducing seizures versus the cannabinoids independently. It is also worth noting that CBG at 2 μM increased seizure index compared to 1 μM, but then decreased again at 4 μM (Kollipara *et al.* 2023).

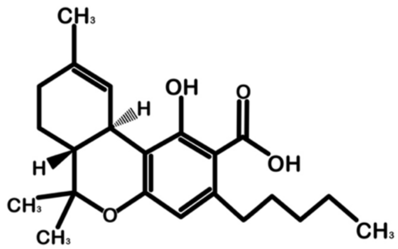
It appears that cannabinoids represent a promising treatment option for seizure relief, and that CBD and CBG may offer increased relief similar to CBD and **Δ**9-THC, but one might predict they would lack the psychoactive effects of **Δ**9-THC.

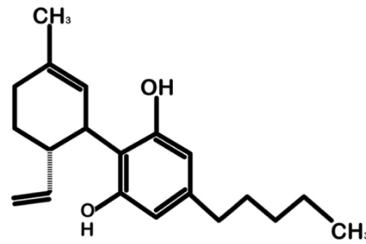
1.7 Objectives

After decades of prohibition, the 'treasure trove' of potential pharmaceuticals derived from the *Cannabis sativa* plant are now much more accessible to researchers interested in testing and understanding their alleged abilities to antagonize seizures, ameliorate anxiety, protect against inflammation, relieve pain, help with psychological disorders, and open therapeutic avenues for myriad diseases causing untold suffering and economic loss. But to do so, we need to better understand the underlying biology, and to explore the vast combinations of cannabinoids and other secondary metabolites like terpenes and other alkaloids that may synergize with or modulate the effects of cannabinoids on the vertebrate nervous system and other tissues. The zebrafish provides both an excellent model system for fundamental biomedical research into the underlying molecular biology and physiology of the effects of cannabinoids and the opportunity to use high-throughput behavioural biology to efficiently analyze their behavioural effects with statistical rigor.

In this thesis I attempt to replicate the studies reported by Achenbach *et al.* (2018) and Samarut *et al.* (2019) and build upon them to investigate the therapeutic potential of isomers and metabolites of THC and CBD, as well as some other poorly studied cannabinoids, such as CBN, using the high-throughput larval zebrafish behavioural analysis platform. I begin by establishing the assay, which proved to be challenging due to the many (and often poorly documented) parameters that can significantly alter the behaviour of zebrafish larvae in this assay. These range from fairly obvious (e.g., temperature, vibration, size of the wells, age of the larvae, etc.), to subtle (e.g., motion of the water that larvae are raised in prior to being used in the assay, time of day, etc.), to difficult to control (e.g., inadvertent selection of slower-swimming larvae when setting up the assay). Having established the assay and statistical analysis pipeline, in Chapter 2, I compare the effects on basal locomotion and visual startle of some isomers and metabolites of THC to **Δ**9-THC and find that I am able to largely reproduce the effects of **Δ**9-THC reported by Achenbach *et al*. (2018). Interestingly, I observe that both **Δ**8-THC and 11-hydroxy-**Δ**9-THC have distinctly different behavioural effects on zebrafish larvae. Unfortunately, in my effort to reproduce the PTZ seizure assay developed by Samarut *et al.* (2019), I discovered a limitation of our instrument which prevented me from testing the potential of these THC variants in antagonizing seizures. Furthermore, and most frustratingly, my investigations of potential synergistic effects of combining various THCs with CBD or CBN revealed completely unexpected and previously unreported deleterious effects of these compounds on zebrafish larvae. These may be due to previous researchers using intermediate dilutions of these compounds, which were likely above their solubility limits and therefore may have resulted in some loss of the compounds by precipitation. However, the concentrations I observe in the tissues of my treated larvae are consistent with (and sometimes even lower than) previously reported results, so this remains somewhat of a mystery.

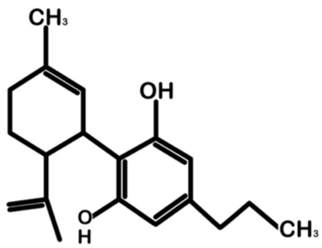
I conclude that this high-throughput behavioural analysis approach to studying the effects of potentially therapeutic compounds using zebrafish larvae has great potential for drug discovery, but is not as straight-forward as one might imagine.

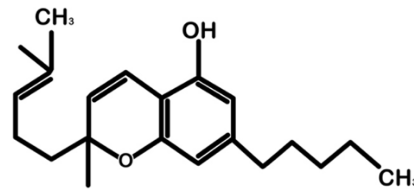




(-)-**Δ**9-tetrahydrocannabinolic acid (THCA)

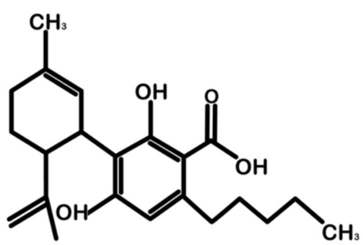
(-)-Cannabidiol (CBD)

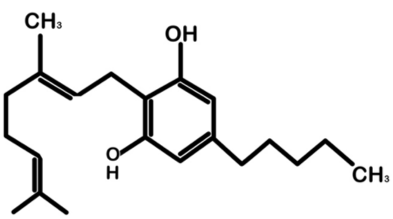




Cannabidivarin (CBDV)

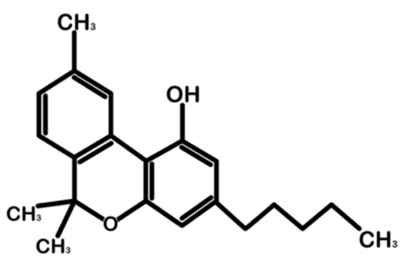
Cannabichromene (CBC)

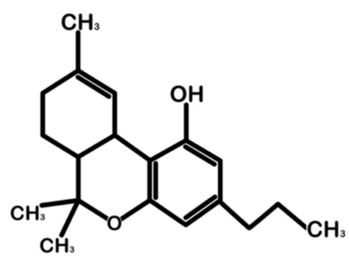




Cannabidiolic Acid (CBDA)

Cannabigerol (CBG)

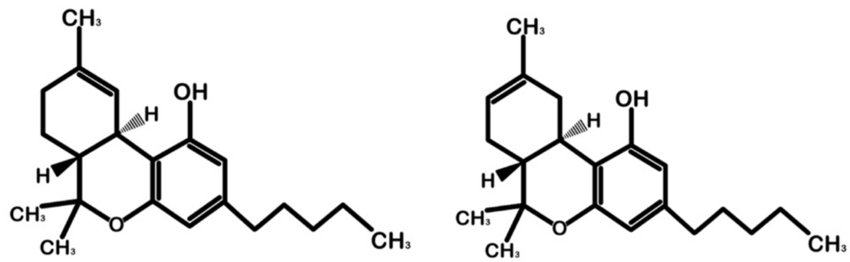




Cannabinol (CBN)

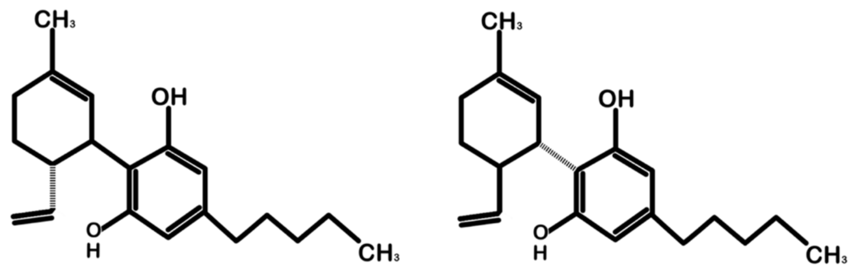
Tetrahydrocannabivarin (THCV)

Figure 1.1. Cannabinoid Structures



(-)-Δ9-tetrahydrocannabinol (-)-Δ8-tetrahydrocannabinol

Figure 1.2. Structural Isomers of Tetrahydrocannabinol (THC)



(-) Cannabidiol (+) Cannabidiol

Figure 1.3. Stereoisomers of Cannabidiol (CBD) (Leite *et al.* 1982).

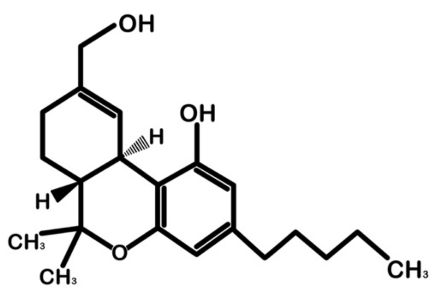
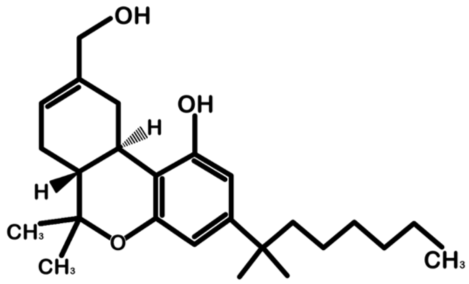
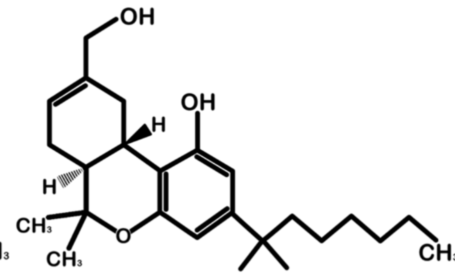


Figure 1.4. Chemical Structure of 11-hydroxy-Δ-9-tetrahydrocannabinol.

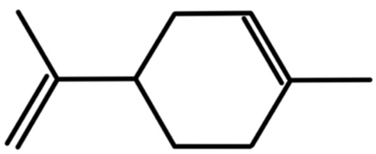


(-)-1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol

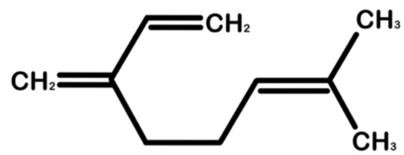


(+)-1,1-Dimethylheptyl-11-hydroxy-tetrahydrocannabinol

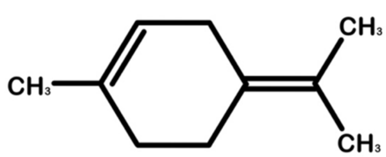
Figure 1.5. Structure of two Synthetic Cannabinoids. HU-210 (left) and HU-211 (right) are stereoisomers that have dramatically different behavioural effects in humans.



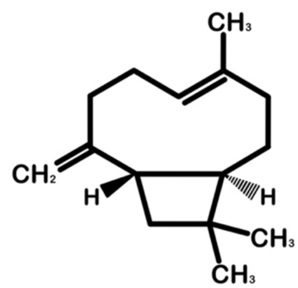
Limonene



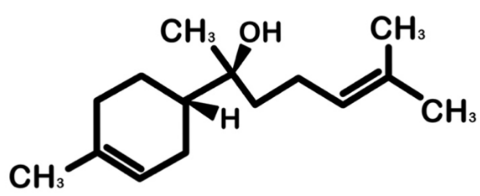
Myrcene



Terpinolene



Carophyllene



Bisabolol

Figure 1.6. Chemical structures of some terpenoids found in *Cannabis.*

2. Materials and Methods

2.1 Animal Care and Husbandry

Zebrafish were maintained on a 14-hour light / 10-hour dark cycle at between 25°C and 28°C in 3 to 8L tanks supplied with flow-through water and aeration and fed standard zebrafish pellet food (GEMMA Micro 500, Skretting) twice daily. Embryos were collected from Tübingen (wild type) adults by natural spawning at the beginning of the light cycle over trays of marbles placed in the tank the night before. Embryos were raised in rearing baskets consisting of reusable coffee filters suspended in Styrofoam rings floating in de-chlorinated water kept at 28.5°C. All work with zebrafish embryos, larvae and adults was done under the supervision and with the approval of the University of New Brunswick's Animal Care Committee and in accordance with the Canadian Council on Animal Care guidelines.

2.2 Chemicals

All cannabinoid solutions were sourced from Sigma-Aldrich and supplied as 1 mg/ml solutions in methanol (Table 2.1). They were diluted directly into E3 medium (4970 μM NaCl, 178 μM KCl, 328 μM $CaCl_2 \cdot 2\ H_20$, 801 μM $MgCl_2 \cdot 6\ H_20$) on the day of the experiment.

Table 2.1. Experimental Solutions sourced from Sigma-Aldrich.

Compound Name	Molarity of Stock Solution	Catalogue Number	Lot Number
Δ-9-THC	3180 µM	T-005	FE04222001
Δ-8-THC	3180 µM	T-032	FE04041901
11-Hydroxy-**Δ**-9-THC	3026 µM	H-027	FE08201801
CBD	3180 µM	C-045	FE10071912
CBN	3221 µM	C-046	FE05052008
PTZ	25 mM	P-6500	MKCM4261
48-well Plate	N/A	748011	100418A004

2.3 Larval Zebrafish Behavioural Assay

My analysis of the effects of compounds on larval zebrafish behaviour is based on the protocol of Achenbach *et al.* (2018). Data were collected using a ZebraBox automated behavioural analysis system (Viewpoint, Inc.) in a controlled environment chamber at 30˚C (water temperature was measured at 28.5˚C in experimental wells, due to evaporative cooling). Recordings of experimental runs were quantified using ZebraLab Behavioural Tracking Software Version 5.18.0.0 (Viewpoint, Inc.) using 60-second time bins (i.e. the run is broken into 1-minute intervals and the total distance traveled by each larva in each well is recorded for each minute), with background refresh rate set to 60, animal colour set to black, low detection threshold set to 30, skip image count set to 1, X min size set to 0, and thresholds all set to 0.

Runs were done using 120 hpf zebrafish larvae, which were transferred to a fresh 48-well polystyrene microtiter plate with one larva per well in 1000 µl of E3 medium. To prevent contamination a fresh 48-well plate was used for each trial/ run. The plate loaded with larvae in E3 was mounted in the ZebraBox with full illumination, and the larvae given 30 minutes to acclimate. During the acclimation period stock solutions were retrieved from the freezer and warmed to room temperature. The appropriate volume of cannabinoid stock solution (0.6 µl) or vehicle control (HPLC-grade methanol) was added directly to each well, and then gently mixed using a plastic transfer pipette. Experiments testing THC isomers and an isomer metabolite were conducted over five different runs on five different days. Each run consisted of 12 replicate wells of each experimental treatment and 12 replicate control wells. The recordings were started immediately after applying the test compounds. For all assays, the recording consisted of 4 hours of continuous light (such that larvae exhibit basal activity), followed by a series of three 10-minute dark / 10-minute light stimuli, which triggers startle and anxiety behaviour (Figure 2.1). At the end of each run all larvae were examined microscopically to verify viability (i.e. that they still had normal heart beats, orientation and/or swimming activity). Twelve vehicle control and treatment larvae were assayed in each run of the experiment, with five replicate runs, such that there were 60 replicates of each treatment and 60 vehicle controls. While there are occasional periods during which the average activity of a group of either vehicle control or treatment larvae is significantly different than another group, these were sufficiently rare and brief that I felt confident in pooling the data from all runs to increase the statistical power of the analysis. The pooled behavioural effect of each compound is shown in a separate graph with the pooled vehicle control data collected from the same runs.

For statistical analysis each larva was considered an independent record. Sample sizes were 60 for each compound and control ($n = 12$ per experimental run, 5 replicate experiments). Statistical significance was determined by a two-way repeated measures analysis of variance (ANOVA) testing the effects of compound and time on distance traveled, followed by a Bonferroni post-hoc test when significant effects were found (at $p < 0.05$) using GraphPad Prism version 9.1.2 for Mac (GraphPad Software, San Diego, California USA).

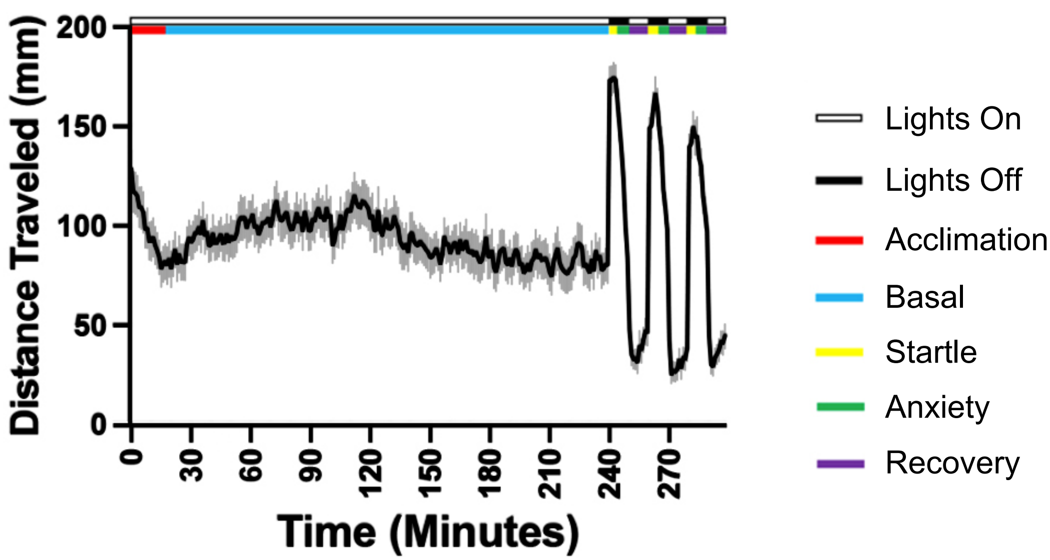


Figure 2.1. Normal behaviour of 120 hpf zebrafish larvae in a 48-well plate in E3 (embryo medium). Activity pattern was measured as average distance traveled (60s time-bins; $n = 60$). Shaded area shows standard error of the mean (SEM).

2.4 Trouble Shooting

Despite the growing popularity of the zebrafish larva for automated high-throughput behavioural analysis using the ZebraBox and similar instruments, I found replicating published work required a great deal of troubleshooting and establishing experimental parameters that were not well documented, or even reported at all. The first problem I had was that the basal activity of 120 hpf larvae were much lower in my hands than reported by others (Figure 2.2). I undertook a series of experiments to find conditions that increases the basal activity of larvae in our system; I concluded that conducting the experiments in 48-well plates as opposed to 96-well plates significantly increased the basal distance traveled yielded basal activity rates of about 100 mm/min, consistent with Achenbach *et al.* (2018) and other reports (Figure 2.3). I also switched from using ERM to using E3 Medium to be consistent with previously published research.

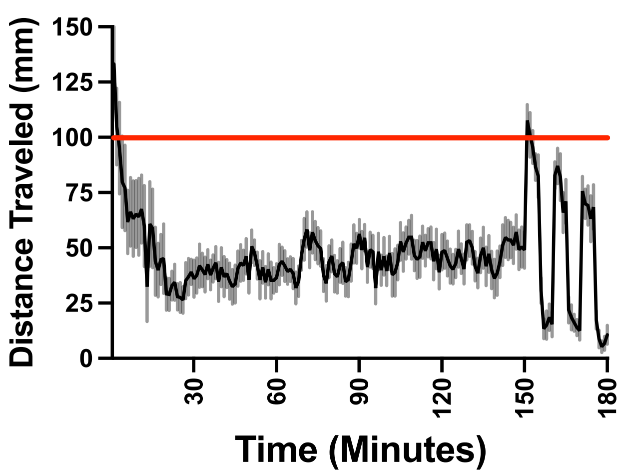


Figure 2.2. Behavioural response of zebrafish larvae at 120 hpf in 96-well plates in ERM (embryo media). Activity pattern was measured as average distance traveled (60s time-bins; n=12). Shaded area shows standard error of the mean (SEM). Red line is average for larvae in vehicle controls from Achenbach *et al.* (2018).

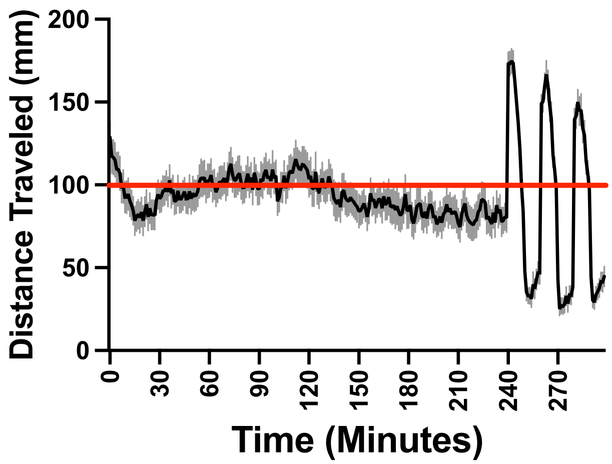


Figure 2.3. Behavioural response of zebrafish larvae at 120 hpf in 48-well plates in E3 (embryo medium). Activity pattern was measured as average distance traveled (60s time-bins; n=60). Data is the same as what is shown in Figure 2.1. Shaded area shows standard error of the mean (SEM). Red line is average for larvae in vehicle controls from Achenbach *et al.* (2018).

Having established a testing protocol that yielded results consistent with those reported by Achenbach *et al.* (2018) and others, I began testing the effects of THC isomers. Again, I was not initially able to replicate the previously reported results; larvae exposed to 2 μM **Δ**9-THC exhibited hyperactivity with respect to both vehicle control and E3 medium control (as opposed to the hypoactivity reported by Achenbach *et al.* 2018), and the vehicle appeared to have a depressing effect (Figure 2.4).

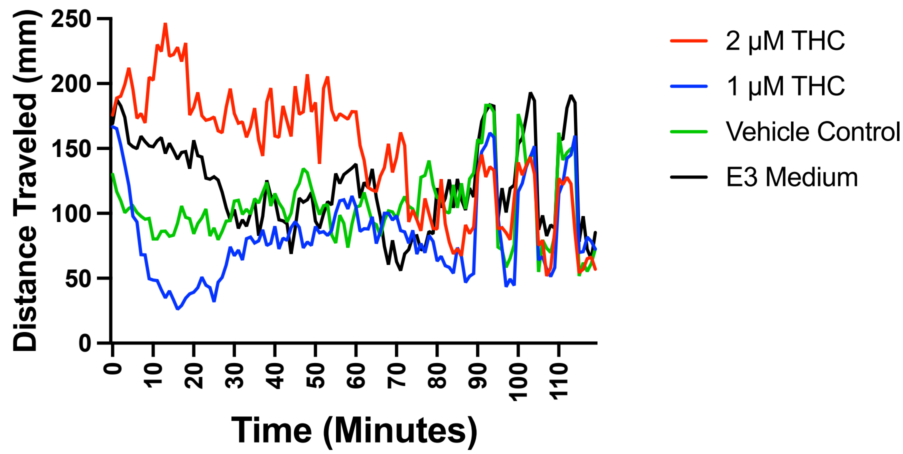


Figure 2.4. Behavioural response of 120 hpf zebrafish larvae exposed to Δ9-THC. Activity pattern was measured as average distance traveled (60s time-bins; n=12, per treatment). SEM was calculated but not shown for legibility.

To prepare the desired concentrations of THC from the stock solution (3.2 mM) I initially made intermediate 10X stock solutions at 20 µM, 10 µM, and 5 µM diluted in E3 medium as reported by Achenbach *et al.* (2018), Samarut *et al.* (2019) and others (French, Memorial University of Newfoundland, personal comm). I chose to use 1% MeOH in the test groups because that was the concentration of MeOH used in the 10X stocks of cannabinoids made by Achenbach *et al.* (2018) (Figure 2.5). This intermediate step also avoided the inconsistencies of pipetting small amounts directly from stock solution. However, the aqueous solubility of THC is only 8.9 µM (Yalkowsky *et al.* 2010), which is below the concentration used in previous reports, leading me to suspect that a significant fraction of the THC in the intermediate '10X' stock was precipitating out of solution. I hypothesized that the low solubility of THC would result in a much lower effective concentration of THC in these assays. I tested my hypothesis by analyzing solutions of cannabinoids after various amounts of time using liquid chromatography-mass spectrometry (LC-MS).

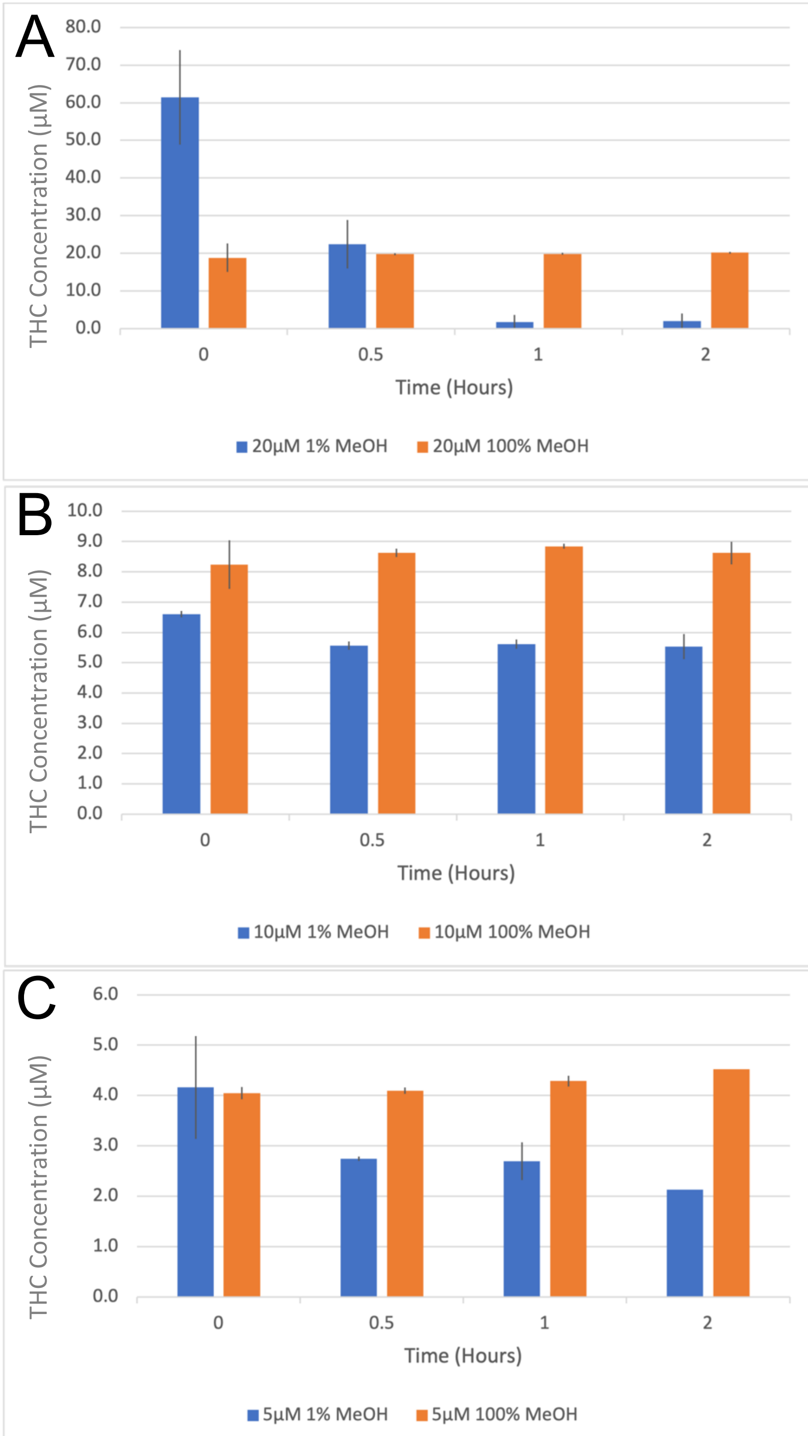


Figure 2.5. THC is more stable in methanol than in aqueous solutions. LC-MS data showing the concentration of **Δ**9-THC in a 48-well plate over 2 hours. Blue bars represent THC solutions made in 1% MeOH / 99% E3 whereas orange bars represent THC solutions made in 100% MeOH (mean ± standard deviation; n=3 per treatment, in all cases). Concentrations are supposed to be A = 20 µM, B = 10 µM, C = 5 µM.

I subsequently avoided this issue by going straight from aliquots of undiluted 1 mg/ml stock in 100% methanol (MeOH) into the media with larvae. Since I had also switched from 96-well plates (containing 300 µl of medium per well) to 48-well plates (containing 1000 µl of medium per well), this entailed pipetting 0.3 µl (1 µM) or 0.6 µl (2 µM) volumes of the stocks, which while not ideal is perfectly feasible, and obviously preferable to using intermediate dilutions with unknown concentrations, and/or adding additional methanol to the treatments.

Even after solving the problem with precipitating THC, I continued to see increased basal activity in the 2 µM **Δ**9-THC-treated larvae for longer than previously reported. Achenbach and colleagues reported a short (~30-minute) period of hyperactivity immediately after addition of 2 µM **Δ**9-THC followed by reduced basal activity, a relatively normal startle response with an attenuated anxiety response (see Figure 1A in Achenbach *et al.* 2018). In my hands, larvae treated with 2 µM **Δ**9-THC exhibit increased activity for about an hour after exposure to the drug, followed by somewhat attenuated startle and anxiety responses (Figure 2.6). However, when I ran my assays longer, initially simply by repeating the protocol immediately after it ended, I found that the larvae treated with 2 µM **Δ**9-THC became significantly less active, and when the second round of lights-off/lights-on stimuli occur, they exhibit an attenuated startle and anxiety response (Figure 2.6). It therefore occurred to me that the speed with which I was distributing the drug (into 48- rather than 96-well plates) and starting the protocol might be significantly faster than previous researchers had been completing the process, and that I might see more comparable data if I simply elongated the continual light (basal activity) phase of the protocol such that the depressive effect of 2 µM **Δ**9-THC had time to manifest.

As predicted, behavioural analysis runs with larvae treated with 2 µM THC, using extended continuous-light basal activity periods of 240 minutes followed by three ten-minute lights-off-lights-on cycles, yielded behavioural profiles with a short initial period of hyperactivity followed by basal activity, a somewhat attenuated startle response, and reduced anxiety activity, consistent with previous reports (see Results below). All further experiments were conducted using this analysis paradigm.

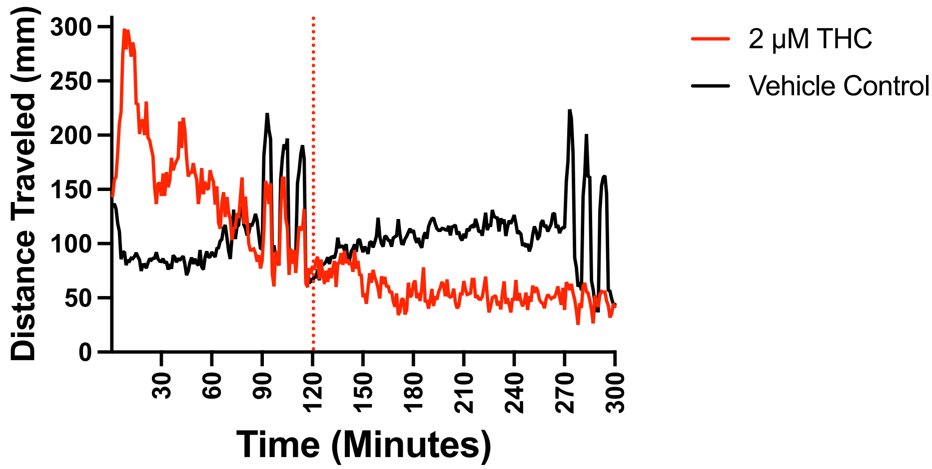


Figure 2.6. Behavioural response of 120 hpf zebrafish larvae following acute exposure to 2 µM THC. Activity pattern was measured as average distance traveled immediately after introduction to **Δ**-9-THC (60s time-bins; n=24, per treatment). Red dotted line is where original protocol would have ended.

2.5 Measuring Concentrations of Cannabinoids in Larval Zebrafish Tissue

My analysis of measuring the concentrations of cannabinoids inside the larval zebrafish is based on the protocol of Achenbach *et al.* (2018). Once the behavioural assay was completed and the larvae were removed from their wells and washed in E3 medium five times. Once the wash was completed all the liquid was moved from the tube and six larvae were placed in the same tube. The tube containing six larvae was then submerged in liquid nitrogen, instantly freezing the larvae. The larvae were then homogenized inside the tubes and 550 µL of MeOH was added to each tube. The tubes were then placed in the -80˚C freezer until the day of analysis. On the day of analysis samples were retrieved from the -80˚C freezer and taken to the lab. They were then filtered and diluted with equal volumes of sample and MeOH. 10 µl of each sample was then injected into the LC-MS, and the amount of THC calculated was based on a standard curve.

3. Results

3.1 Experiment Controls

3.1.1 Methanol Does Not Affect Larval Behaviour Below 0.5 %

Having established a protocol that generated results consistent with previous reports (Figure 3.1), I verified that my vehicle controls were representative of normal behaviour. I treated larvae with 0%, 0.1%, 0.5%, and 1% methanol (MeOH) in E3 medium, and found no significant differences in behaviour of larvae treated with MeOH below 0.5% (Figure 3.2), providing me with confidence that the 0.06% methanol in my vehicle control experiments was not affecting my results (Figure 3.2).

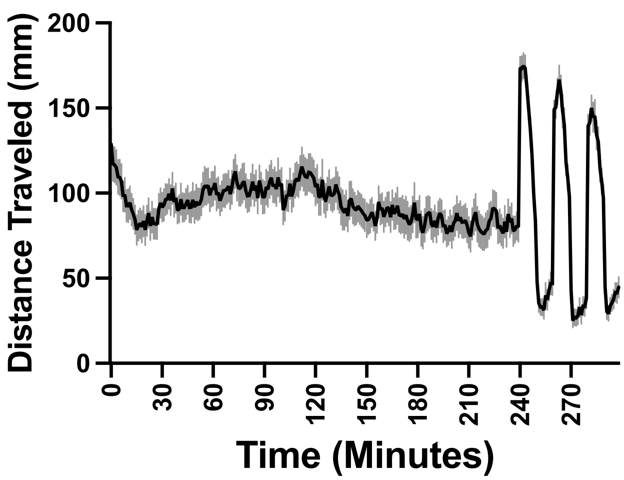


Figure 3.1. Behavioural response of 120 hpf zebrafish larvae in E3 (embryo medium) with 0.06% MeOH. Data is the same as what is shown in Figure 2.1. Activity pattern was measured as average distance traveled (60s time-bins; n=60). Shaded area shows standard error of the mean (SEM) for each time bin.

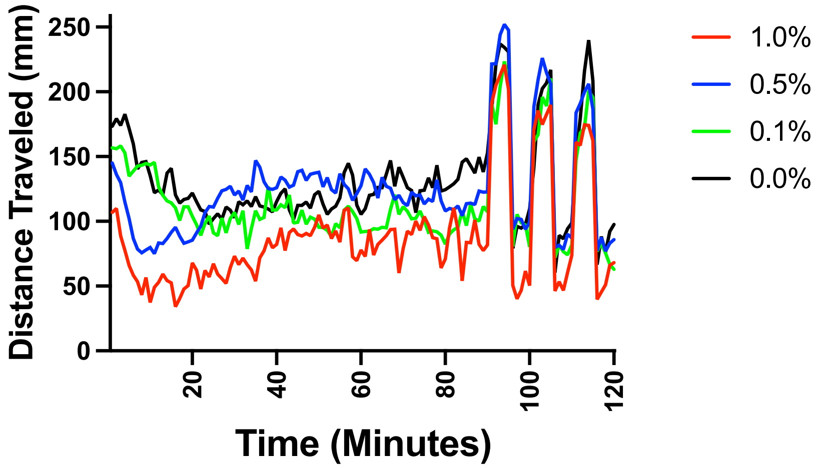


Figure 3.2. Behavioural response of 120 hpf zebrafish larvae following acute exposure to various concentrations of methanol. Activity pattern was measured as distance traveled immediately after introduction to MeOH (60s time-bins; n=12, per treatment). SEM was calculated but not shown for legibility.

3.2.1 Δ-9-Tetrahydrocannabinol

The initial effect of an acute exposure to **Δ**9-THC on the zebrafish larvae is an increase in their distance travelled (Figure 3.3). This period of hyperactivity lasts approximately 100 minutes, after which the activity of larvae exposed to THC subsides to levels comparable to vehicle controls and continues to decrease such that, by about 2 hours after the beginning of the trial, larvae in **Δ**9-THC are significantly less active than controls and remain so for the remainder of the experiment. Unlike controls, larvae in **Δ**9-THC exhibit an attenuated startle/escape response and anxiety-provoked increased activity when the lights go off for ten-minute intervals at the end of the run.

3.2.2 Δ-8-Tetrahydrocannabinol

Like **Δ**9-THC, **Δ**8-THC induces a period of hyperactivity in zebrafish larvae, but this excitatory effect lasts only about 60 minutes, after which the distance traveled by larvae drops to what is seen in the controls (Figure 3.3). Also, similarly to **Δ**9-THC, the activity of larvae exposed to Δ8-THC drops below the basal activity of controls and remains depressed for the remainder of the experiment. However, unlike larvae treated with Δ9-THC, larvae treated with Δ8-THC exhibit a distinct startle/escape response when the lights go out. Interestingly, despite rapidly increasing their movement when the lights go out, unlike controls they quickly return to basal activity during the 10-minute dark periods, suggesting reduced anxiety compared to controls.

3.2.3 11-Hydroxy-Δ9-Tetrahydrocannabinol

Larvae exposed to 11-hydroxy-Δ9-THC do not exhibit a significant period of hyperactivity at the beginning of the trial; their basal activity is significantly decreased relative to controls almost immediately, in contrast to larvae treated with the same concentrations of either Δ9-THC or Δ8-THC (Figure 3.3). Interestingly, and unlike larvae treated with Δ9-THC, both the startle/ escape response (i.e. the initial spike in movement when the lights go out), and anxiety behaviour (i.e. the elevated activity during the following 10-minute dark period) remain evident in larvae treated with 11-hydroxy- Δ9-THC, unlike those treated with Δ9-THC or Δ8-THC, with respect to the anxiety behaviour.

3.2 Behavioural Response to THC Isomers

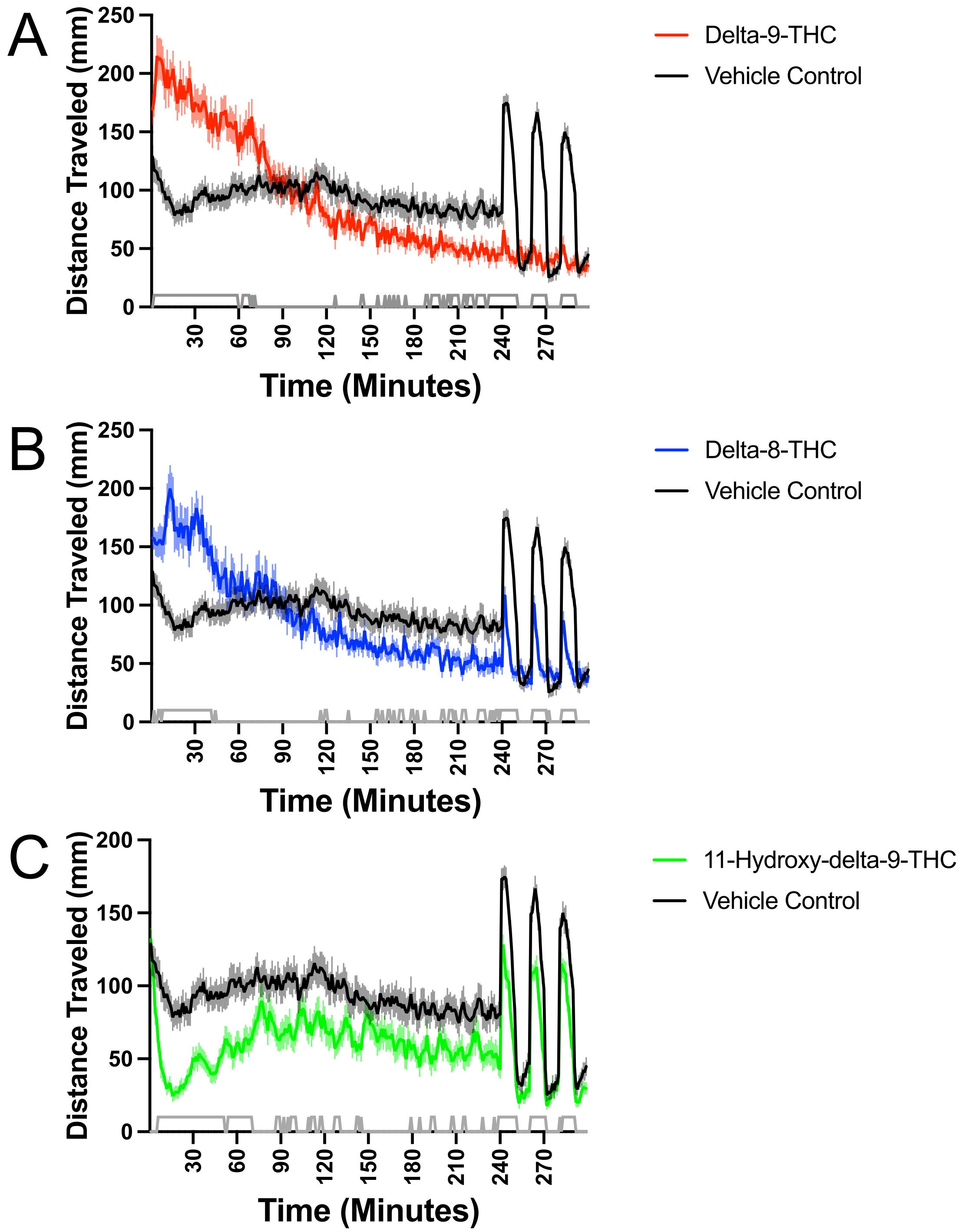


Figure 3.3. Behavioural response of 120 hpf zebrafish larvae following acute exposure to isomers of THC. Activity patterns were measured as average distance traveled during exposure to 2 µM **Δ**9-THC (A), 2 µM **Δ**8-THC (B), 2 µM 11-hydroxy-**Δ**9-THC (C) and Vehicle Control (0.06 % MeOH) (60s time-bins; n=60, per treatment).

Shaded area shows standard error of the mean (SEM) for each time bin. Vehicle control data is the same as shown in Figure 2.1. Significant differences ($p<0.05$) with respect to vehicle controls at any given time is indicated by grey bars along the bottom of the plot (10 = significant, 0 = not significant).

3.3 CBD and CBN are Deleterious When Applied at Reported Concentrations

CBD is reported to blunt the psychoactive effects of THC by competing with THC for binding sites at the CB_1 receptor (Bhattacharyya *et al.* 2010). CBN is also able to bind the CB_1 receptor and does so with a greater affinity than either CBD or even THC (Rosenthaler *et al.* 2014). This led me to predict that CBN might mitigate the psychoactive effects of THC isomers even better than CBD. Having established a behavioural assay and having used it to assess the effects of THC isomers on larval zebrafish behaviour, I wanted to investigate how CBD and CBN might modulate these effects. I began by trying to reproduce the effects of CBD in isolation, which were reported to be mild or undetectable (Achenbach *et al.* 2018).

Surprisingly, in initial experiments, all the larvae treated with 2 µM CBD died and/or exhibited extensive tissue damage (this never occurred in vehicle controls or THC treatments). I see similar deleterious effects when treating larvae with 2 µM CBN (Figure 3.4). Because this is inconsistent with results in zebrafish reported by others (e.g., Achenbach *et al.* 2018; Samarut *et al.* 2019; Kollipara *et al.* 2023), I repeated this 18 times, using three different sources of CBD and 2 different sources of CBN. While not every larva was killed in every treatment, most larvae treated with either of these cannabinoids (CBD or CBN) were either killed or severely damaged and completely inactive and unresponsive to touch by the end of the experiment; the behavioural assays consequently show dramatically reduced or zero activity in these treatment groups

(Figure 3.5) (Table 3.1). In my hands, both CBD and CBN and other combinations of cannabinoids with CBD and CBN are clearly deleterious to zebrafish larvae over a period of only a few hours (Table 3.1).

This unexpected, but consistently reproducible, deleterious effect of cannabinoids led to the obvious question of why this had not been reported previously. After ruling out contamination of my CBD and CBN stocks by analyzing them by mass spectrometry (Table 3.2), and repeating this using CBD from other labs, I began to wonder if previous researchers might have failed to account for CBD's insolubility in aqueous solutions; if researchers had been making '10X stocks' as intermediates prior to diluting CBD into the media for testing (as reported by Achenbach *et al.* 2018), these intermediate stocks would have to have been nearly 40 µM, which is well above the highest reported aqueous solubility of CBD at 0.2 µM (Lv *et al.* 2019). So, it would seem likely that the CBD would precipitate out of these intermediate stocks, dramatically lowering the effective dosages in the treatments, thus explaining the negligible effects observed.

I tested this hypothesis by comparing the concentrations of CBD in the tissue of larvae exposed to CBD after direct addition of the compound to the media (i.e. using my protocols, which result in deleterious effects) to those reported by previous researchers, who observed no deleterious effects and only mild behavioural effects. Achenbach *et al.* (2018) ran several uptake analyses following bathing exposure. After a 300-minute exposure to a reported 1.5 µM CBD they measured the concentration of CBD at ~26 mg/kg by LC-MS. After a 300-minute exposure to 2 µM CBD, I observe by LC-MS that larvae have a comparable 36 mg/kg CBD in their tissues. Similarly, after a 300-minute exposure to 1.5 µM THC Achenbach *et al.* report larvae had ~55 mg/kg of THC in their tissues. After a 300-minute exposure to 2.0 μM THC I observe a comparable 32.6 mg/kg of THC in larval tissues. I also looked at the concentration of CBN in larval tissues after 300 minutes in 2 μM CBN and detect 34.6 mg/kg (Table 3.2). Rats and mice are given similar concentrations when exposed to cannabinoids orally and intraperitoneally for experimental trials. For example, in research exploring obsessive-compulsive behaviour, Deiana *et al.* (2011) exposed rats and mice to 120 mg/kg of CBD and CBG, and 30 mg/kg of **Δ**9-THCV, orally and intraperitoneally and observed that in a time dependent manner, CBD inhibited obsessive-compulsive behaviour. (Deiana *et al.* 2011). So, while not identical, the concentrations of these cannabinoids in the larval tissues of zebrafish following my treatments are not wildly different than those reported by Achenbach and colleagues (2018), nor are they completely inconsistent with dosages used in mammalian studies. While this does not support the idea that the dramatically different results of our treatments (deleterious effects and death at 2 μM, versus mild behavioural effects at up to 3.75 μM), this discrepancy may be explained by the loss of the compounds during the preparation of intermediate dilutions used by other researchers. Apart from the existence of unknown contaminating toxins that are not detectable by LC-MS, and which are present in multiple independently sourced aliquots of these cannabinoids, the only explanation I can think of to explain these difference is that, because I am pipetting small volumes of concentrated cannabinoids directly into the wells with the larvae whereas other researchers have been pipetting from intermediate dilutions (which may have precipitated), the larvae in my experiments may be subject to injury due to the compounds forming crystals in/on the larvae themselves. I speculate that the larvae in these experiments are being exposed to essentially the saturated solutions of cannabinoids, as the aqueous solubility of these compounds is in the low micromolar range, and so what is different is that the larvae in my experiments may have been serving as the nucleation sites for these compounds to precipitate out of solution, and rather than chemical toxicity being the cause of the death and tissue damage That is, rather than chemical toxicity, the tissue damage I observe may be due to mechanical injury caused by formation of crystals. So just like water is not toxic, but cells are killed by freezing, it may be that these cannabinoids are not toxic, but their application in saturated solutions are damaging to living tissues.

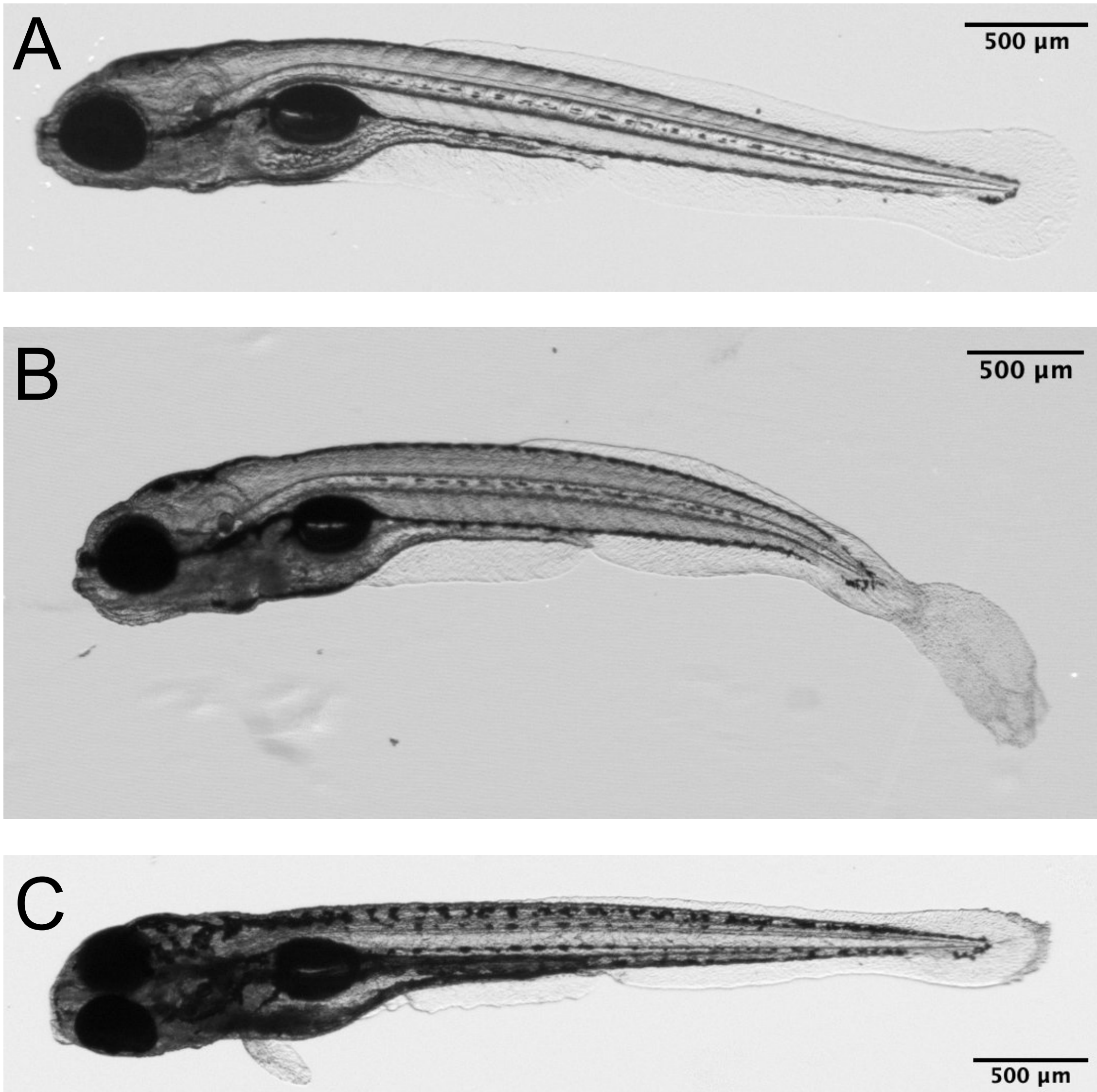


Figure 3.4. 120 hpf zebrafish larvae exposed to cannabinol (CBN) and cannabidiol (CBD) have tissue necrosis on their fins. Micrographs of larvae exposed to 2 μM (0.06%) methanol (showing normal morphology) (A), 2 μM CBN (showing severe tissue damage) (B), and 2 μM CBD (showing moderate tissue damage) (C). Scale bar represents 500 μm.

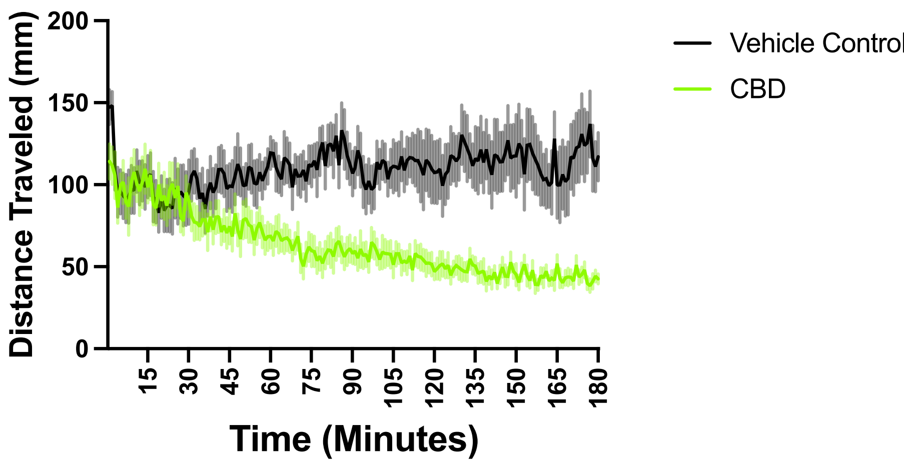


Figure 3.5. Behavioural response of 120 hpf zebrafish larvae following acute exposure to CBD. Activity pattern was measured as average distance traveled immediately after introduction to 2 μM Cannabidiol (60s time-bins; n=12, per treatment). Shaded area shows standard error of the mean (SEM).

Table 3.1. Larval health status after 5 hour behavioural runs.

Cannabinoid	Normal (%)	Moribund (%)	Dead (%)
VC (n = 100)	99%	0%	1%
CBD (2 μM) (n = 142)	6.3%	92.3%	1.4%
CBN (2 μM) (n = 60)	8.3%	81.7%	10%
Δ8-THC (2 μM) + CBD (2 μM) (n = 84)	1.2%	73.8%	25%
Δ8-THC (2 μM) + CBN (2 μM) (n = 96)	0%	54.2%	45.8%

Table 3.2. Comparing larval concentrations of cannabinoids in 120 hpf zebrafish larvae to previously published work.

Cannabinoid	Treatment	Measurement in Tissue	Reference
Δ9-THC	1.5 µM	55 mg/kg	Achenbach *et al.* 2018
	2.0 µM	32 mg/kg	This work
CBD	1.0 µM	26 mg/kg	Achenbach *et al.* 2018
	2.0 µM	36 mg/kg	This work
CBN	2.0 µM	34 mg/kg	This work

4. Discussion

I set out to investigate the pharmaceutical potential of cannabinoids, particularly with respect to the treatment of seizure disorders, and with a particular interest in less well-studied isomers of THC and their potential entourage effects with other cannabinoids, using a high-throughput zebrafish larval behavioural analysis approach. While the zebrafish behavioural model is popular for drug discovery, toxicological testing, and basic neurobiological research, I found replicating published results challenging, and discovered that there are subtle and difficult to control factors that can confound these analyses. However, I was ultimately able to closely reproduce the effects of **Δ**9-THC reported by Achenbach *et al.* (2018), and to extend upon this by characterizing the effects of **Δ**8-THC and 11-hydroxy-**Δ**9-THC, both of which have subtly distinct effects on zebrafish behaviour and may represent promising alternatives to **Δ**9-THC from a pharmacological point of view. Instrumental limitations prevented me from assessing the effects of these cannabinoids on a chemically induced seizure model (Appendix A), but it would be very interesting to see if the THC isomers that I have characterized here retain seizure-antagonizing properties, and to further explore their potential as treatments for seizure disorders in humans. Kollipara and colleagues recently tested CBD in combination with several other cannabinoids and their ability to antagonize seizures using the PTZ-induced seizure model in a very similar experimental design. They demonstrated that cannabinoids such as CBN and CBC effectively antagonize seizures at lower concentrations than CBD, and that CBG may work synergistically with CBD to further reduce seizures (Kollipara *et al.* 2023). However, I observe very different effects that have been previously reported when treating zebrafish larvae with CBD and CBN; in my hands these compounds seem to be deleterious, but hundreds of other studies have reported them as being relatively benign and often beneficial. I speculate that these inconsistencies arise from the fact that cannabinoids, and CBD in particular, are sparingly soluble in aqueous conditions. This paradox remains a puzzle and will need to be resolved in order to understand the results reported by other researchers working with cannabinoids in zebrafish.

4.1 Replicating Previously Published Results (Achenbach *et al.* 2018)

When exposed to **Δ**9-THC zebrafish initially exhibit hyperactivity (i.e. an increase in distance traveled), followed by hypoactivity (Achenbach *et al.* 2018). After extensive troubleshooting, I was able to observe similar results in the lab at UNB. Achenbach *et al.* (2018) used a previously published multimodal behavioural assay that can distinguish distinct differences between the effects of cannabinoids on larval behaviour. The assay asses the difference in baseline activity rates followed by a series of light-to-dark challenges, which elicit both an initial startle response, followed by anxiety behaviour during the dark period, and finally a return to basal activity when light is restored. I made a few notable adjustments to Achenbach's protocol, which are as follows: I used a 48-well plate in place of a 96-well plate, providing a larger swimming arena for the larvae, which I found was necessary to get basal activity levels comparable to previous reports; I shortened the acclimation period from 2 hours to 30 minutes, to allow completion of experiments in one day; I added the cannabinoids to the E3 medium in the wells from undiluted aliquots at 1 mg/ml in methanol, rather than making '10x' intermediate dilutions as reported by previous researchers because I found the cannabinoids precipitated at higher concentrations; I extended the observation of basal activity under continuous illumination to 4 hours instead of 2 hours, which allowed me to reproducibly detect the hypoactivity of larvae exposed to cannabinoids; and finally I extended the dark challenges from 5 minutes to 10 minutes, to reproducibly observe both the startle response as well as the anxiety response of the larvae.

Consistent with previous reports (Achenbach *et al*. 2018), I observe that exposure to **Δ**9-THC results in an initial increase in larval activity, followed by hypoactivity. However, I do not see a decrease in distance traveled for the first 5-10 minutes of the experiment; in my hands larvae become hyperactive immediately following treatment. The period of hyperactivity reported by Achenbach *et al.* (2018) lasted approximately 45 minutes, but I saw hyperactivity lasting for approximately 80 minutes. During the following period of hypoactivity, Achenbach *et al*. (2018) report larvae settling to around 35-40 mm/minute, whereas I see basal activity leveling off later in the experiment at around 50 mm/minute. Finally, the previous researchers saw a clear startle response from the light-to-dark stimulus that was repeated three times over half an hour, whereas I see no startle response in larvae treated with **Δ**9-THC. Achenbach and colleagues suggest the reduced startle effects they observe may be due to reduced anxiogenic response rather than sedation. My results argue that the complete loss of the startle response is due to a sedative effect and not a reduced anxiogenic effect (Figure 4.1). Further investigations of the effects of **Δ**9-THC on zebrafish larvae focusing on thigmotaxis (Schnörr *et al.* 2012; Lucon-Xiccato *et al.* 2022), exploratory boldness (Sailer *et al.* 2012) or other indicators of anxiety may help clarify this.

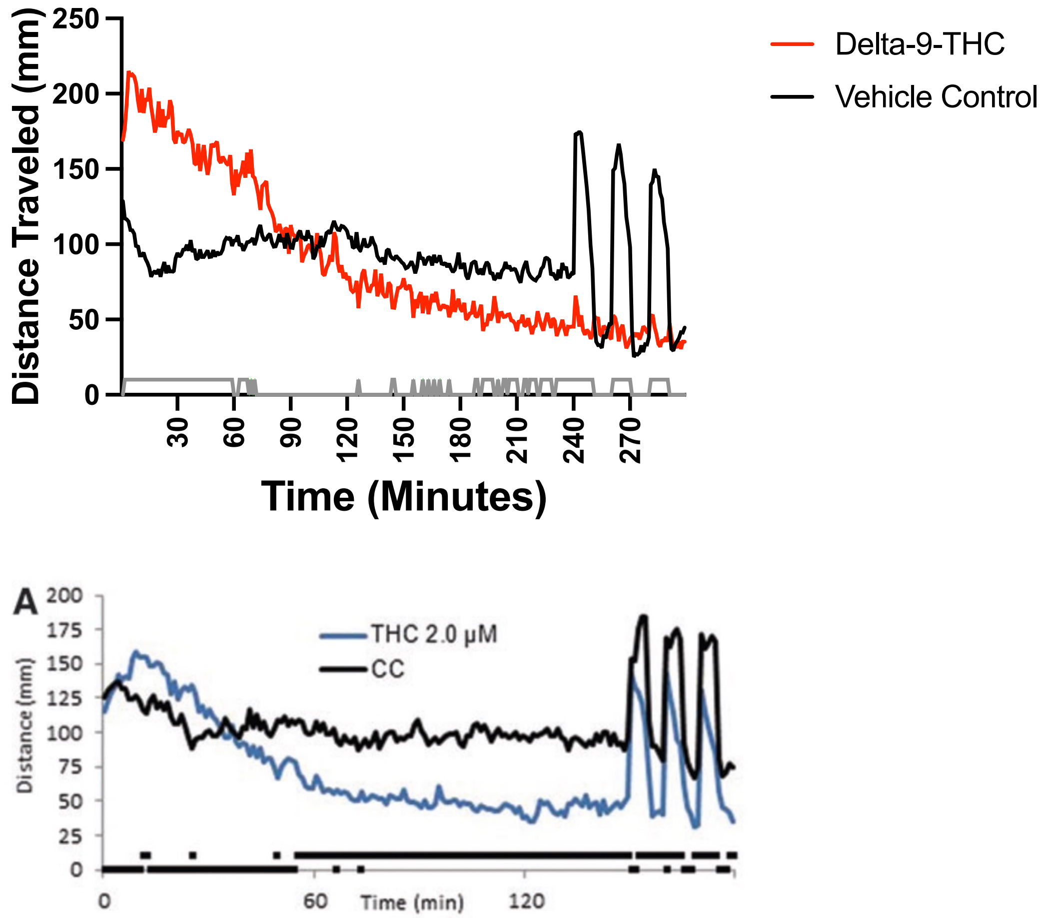


Figure 4.1. Comparison of my findings using Δ9-THC to previously published data from Achenbach *et al.* (2018). Concentration of Δ9-THC for both experiments is 2 µM. n=60, per treatment for my experiments, n=48, per treatment from Achenbach *et al.* (2018).

4.1.1 Different Isomers of Cannabinoids Have Different Effects on Larval Zebrafish Behaviour

There are many isomers and isomer metabolites of cannabinoids. (-)Δ9-THC is the most abundant isomer of THC found in the *Cannabis* plant, followed by Δ8-THC. When Δ8-THC was given intravenously and orally to humans it was reported to be about 2/3 as potent as Δ9-THC (Hollister & Gillepsie, 1973). Very little research has been done on Δ8-THC and it has not been previously given to zebrafish larvae in the context of behavioural analysis. Compared to larvae treated with Δ9-THC, larvae treated with Δ8-THC behave similarly. There is an initial period of hyperactivity, which peaks around 200 mm/min in Δ8-THC, compared to 210 mm/min in Δ9-THC. The period of hyperactivity in Δ8-THC is also slightly shorter, lasting about 70 minutes after exposure, compared to 80 minutes in Δ9-THC. Basal activity rates for both isomers subside to about 50 mm/min, well below the basal activity I see in untreated larvae or larvae subjected to solvent controls. Unlike the larvae treated with Δ9-THC, the larvae given Δ8-THC exhibit a functional, if attenuated startle response, and they returned to basal activity rates much faster than larvae exposed to vehicle controls, suggesting reduced anxiety during the dark challenges.

11-hydroxy-Δ9-THC is a metabolite of Δ9-THC. In 1972, Hollister and colleagues concluded that the placement of the double-bond, changing the length of the side chain, or producing hydroxy metabolites at the 11- or 8- position, does not alter the actions of THC, but can drastically change the potency (Evans, 1991). Very little research has been done on 11-hydroxy-Δ9-THC and it has not previously been given to zebrafish larvae. Interestingly, larvae exposed to 11-hydroxy-Δ9-THC do not exhibit any hyperactivity, and their movement rates drop significantly below what I see in the vehicle control. This is important to note because it refutes the hypothesis that the hypoactivity observed following hyperactivity in larvae treated with other isomers of THC represents simple fatigue. Distance traveled dropped to 30 mm/min after approximately 10 minutes of exposure to 11-hydroxy-Δ9-THC and then slowly increased to 50 mm/min. Basal activity remained between 50 mm/min to 75 mm/min until the dark challenges. Larvae treated with 11-hydroxy-Δ9-THC exhibit normal startle response and increased activity in the dark, although not as high as in the vehicle control. The hypoactivity in 11-hydroxy-**Δ**9-THC suggest it has increased sedative and/or reduced euphoric properties compared to **Δ**9-THC, but it apparently does not blunt the startle response or anxiety triggered by the dark challenges.

4.2 Testing Seizure-Antagonizing Cocktails of Cannabinoids

Between 25%-40% of newly diagnosed epilepsy patients will have seizures that are resistant to current treatments (Schmidt & Sillanpaa, 2012). Advancements have stalled with no major advances over the last 40 years (Samarut *et al.* 2019). Finding potential new drugs to aid with seizures is of utmost importance, and cannabinoids could potentially be used as a therapeutic treatment. Previous findings from a multitude of studies using both *in vivo* and *in vitro* models of seizures show that activation of the pre-synaptic CB_1 receptor suppresses epileptic activity (Blair *et al.* 2015).

One of the main objectives of this thesis was to determine if previously untested mixtures of cannabinoids could oppose seizures while causing less behavioural disruption. Unfortunately, due to time and equipment limitations I was not able to use the PTZ-induced seizure model and the assay developed by Samarut *et al.* (2019) (Appendix A). However, at the same time I was working on this, Kollipara and colleagues at Memorial University of Newfoundland were using this approach with different instrumentation and were successful in finding cannabinoids that antagonize PTZ-induced seizures (Kollipara *et al.* 2023). They concluded that CBD, **Δ**9-THC, **Δ**8-THC, CBN, CBC, and CBG all independently lower seizure index. They also show combinations of CBD and **Δ**9-THC, CBD and CBG, as well as CBD and **Δ**8-THC further reduce seizure index more than the independent cannabinoids (Kollipara *et al.* 2023). The mechanism(s) by which CBD antagonizes seizures is still unknown (Zhang *et al.* 2023). Using the power of the zebrafish model we may be able to solve this mystery.

4.3 The Deleterious Effects of CBD, CBN, and Other Problems

CBD has previously been used to oppose chemically induced PTZ seizures. Samarut *et al.* (2019) was able to reduce total activity levels of larvae treated with PTZ to levels that were not significantly different than the control by giving them CBD. They conclude that this indicates that the effects of CBD are not because of sedation but rather it opposes fast-darting activity and has little effect on underlying normal movements (Samarut *et al.* 2019). They were able to see these effects when larvae were given 1 μM, 1.5 μM, and 2 μM CBD.

When I treated larvae with 2 μM CBD I saw vastly different results. I observed rapidly declining activity, which is due to severe tissue necrosis and death of the larvae during the experiment. While some previously published papers have reported negative side effects (i.e. reduced heart rates, axial malformations, and shorter trunks) of exposing THC and CBD to zebrafish larvae (Ahmed *et al.* 2018), nothing approximating the severity of what I observed has been reported. Zebrafish larvae (120 hpf) exposed to 0.75 mg/ml (2.4 μM) CBN exhibit pericardial edema; larvae are exposed to 1 mg/ml (3.2 μM) CBN exhibit pericardial edema and tail bending, and when larvae are exposed to 1.2 mg/ml (3.9 μM) CBN they observe pericardial edema, yolk sac edema, and tail bending (Chousidis *et al.* 2020). Notably, CBN's solubility in water is estimated

0.0021 mg/L (0.0067 μM) (ChemSpider entry 2447), so all of the reported concentrations are orders of magnitude greater than its maximum solubility.

Another cannabinoid that I attempted to use to inhibit the psychoactive effects of THC was CBN. CBN is commonly found as an artifact after prolonged improper storage of THC and was the first cannabinoid to be isolated and identified (Wood & Spivey, 1899). CBN maintains ¼ of the potency at the CB_1 receptor when compared to THC (Karniol *et al.* 1975). Like THC, CBN has demonstrated therapeutic effects such as anti-inflammatory, antibiotic, anti-convulsant, as well as sedative effects (Rhee *et al.* 1997). Using CBN at 2 μM I also observed rapidly declining activity, which is due to severe tissue necrosis and death of the larvae during the experiment.

I have ruled out contaminants in the cannabinoid stocks I was working with and have demonstrated that the larvae are taking up comparable amounts of these compounds in my treatments, leaving the explanation for these discrepancies unclear.

4.4 Inconsistencies may be Caused by Limited Solubility of Cannabinoids

One possibility that should be explored regarding the discrepancies between studies using zebrafish and other aquatic species to assess the biological effects of cannabinoids is their low solubility in water. Achenbach *et al.* (2018) diluted these cannabinoids in solution at a working concentration up to 37.5 μM CBD, and 20 μM **Δ**9-THC. I have demonstrated that **Δ**9-THC precipitates out of a 20 μM solution in less than 1 hour (Figure 2.5). The initial observed concentration of THC in Figure 2.5 panel A is 3x higher (60 μM) than the starting solution (20 μM). I believe the reason for this discrepancy is the precipitation of THC out of the solution forming “THC clumps”. When the sample was collected, a clump of THC was also collected resulting in a higher concentration. The solubility of **Δ**9-THC is 8.9 µM (Yalkowsky *et al.* 2010), well below the concentration many researchers are using as intermediate dilutions (e.g. 10x stocks) in their experiments. The solubility of CBD is not widely reported, but two recent investigations into using cyclodextrin complexes to improve its aqueous availability independently find its intrinsic phase solubility in water to be 0.2 µM (Mannila *et al.* 2007; Lv *et al.* 2019). CBD is clearly very insoluble in water, yet researchers report making aqueous stock solutions of CBD at nearly 40 µM (Achenbach *et al.* 2018; Samarut *et al.* 2019; Kollipara *et al.* 2023; French, Memorial University of Newfoundland, personal communications). This may explain the wide-spread perception that CBD has little to no behavioural effects; if researchers are treating their experimental animals with vastly lower concentrations than they think they are, they would understandably underestimate the effects of this compound. Something similar may be responsible for the differences in deleterious effects that I observed using CBD and CBN (Figure 3.4), in comparison with others using the same starting material (dissolved in methanol) in the same lab. When adding the small volume of cannabinoid to the 1 ml of embryo medium in a 48-well plate, it may matter *where* in the well the tip of the pipette is placed, both with respect to the sides of the well and the zebrafish larva in it. If the locally high concentration of methanol and cannabinoid expelled from the pipette tip is expelled near the hydrophobic polystyrene wall of the well, significant amounts of the cannabinoid might precipitate on the wall of the well rather than going into solution at near their limit of solubility. Conversely, if the bolus was expelled near the larva, the locally high concentration of cannabinoids may either precipitate in/on the tissues of the larva or be present at higher concentrations in the larval cell membranes than would be the case if the larva were exposed to a more homogeneous solution (or suspension) of the same molecule.

4.5 Future Directions

The ability to distinguish behavioural differences caused by **Δ**9-THC, its structural isomer **Δ**8-THC, and its metabolite 11-hydroxy-**Δ**9-THC, provides an important look into the function of these cannabinoids, and illustrates the power of the zebrafish model system for this type of analysis. Because I was unable to obtain stereo isomers of THC for testing, I was not able to determine if the stark differences in behavioural effects observed for enantiomers of synthetic cannabinoids pertains to naturally occurring cannabinoids, but the assay I have developed here makes this eminently feasible once test compounds are available. It would be very interesting to test the effects of non-psychoactive synthetic cannabinoids like HU-211 in combination with CBD, CBN, CBC, and CBG to determine if there is a synergistic effect with regards to antagonizing seizures. It would also be interesting to see the behavioural effects of mixed doses of these cannabinoids in conjunction to the seizure model.

It is also important to keep solubility in mind when conducting these experiments, and to plan experiments around the solubility limits of these cannabinoids. Cannabinoids are extremely insoluble in water, but there has been a lot of research published on ways to improve CBD solubility (Lv *et al.* 2019), and strategies like nano-encapsulation may not only make these potent pharmacological agents more soluble in aqueous media, but also improve their bioavailability and pharmacokinetics (Crawford, University of New Brunswick, personal comm.).

4.6 Conclusions

Zebrafish can be used as a model organism for studying the effects of cannabinoids (Achenbach *et al.* 2018). They offer a high-throughput model to test cannabinoids, as captive zebrafish spawn all year round and a few adult zebrafish can produce hundreds of embryos each week that grow to swimming larvae in less than 5 days. Using a high-throughput behavioural assay, I was able to reproduce similar effects to those previously reported for **Δ**9-THC. I have used this assay to assess the behavioural effects of another isomer of THC as well as an isomer metabolite, which have not been previously tested in zebrafish larvae. I was unable to assess the ability of these or other cannabinoids to antagonize seizures due to instrument limitations, but I discovered dramatic and highly reproducible deleterious effects on larvae using cannabinoids that have previously been reported as benign or beneficial in the treatment of a huge variety of maladies. I suspect that the extremely limited solubility in water is responsible for these discrepancies, and that future work aimed at delivering cannabinoids packaged in more hydrophilic carriers such as cyclodextrins, micelles, or amphipathic nano-particles will resolve this problem.

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Appendix A: Antagonizing Chemically Induced Seizures Using Cannabinoids in Zebrafish Larvae.

Introduction

Epilepsy is a syndrome of recurrent and unprovoked seizures (Fisher *et al.* 2014). It is one of the most common neurological diseases and has a prevalence of 0.5%-2% in the general population. 30% of patients with epilepsy will be considered treatment resistant (Kwan & Brodie, 2000). New anti-seizure drugs have not resulted in significantly lower rates of seizures and the side effects of these drugs, as well as co-morbidities has led to a significant decrease in the quality of life for these patients. Therefore, the search for new anti-seizure drugs continues (Chen *et al.* 2018).

Previous findings from a multitude of studies both *in vivo* and *in vitro* models of seizures show that activation of the pre-synaptic CB_1 receptor suppresses epileptic activity (Blair *et al.* 2015), so it seems plausible that cannabinoids that bind the CB_1 receptor may function as anti-epileptics. Recently there have been many clinical trials assessing the ability of CBD to oppose seizures. A recent study found a 34.6% decrease in all seizures, 55% decrease for focal seizures, 54.3% decrease for atonic seizures, and 16% decrease for tonic seizures (Devinsky *et al.* 2016).

Using zebrafish larvae Samarut and co-workers show that THC and CBD synergistically antagonize both genetic and chemical models of epileptic seizures (Samarut *et al.* 2019). In pentylenetetrazole (PTZ) induced seizures, both CBD and THC alone reduced fast-twitch swimming representatives of seizures in larvae. Samarut and colleagues also concluded that combining both cannabinoids lead to increased effectiveness in a genetic seizure model (Samarut *et al.* 2019).

In an effort to adapt this approach to investigating the seizure-antagonizing properties of other cannabinoids, I set about trying to replicate this work to establish a seizure model for further work.

Materials & Methods

Adult zebrafish were maintained on a 14-hour light / 10-hour dark cycle with water temperatures between 25°C and 28°C. They were housed in 3L to 8L tanks with continuous flow through water and aeration. They were fed twice daily with Gemma Micro 500 Skretting pellets. Embryos were collected from Tübingen (wild-type) adults with natural spawning at the beginning of their light cycle by placing a container filled with marbles in the tank the night before. The numbers of embryos needed for the experiment, plus a few extra were raised in rearing baskets that consisted of reusable coffee filters suspended in Styrofoam rings floating in de-chlorinated water kept at 28.5°C. All work with zebrafish larvae was done under the supervision and with the approval of the University of New Brunswick's Animal Care Guidelines and in accordance with the Canadian Council on Animal Care.

All cannabinoid solutions were sourced from Sigma-Aldrich and supplied as 1 mg/ml solutions in methanol (Table A.1). They were diluted directly into E3 medium (4.97 mM NaCl, 178 µM KCl, 328 µM $CaCl_2 \cdot 2\ H_20$, 801 µM $MgCl_2 \cdot 6\ H_20$) the day of the experiment.

Table A.1. Experimental solutions sourced from Sigma-Aldrich.

Compound Name	Catalogue Number	Lot Number
Δ-9-THC	T-005	FE04222001
Cannabidiol	C-045	FE10071912
PTZ	P-6500	MKCM4261
48-well Plate	748011	100418A004

My analysis of the effects of compounds on larval zebrafish behaviour is based on the protocol of Samarut *et al.* (2019). Data was collected using a ZebraBox automated behavioral analysis system (Viewpoint, Inc.) in a controlled environment chamber at 30˚C (water temperature was measured at 28.5˚C in experimental wells, due to evaporative cooling). Recordings of experimental runs were quantified using ZebraLab Behavioural Tracking Software Version 5.18.0.0 (Viewpoint, Inc.) using 60 second time bins (i.e. the run is broken into 1 minute intervals and the total distance traveled by each larva in each well is recorded for each minute), with background refresh rate set to 60, animal color set to black, low detection threshold set to 30, skip image count set to 1, X min size set to 0, the large threshold set to 30, and all other thresholds set to 0.

Runs were done using 120 hpf zebrafish larvae, which were transferred to a clean 48-well polystyrene microtiter plate (Nest Scientific) with one larva per well in 1000 µl of E3 medium. The plate loaded with larvae in E3 was mounted in the ZebraBox with full illumination, and the larvae given 30 minutes to acclimate. During the acclimation period stock solutions were retrieved from the freezer and warmed to room temperature. The appropriate volume of cannabinoid stock solution (usually 0.6 µl) or vehicle control

(HPLC-grade methanol) was added to each well, and then gently mixed using a plastic transfer pipette. For each experimental condition at least 12 replicate wells were used for each compound. After the addition of cannabinoids, larvae were acclimated for another 90 minutes. After the second acclimation period PTZ was added, and then gently mixed with a transfer pipette. The recordings were started immediately after applying the PTZ compounds. For all assays, the recording consisted of 3 hours of continuous light (such that larvae exhibit basal activity). At the end of each run all larvae were examined microscopically to verify viability (i.e. that they still had normal heart beats, orientation and/or swimming activity).

Results

PTZ-induced seizures increase the movement of larvae, however this is not what is reflected in the data I obtained using the ZebraBox. In my experiments larvae exposed to PTZ exhibit a lower distance traveled than the vehicle control for the first 75 minutes. Between 75-90 minutes they exhibit a similar distance to the vehicle control, and after 90 minute their distance traveled is slightly elevated. When larvae were exposed to both PTZ and CBD, they displayed the same distance traveled as the CBD control. Larvae that were exposed to PTZ, CBD, and THC exhibited no hyperactivity, and a lowered distance traveled compared to the vehicle control, and PTZ control (Figure A.1 and A.2).

Upon closer examination I determined that this was due to a limitation of the ZebraBox instrument and not that the larvae were unresponsive to PTZ. When larvae were put into the ZebraBox after being exposed to PTZ, I could observe their swimming activity increase compared to the control, but the software was not tracking it. When the larvae swam fast the software would stop following them until they slowed down to a normal speed again. After consultation with ViewPoint Technologies, we determined this was due to the frame-rate of the camera (60-fps (frames per second)), which is not fast enough to track the rapid movement of the larvae undergoing PTZ-induced seizures. Other researchers using the Noldus behavioural analysis system, which includes a 500-fps camera (e.g., Samarut *et al.* 2019; Kollipara *et al.* 2023) have been successful with this protocol, but no combination of settings would allow me to pursue this approach using the ZebraBox system.

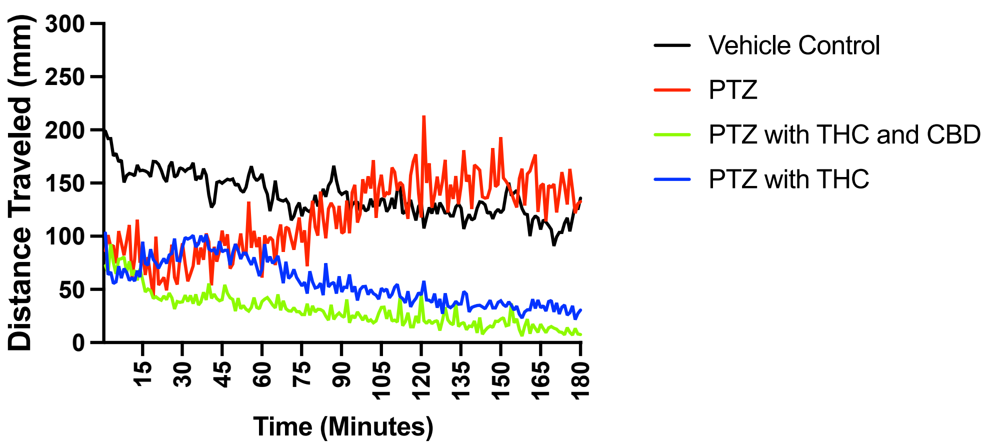

Figure A.1. Behavioural response of 120 hpf zebrafish larvae following acute exposure to PTZ followed by THC, CBD, or both. Behaviour activity patterns are measured by average distance traveled in 60 second time bins, 90 minutes following exposure to 2 µM **Δ**9-THC, 2 µM CBD and Vehicle Control and immediately following exposure to 2.5 mM PTZ. n=9-24, per treatment.

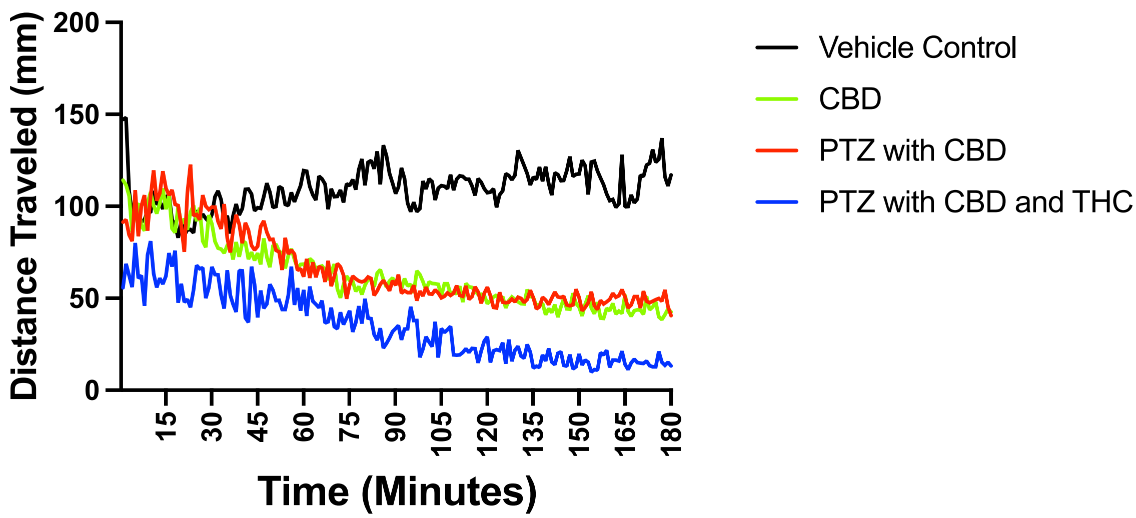

Figure A.2. Behavioural response of 120 hpf zebrafish larvae following acute exposure to PTZ followed by CBD, THC or both. Behaviour activity patterns are measured by average distance traveled in 60 second time bins, 90 minutes following exposure to 2 µM **Δ**9-THC, 2 µM CBD, and Vehicle Control and immediately following exposure to 2.5 mM PTZ. n=10-24, per treatment.

Discussion

I set out to investigate the pharmaceutical potential of differing isomers of cannabinoids and their potential entourage effects with other cannabinoids. with respect to seizure disorders. I used a high-throughput zebrafish larval behavioural model approach, which is becoming increasingly popular in the drug discovery field. I found replicating previously published results challenging and discovered that there are difficult to control and innumerable subtle factors that confound these analyses. Limitations with the instrument prevented me from assessing the effects of cannabinoids using a chemically induced seizure model by direct visualization of larval movement, and the development of other assays to quantify seizures in larval zebrafish was beyond the scope of this work.

Appendix A: References

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Appendix B: LC-MS Chromatograms of THC in Solution Over Time

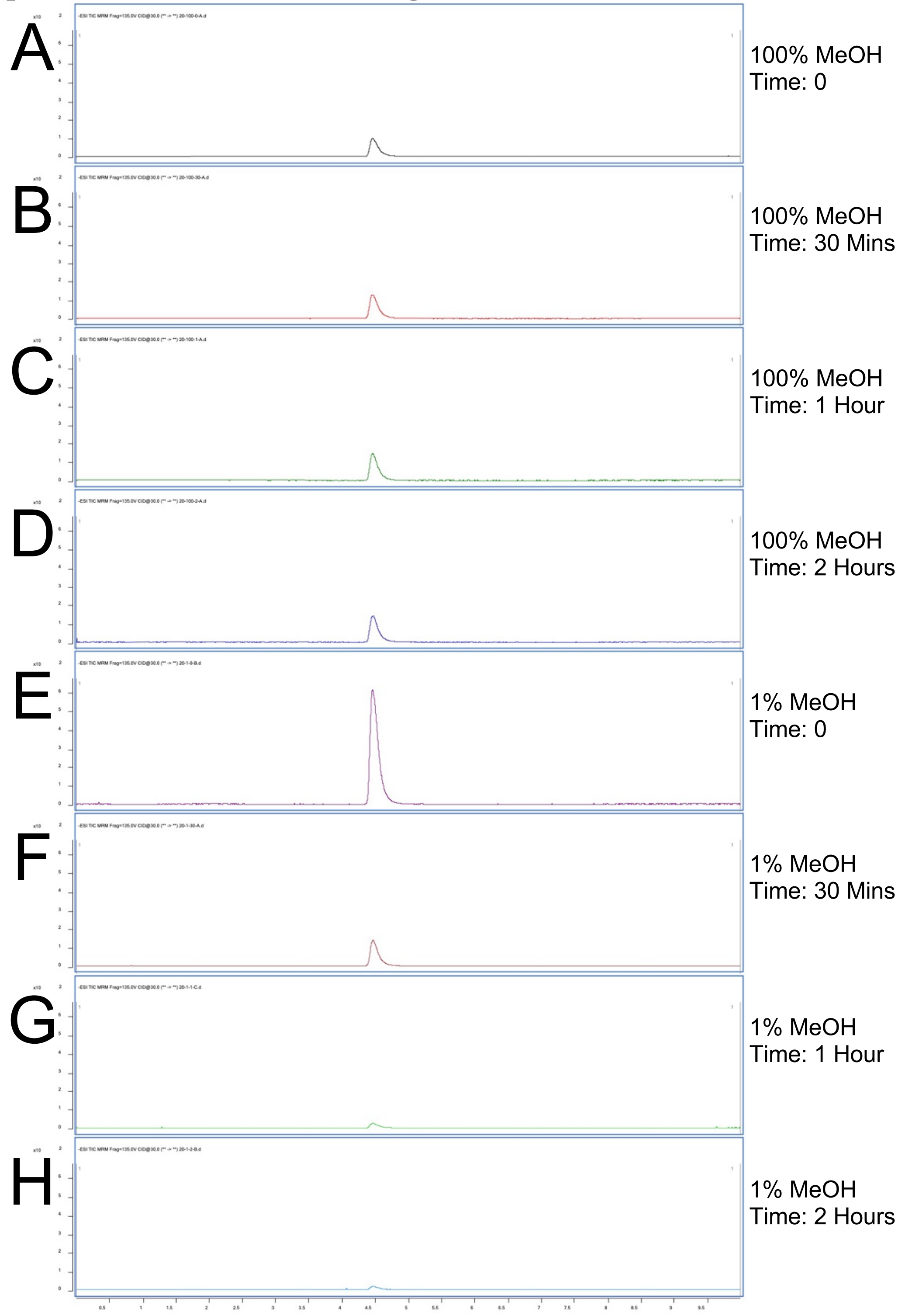

Figure B.1. Chromatograms showing that THC precipitates out of a 20 µM solution in E3 Medium with 1% MeOH over 2 hours. Note the absence of peaks suggesting chemical modification of the compound

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