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Early Life Δ9-Tetrahydrocannabinol Exposure of F0 Zebrafish Causes Hyperactivity in F1 Offspring

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A thesis submitted to the faculty of the University of Mississippi in partial fulfillment of the requirements of the Sally McDonnell Barksdale Honors College.

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ABSTRACT

As cannabis increases in its accessibility, potency, and acceptance across the United States, investigation into the multigenerational effects of Δ^9-tetrahydrocannabinol (THC) exposure during key stages of development is critical. The aim of this study was to evaluate whether there would be behavioral impacts in the F1 offspring following a dose-response of THC exposure (0.08, 0.4, or 1 μM) during development in the F0 generation. Zebrafish (*Danio rerio*) were utilized in this study. Behaviors (locomotive activity and anxiety-like behavior) in the F1 generation were evaluated at 120 hours post-fertilization (hpf) with the larval photometer response (LPR) assay, and at 3, 11, and 24 weeks post-fertilization (wpf) in an open field test (OFT). F1 zebrafish at 120 hpf showed hyperactivity compared to controls in the dark-phase of the LPR assay following F0 exposure to 0.4 and 1 μM THC. In the OFT at 3 wpf, velocity, freezing duration, and time spent in the periphery were not significantly altered compared to controls. At 11 wpf, freezing duration, but not velocity or time spent in the periphery, was significantly increased following parental 0.4 μM THC exposure. At 24 wpf, velocity was significantly increased in the F1 males whose parents were developmentally exposed to 0.08 and 1 μM THC compared to controls. Time spent in the periphery and freezing duration was not significantly altered in F1 males or females at 24 wpf. These results suggest that exposure to cannabis during critical periods of development have multigenerational physiological implications for F1 generations that persist in zebrafish to adulthood, although these effects are not as consistent as observed in F0 following direct exposure.

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LIST OF ABBREVIATIONS

wpf	Weeks post-fertilization
hpf	Hours post-fertilization
THC	Δ^9-Tetrahydrocannabinol
CBD	Cannabidiol
CBR	Cannabinoid Receptors
CB1R	Cannabinoid Receptor 1
CB2R	Cannabinoid Receptor 2
CNS	Central nervous system
ECB	Endocannabinoid
AEA	Arachidonylethanolamide
2-AG	2-arachidonoylglycerol
NAPE-PLD	*N*-acylphosphatidylethanolamine-specific phospholipase D
PCE	Prenatal Cannabis Exposure
OFT	Open Field Test
LPR	Larval Photomotor Response

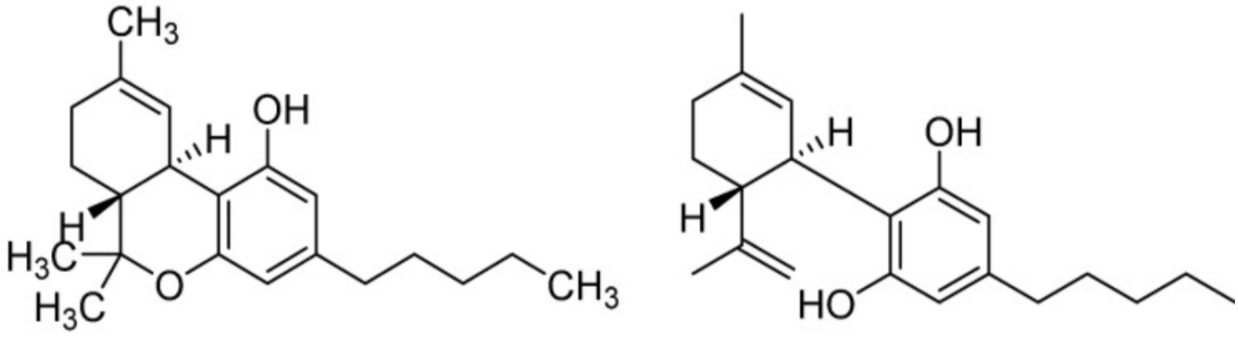
I. INTRODUCTION

1.1 Ramifications of Rapid Cannabis Legalization in the United States

Public perception of marijuana consumption has shifted in support of the drug in recent years, driving support for widespread recreational and medicinal legalization of cannabis across the United States despite potential health risks (Centers for Disease Control and Prevention, 2019). As of January 2023, the recreational use of cannabis has been legalized in twenty-one states, and cannabis remains the most prominently used federally illicit drug in the United States. The increasing legalization of cannabis and the rising acceptance of its use in light of its medical benefits have created a strong market for the drug. According to a market analysis report conducted by Grand View Research, the U.S. cannabis market size was valued at USD 10.8 billion in 2021, and is expected to expand at a compound annual growth rate of 14.9% from 2022 to 2030 (Grand View Research, 2021). It is clear that marijuana legalization has growing support, with more than two in three Americans (68%) supporting legalization in both 2021 and 2022 (Gallup, 2022). As the market for cannabis booms, an increased demand and production of higher potency Δ^9-tetrahydrocannabinol (THC) has been observed. A study of nearly 40,000 cannabis samples received and analyzed between January 1995 and December 2014, showed that the potency of Δ^9-THC present in illicit cannabis plant material has consistently risen over time from approximately 4% THC in 1995 to approximately 12% THC in 2014 (ElSohly et al., 2016). Conversely, increased concentration of THC in cannabis products results in substantially lower levels of CBD (cannabidiol), a major non-psychoactive component of cannabis. Given cannabis' reputation for pain and nausea management, usage of the drug by pregnant women has more than doubled in the past ten years, and is used more frequently than any other drug amongst pregnant women (Volkow et al., 2019). Pregnant women most commonly report using cannabis most

frequently during the first trimester (Volkow et al., 2019) as an analgesic for morning sickness (Dickson et al., 2018). Concern for adverse fetal and neonatal outcomes following THC exposure are validated by THC's ability to readily cross the placenta and further access and bind cannabinoid receptors present in the placenta and fetal brain (Lo et al., 2022).

Cannabinoid is a term used to describe compounds with a chemical structure derived from the *Cannabis sativa* plant. Cannabis is an incredibly complex plant and contains more than five hundred compounds, including more than 100 cannabinoid compounds which have varying effects (Atakan, 2012). The two primary cannabinoids present in cannabis are Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD). Δ^9-Tetrahydrocannabinol, commonly known as THC, is the psychoactive constituent of marijuana and produces behavioral effects. THC targets the endocannabinoid (ECB) system, which regulates many of the key biological processes involved in development and maintenance of neuroplasticity (Smith et al., 2020).



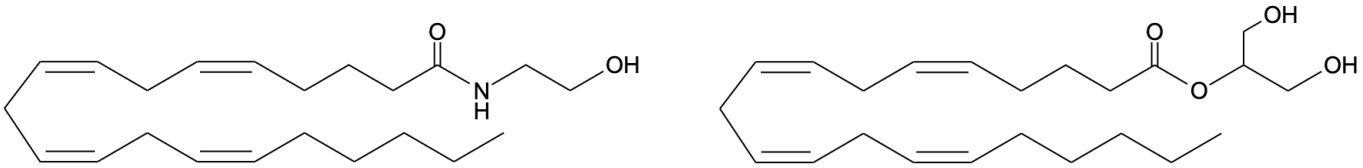
Δ^9-Tetrahydrocannabinol; THC. **Cannabidiol**; CBD.

Figure 1: Chemical structure of two primary biologically active components of cannabis: Δ^9-Tetrahydrocannabinol (THC) and Cannabidiol (CBD). Figure made using ChemDraw.

1.2 Endocannabinoid System

Following the novel discovery of THC, further research into the elusive compound and its biological mechanism led to development of various synthetic cannabinoids, as well as the later identification and cloning of two G-protein coupled cannabinoid receptors (GCPR): cannabinoid receptors 1 and 2 (CB1R and CB2R; Cnr1 and Cnr2 in fish) (Mackie, 2008). CBR are found in most tissues and are among the most abundant GPCR in the body, and thus play a critical role in modulating numerous biological processes and in regulating health and disease (Pacher et al., 2006). CB1R are encoded by the gene CNR1 (472 aa) in humans, and primarily exist in neural structures of the central nervous system (CNS) such as brain, spinal cord, and peripheral nerves. CB2R are encoded by the gene CNR2 (360 aa) in humans and predominantly exist in immune cells, namely leukocytes and keratinocytes (Romero-Sandoval et al., 2008). CBRs and ECBs comprise the endocannabinoid system, which is crucial in the physiological functions associated with pain (Fine and Rosenfeld, 2013), cardiovascular (Montecucco and Di Marzo, 2012), and reproductive systems (Correa et al., 2016).

ECBs derive their name from their ability to activate the same receptors as cannabinoids while originating from within the body. The brain produces its own organic cannabinoids, made of lipid ligands and cannabinoid receptors, which modulate action of THC. ECBs are lipid mediators, isolated from brain and peripheral tissues which include functional groups such as amides, esters, and ethers of long chain polyunsaturated fatty acids; the two most biologically active ECBs are anandamide (arachidonylethanolamide; AEA) and 2-arachidonoylglycerol (2-AG), depicted in Figure 2. ECBs are released from membrane phospholipid precursors: the major enzyme responsible for AEA production is largely considered to be *N*-acylphosphatidylethanolamine-specific phospholipase-d (NAPE-PLD) (Okamoto et al., 2009).



Anandamide; arachidonylethanolamide; AEA. **2-arachidonoylglycerol**; 2-AG.

Figure 2: Chemical structure of two primary biologically active endocannabinoids: anandamide (arachidonylethanolamide) and 2-arachidonoylglycerol; AEA, 2-AG. Figure made using ChemDraw.

2-AG synthesis can be attributed to a specific phospholipase C followed by the activity of the *sn*-1-diacylglycerol lipase (DAGL) (Ueda et al., 2011). The ECB system is ubiquitous in the body, cell membranes of the brain, organs, connective tissues, glands and immune cells. Further, the ECB is found at the intersection of several other biological systems, allowing for coordination and communication between different systems of organization within the body. Within the central nervous system (CNS), ECBs are synthesized in response to stimuli such as depolarization of the neuron or elevated intracellular calcium signaling (Di Marzo et al., 1994; Kondo et al., 1998; Petrocellis et al., 2004), which activates the enzymes contained in the ECB system responsible for synthesis, such as the 2-AG signalosome whose initiation is essential to turning precursor biological molecules into their respective ECB (Krug and Clark, 2015). Following their biosynthesis, ECBs are released. THC is an agonist to CB1R and CB2R and mimics ECB action when ingested (Lo et al., 2022). Cannabinoid receptors are localized in the adult brain in regions playing critical roles in physical movement, cognition, emotion, attention,

and memory throughout development at all stages (Jutras-Aswad et al., 2009). CB1R was found in one study to be expressed in the human fetal brain as early as 9 weeks (Bara et al., 2021), and male mitotic germ cells were found to express a high level of CB2R that when activated promotes cell differentiation and spermatogenesis (Innocenzi et al., 2019) (Figure 3).

The ECB system remains a dynamic and essential component in facilitating neurodevelopment throughout adolescence as CBR levels fluctuate, and its pathways are tightly regulated to ensure correct brain function via proper signaling (Smith et al., 2020). In both rodents and humans, there is a transient presence of CB1Rs on white matter neuronal fibers during embryonic stages of development, potentially reflecting CB1R on axons as they migrate to their final site in establishing neuronal pathways, or their presence on non-neuronal cells such as astrocytes and oligodendrocytes that guide neuronal and axonal elongation (Bara et al., 2021). Further, CB1R expression directly correlates with neural differentiation (Harkany et al., 2007), and the expression of the receptor by progenitor cells can control the neuron:glia ratio in the brain. Alterations to CB1R expression during fetal development via introduction of exogenous cannabinoids can modify the connectivity between regions of the brain critical to motor function and memory, such as the cerebral cortex and hippocampus (Berghuis et al., 2007). Various studies have provided evidence suggesting that ECB signaling cascades mediated by CBRs regulate cellular function in localized tissues via epigenetic alterations in DNA and histone methylation, as well as in miRNAs (Szutorisz and Hurd, 2018) which suggest a significant role of the ECB system in epigenetic modification. Data presented in these studies further suggest that modulation of these mechanisms by introduction of cannabis use may have long-term neurobiological impact. Thus, thorough research is needed to confirm the effects of exposure to

THC during critical windows of development as exposure to the compound has the potential to disrupt the action of the ECB system.

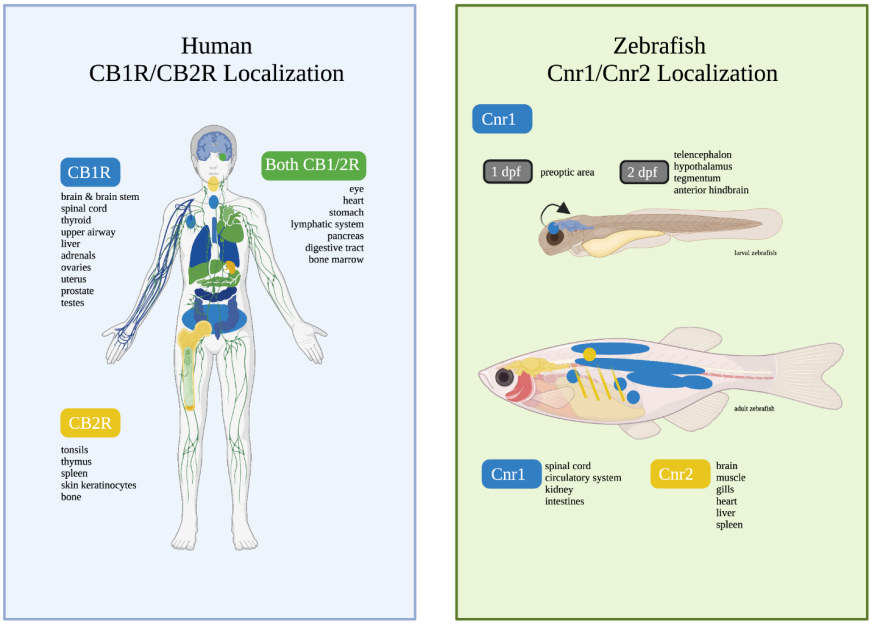


Figure 3: Endocannabinoid Receptor System. Major localization sites of CB1/2R in humans and Cnr1/Cnr2 in zebrafish. Made using BioRender.

1.3 Effects of Prenatal and Developmental THC Exposure

As cannabis becomes more readily attainable in light of loosening cannabis laws, there has been a subsequent increase in pregnant women reporting daily or recent (within the last month) cannabis use, as well as an increase in the average number of days during pregnancy that women reported cannabis consumption (Volkow et al., 2019). Brain development is an active, vital process that persists past the gestation period, and the ECB system has been confirmed by various studies to play an important role throughout development in humans and other animals. CBR expression begins in the CNS during embryonic development (Buckley et al., 1998) and remains active in varying capacities throughout adolescence. Several studies have confirmed adverse effects of interference with the ECB system in germ cells by exogenous cannabinoids (Hines et al., 2021; Innocenzi et al., 2019; Lo et al., 2022), suggesting that THC exposure during

critical periods of development may impair placental and embryonic growth via epigenetic action (Innocenzi et al., 2019).

Due to its lipophilic nature, THC has the ability to readily cross the placenta (Grant et al., 2018). Various studies have provided evidence that cannabis exposure during gestation can be correlated with low birth weight, prematurity, intrauterine growth restriction, and increased risk of miscarriage, although mechanisms for interference remain unclear (Maia et al., 2019). ECBs and ECB signaling in reproductive tissues are essential for the modulation of many key processes during pregnancy. Various studies have confirmed the presence of all active components of the ECB system (CB1R, CB2R, NAPE-PLD, FAAH) in rodent and human ovarian tissue (El-Talatini et al., 2009), oviduct (Wang et al., 2003), uterus (Paria et al., 2001; Scotchie et al., 2015), and testis (Nielsen et al., 2019). Further, CB1R and CB2R have been detected in oocytes at all stages of development, where NAPE-PLD and FAAH are expressed at high levels in growing secondary and tertiary follicles, and corpora lutea. (El-Talatini et al., 2009).

In male reproductive tissues, germ cells contain the entire ECB system which is modulated during spermatogenesis, suggesting that activation and suppression ECBs may play a role in the regulation of human spermatogenesis (Barchi et al., 2019). Any alteration of this system negatively affects male reproductive efficacy and fertility; in humans, exogenous cannabinoid exposure has been demonstrated to over-activate the system and is associated with impotence, decreased testosterone production, and the reduction of sperm motility and viability (Barchi et al., 2019; Nielsen et al., 2019). Further, recent studies have associated disruption of the ECB system with alteration in the sperm epigenome in humans, rats (Murphy et al., 2018; Schrott and Murphy, 2020), and mice (Innocenzi et al., 2019).

1.4 Multi-Generational Exposure

Multi-generational epigenetic effects occur when an external environmental trigger induces epigenetic change that can be observed in at least one of the subsequent generations (Szutorisz and Hurd, 2016). Until the last decade, it was widely believed that the neurobiological effects of environmental toxins and drugs of abuse, such as cannabis, alcohol, cocaine, and opiates, would be reprogrammed across most of the genome during embryonic development from parents to offspring (Cantone and Fisher, 2013). Several recent studies have shown that the effects of exogenous disturbances of this variety during parent lifetime were inherited through the germline from parent to child (Chastain and Sarkar, 2017; Szutorisz et al., 2014; Vassoler et al., 2013; Yohn et al., 2015; Zeid and Gould, 2020). In humans, a gestating female exposure results in both the F1 and F2 generation germline being exposed to the environmental toxin through the reprogramming of the germline epigenome, specifically in the processes of embryonic gonadal development and germline differentiation (Skinner, 2008). The process by which environmental compounds promote transgenerational germline reprogramming remains to be elucidated, but their ability to do so is the causal factor in epigenetic transgenerational phenotypes (Figure 4).

In one study, adolescent exposure of rats to THC resulted in behavioral and neurobiological abnormalities in the subsequent generation as a result of parental germline exposure to the drug. Adult F1 offspring which were not directly exposed to THC exhibited compulsive heroin-seeking tendencies, with enhanced stereotyped behaviors during the withdrawal phase. Molecularly, parental exposure was affiliated with changes in the mRNA expression of cannabinoid, dopamine, and glutamatergic receptors involved in neural circuits responsible for mediating compulsive behaviors and reward sensitivity (Szutorisz et al., 2014). These findings show that parental THC exposure affects the molecular characteristics of the

brain and behavior in subsequent generations; additionally, the question is raised as to how parental exposure impacts the development of psychiatric disease in adult offspring as a result of epigenetic alteration. In depth investigation is still necessary to fully understand the mechanism of interference underlining the inheritance of effects through the germline, as well as the extent of transmission through generations (F2 and beyond).

Several studies have provided evidence that the two primary ECBs, AEA and 2-AG, and the signaling cascades mediated by CB1Rs and CB2Rs utilize epigenetic alterations to regulate cellular functions in various tissues. For example, epigenetic alterations to the ECB system have been observed in DNA methylation via cell differentiation in human keratinocytes and epidermal cells (D'Addario et al., 2013); in miRNA via regulating cells involved in inflammatory response (Jackson et al., 2014); and in histone methylation via differentiation and inhibition of gliomagenesis (Aguado et al., 2007). This data suggests that the ECB system is essential in regulating a wide range of cellular functions through various mechanisms of epigenetic modifications, and that interference with ECB mechanisms by exogenous cannabinoids may have multi-generational effects.

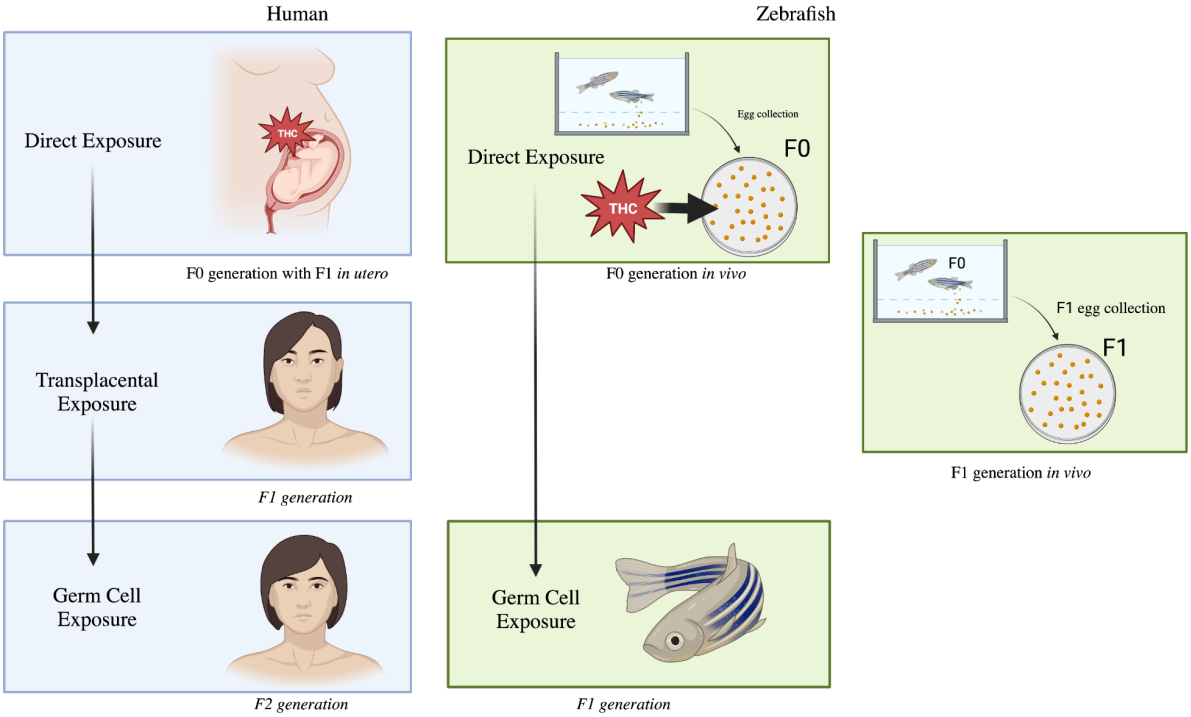


Figure 4: Multi-Generational Exposure: Schematic representation of *in utero* exposure of humans compared to *in vivo* exposure of zebrafish to THC. In humans, transplacental THC exposure of an F1 generation *in utero* results in germ cell exposure of the F2 generation. In zebrafish, direct THC exposure of an F0 generation *in vivo* results in germ cell exposure of the F1 generation. Made using BioRender.

1.5 Zebrafish as a Model to Study Cannabinoid Toxicity

The zebrafish has become an important and useful vertebrate model organism for molecular biology, developmental biology, genetics, neuroscience, cancer and toxicology research throughout the last twenty years (Demin et al., 2018; Kawakami et al., 2016; Metscher and Ahlberg, 1999; Mione and Trede, 2010; Steenbergen et al., 2011; Stewart et al., 2014; Vaz et al., 2019). The zebrafish models' advantages are vast, including their genetic tractability, accelerated rate of development, external fertilization, small size, and easy maintenance (Demin et al., 2018; Stewart et al., 2015). Studies have shown that the signals which drive avoidance and thigmotaxis in zebrafish larvae are conserved and are similar to those which control anxiety in

humans (Richendrfer et al., 2012). Thigmotaxis is a behavior observed in animals, including zebrafish, in which they exhibit a tendency to stay in close proximity to physical boundaries, such as walls or other objects in their environment. This behavior is often seen as a means of seeking refuge or security, as the animals are able to use the boundaries to protect themselves from predators or other threats. In zebrafish, thigmotaxis is commonly observed in experimental settings, where the fish are placed in a tank or other enclosure with a defined boundary. The fish will often swim along the perimeter of the tank, maintaining contact with the walls, rather than exploring the open space in the center. The majority of existing literature on THC, ECB, and the ECB system utilize in vitro or rodent models, but zebrafish have emerged as a strong model for research exploring multi-generational exposure. Unlike rodent models, fertilization and embryonic development occur *ex utero* in zebrafish, and small molecules (such as THC) readily travel across the embryo membrane, making zebrafish a fit subject for assessing developmental effects of early-life exogenous THC exposure (Krug and Clark, 2015; Vaz et al., 2019). Zebrafish also have complex, well defined behavioral patterns in developmental stages from larvae through adulthood (Kalueff et al., 2013), and their small size and optical transparency allow for *in vivo* visualization of internal processes throughout maturation to adulthood (Kozol et al., 2016).

Zebrafish possess 26,206 protein-coding genes, with 71.4% of human genes and 82% of disease-causing human proteins having direct orthologs in the zebrafish system (Howe et al., 2013). All but one human gene implicated in cannabinoid signaling have direct analogs in zebrafish: select ECB system homologs and their protein identity rates (HomoloGene database) are summarized below in table 1. Like other teleost fish, the entire zebrafish genome undergoes an additional round of replication, resulting in multiple gene duplicates (Stewart et al., 2015), further complicating the intricate field of ECB research. No homologs have been identified for

N-acylethanolamine acid amidase, one of many enzymes known to hydrolyze AEA in mammalian systems (Klee and Schneider, 2011). Additionally, four human genes have two zebrafish homologs each (Klee and Schneider, 2011). Zebrafish possess an enhanced conservation of pharmacological properties and molecular targets (Milan et al., 2003) and are highly susceptible to genetic manipulation, making them a uniquely malleable model for behavioral research following exposure to exogenous cannabinoids.

Despite being a non-mammalian model, adult zebrafish share many similarities with mammals in terms of brain development and structure, while it is significant to note that the zebrafish varies from the mammalian brain in terms of complexity and some neural structures are formed differently. Similar structures include the hippocampus, diencephalon, tectum and tegmentum, and the cerebellum; these are of the same cell types, and their mechanisms to maturation and pathways of differentiation are similar to those of mammals (Vaz et al., 2019). The zebrafish brain is simple in comparison to the mammalian brain as zebrafish lack the trisynaptic neural potential essential for complex learning and memory, but previous studies have shown zebrafish to be capable of a variety of simple learning tasks such as avoidance and olfactory or appetitive conditioning (Braubach et al., 2009; Xu et al., 2007). Larvae hatch from their chorion in 2-3 days post-fertilization, and begin to display a range of behaviors including hunting, avoidance, escape, phototaxis, and thigmotaxis (Richendrfer et al., 2012). There have been limited studies conducted on the expression of ECB in zebrafish, but RT-PCR analysis has allowed for confirmation of the presence of these homologs as well as their localization throughout development, although the function of these receptors in relation to zebrafish behavior remains insufficiently understood (Luchtenburg et al., 2019). Cnr1 is first observed in zebrafish at 24 hpf in a small region of the preoptic area of the diencephalon, and the distribution

of cnr1 continues throughout development with substantial density in the hypothalamus, tegmentum, telencephalon, and anterior hindbrain (Lam et al., 2006); Cnr2, similarly to other vertebrates, is primarily expressed in peripheral tissues in zebrafish (Rodriguez-Martin et al., 2007).

Table 1. Sequence alignment homology results of selected proteins related to the endocannabinoid systems between humans and zebrafish, based on the HomoloGene database.

Human genes	Zebrafish genes	Zebrafish protein	Protein identity, %
CB1R	*Cnr1*	Cannabinoid receptor 1	75
CB2R	*Cnr2*	Cannabinoid receptor 2	46
CB2R	*LOC1018854336*	Cannabinoid receptor 2-like	45
CNRIP1	*Cnrip1b*	Cannabinoid receptor interacting protein 1b	61
NAPE-PLD	*Napepld*	N-acyl phosphatidylethanolamine	65
DAGLA	*Dagla*	Diacylglycerol lipase, alpha	72
DAGLB	*Dagb*	Diacylglycerol lipase, beta	59

1.6 Study Goals & Hypotheses

The goals of this study are to determine the behavioral effects in the F1 offspring following an F0 early-life THC exposure. Specifically, we will determine if there are dose- or sex-dependent effects and if these effects persist into adulthood in the F1 offspring.

The hypotheses of this study are:

1. F1 behavior will exhibit dose-dependent alteration as a result of THC exposure to F0 zebrafish.
2. Additionally, F1 hyperactivity will persist into adulthood as was observed in F0 zebrafish following THC exposure.

II. MATERIALS AND METHODS

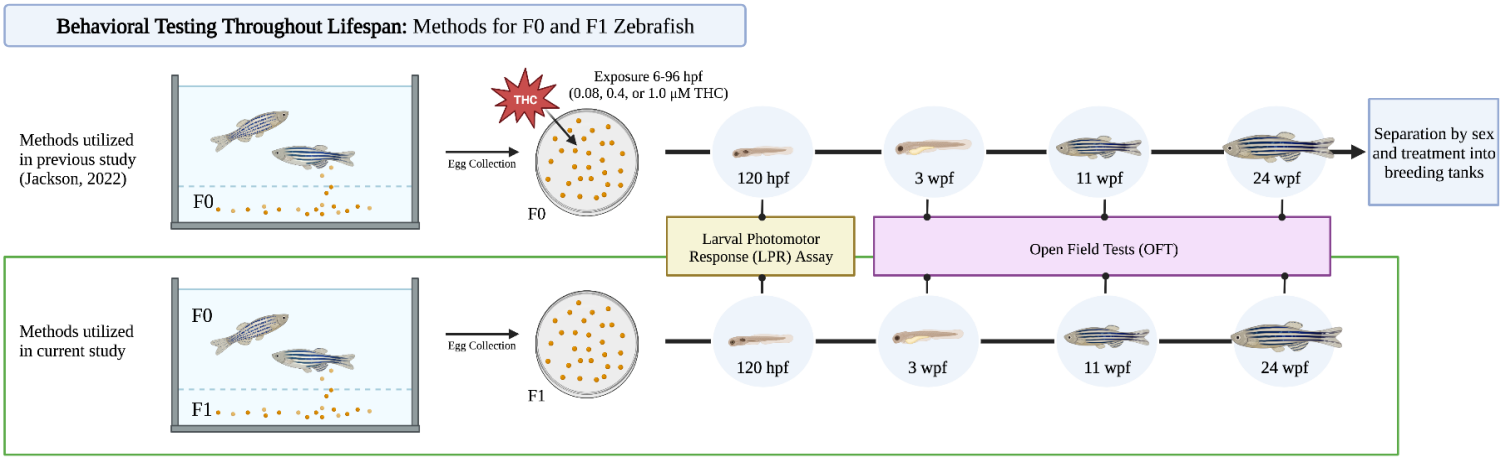


Figure 5: Behavioral Methods. Schematic representation of behavioral assay methods and time endpoints utilized for F0 and F1 zebrafish. In a previous study (Jackson, 2022), the F0 generation was bred and exposed *in vivo* to 0.08, 0.4, or 1.0 µM THC, and were further assessed for alteration in locomotive behavior throughout lifetime at 120 hpf, and 3, 11, and 24 wpf. At 120 hpf, larval photomotor response (LPR) was used; at 3, 11, and 24, fish behavior was assessed via open field testing (OFT). At the onset of reproductive maturity, F0 zebrafish were separated by sex and treatment into breeding tanks to yield an F1 generation. F1 zebrafish are exposed to THC as germ cells, and their behavior was assessed using the methods previously described for F0 zebrafish. Green box indicates research described in this thesis. Made using BioRender.

2.1 Δ^9-Tetrahydrocannabinol Exposures to 5D F0 Zebrafish

In this study, the 5D zebrafish strain was utilized. Wild type 5D zebrafish were obtained from Dr. Robyn Tanguay at Oregon State University. At all times, guidelines set in place by the Institutional Animal Care and Use Committee were adhered to. Healthy adult zebrafish were kept in Aquatic Habitats Zebrafish Flow-through System (Aquatic Habitats, Apoka, Florida) under ambient conditions (pH 7.5-8.0, dissolved oxygen 7.2-7.8 mg/L, conductivity 730-770 mS, and temperature 27°C- 29°C, 14:10 light:dark.) In a previous study, 5D F0 zebrafish were exposed to 0 (0.05% DMSO), 0.08, 0.4, or 1.0 µM THC (obtained from the NIDA Drug Supply

Program (Research Triangle Park, North Carolina)) from 6 to 96 hours post-fertilization (hpf). Following the F0 exposure, fish were raised in clean culture water until adulthood.

2.2 F0 Reproduction to obtain F1 Zebrafish

Adult F0 fish were bred to produce an F1 generation by placing a 1:1 ratio of male and female adult fish into breeding tanks overnight. The next morning, debris and waste were removed, and fertilized embryos were collected into petri dishes that contained egg embryo water (pH 7.4-7.4; 60 ppm Instant Ocean, Cincinnati, Ohio). F1 embryos were not directly exposed to THC.

2.3 Larval Photometer Response Assay

Larval photometer response (LPR) assay was conducted in F1 as a means of analyzing larval zebrafish behavior in response to light and dark exposure. The assay was performed at 120 hpf. Behavior was measured using a ViewPoint ZebraBox (ViewPoint, Montreal, Canada). Larvae were pipetted into a 96-well plate where one fish was placed in each well (300 μL embryo water per well). The plate was placed in the ViewPoint ZebraBox in a temperature controlled room (27°C- 28°C). Following ten minutes of acclimation in light [8000 lux], the assay consisted of an additional ten minutes in the dark [0 lux], followed by ten minutes in the light, followed by another ten minutes in the dark, and finishing with ten minutes in the light for a total assay time of 50 minutes. In each phase of the assay, the locomotor activity (total distance traveled) was measured and analyzed every two minutes for each fish.

2.4 Open Arena Tests

In order to determine the breadth and persistence of the effects of multigenerational exposure to THC in regard to locomotor activity and as a measure of thigmotaxis, open field tests (OFT) were conducted at 3, 11, and 24 weeks post-fertilization (wpf). OFTs were conducted in an empty arena in a temperature-controlled room, utilizing system water from the primary zebrafish culture unit (Aquatic Biosystems). Each arena contained an inner and outer zone, where the inner zone and periphery were equal in area. In behavioral zebrafish testing, the location where a fish swims within an arena can be indicative of its behavior and state. When a zebrafish swims in the inner zone, it is considered to be exploring its environment and exhibiting normal exploratory behavior. On the other hand, when a zebrafish spends more time swimming in the peripheral zone, it may be exhibiting thigmotaxis. Thigmotaxis in zebrafish is characterized by a preference for swimming close to the walls or other boundaries of the arena. Researchers often use time spent in the periphery as a measure of thigmotaxis in zebrafish. If a fish spends a significant amount of time in the peripheral zone, it suggests that the fish is exhibiting a preference for the boundaries of the arena and may be experiencing stress or anxiety. By contrast, if a fish spends more time in the center zone, it suggests that the fish is exploring its environment and is less stressed or anxious. For adult fish, fish were separated by sex the day prior to testing. The fish were given a ten minute acclimation period prior to testing to adjust to temperature and lighting conditions present in the room.

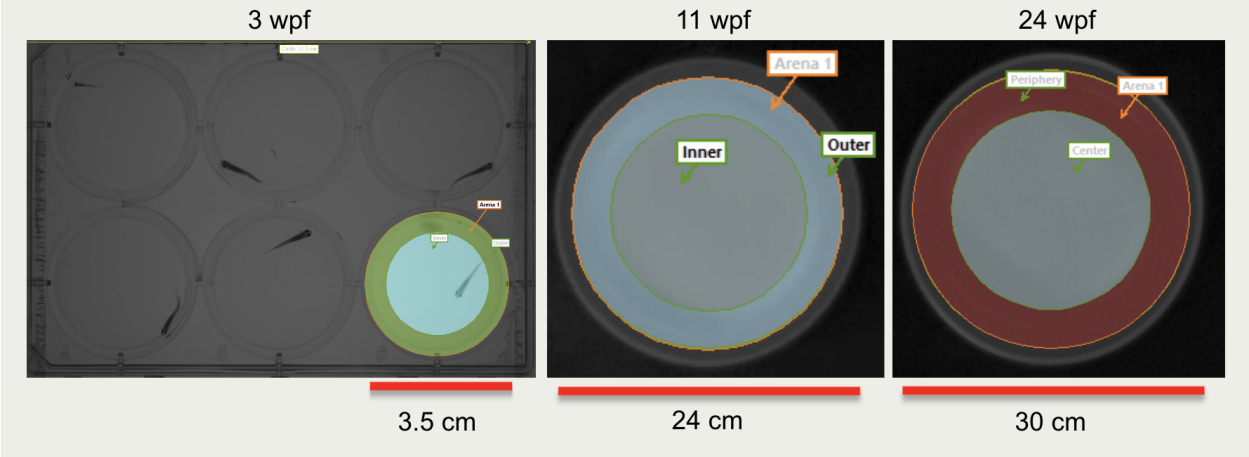


Figure 6: OFT. Arenas and their respective dimensions for each stage of OFT assays: 3 wpf, 6 well plate, 3.5 cm/diameter; 11 wpf, 2-gallon bucket, 24 cm/diameter; 24 wpf, 5-gallon bucket, 30 cm/diameter.

At 3 wpf, juvenile zebrafish were tested in a 6 well plate, 3.5 cm in diameter filled with 10 mL of water (Figure 6). At 11 wpf, zebrafish at the onset of sexual maturity were tested in a 2-gallon bucket, measuring 24 cm in diameter and 27 cm in height and containing 4 L of water to a height of 12 cm. At 24 wpf, adult zebrafish were tested in a 5-gallon bucket, measuring 28 cm in diameter and 37 cm in height and containing 10 L of water to a height of 17 cm.

Each arena was divided into two zones: the inner zone or center and outer zone or periphery that are equal in area (i.e. for the 24 wpf arena, the inner zone was 20 cm diameter circle and the outer zone was 4 cm from arena wall to the inner zone) (Figure 6). Treatments were monitored and designed to avoid diurnal variation in zebrafish through randomization of treatment tanks, and consistent testing conducted between 12 pm and 5 pm. Fish were transferred one at a time into the arena and assessed for five minutes. Following each test, fish were returned to their original tanks. Following each tank's complete run through the open arena tests, the water was changed in the bucket. EthoVision software was used to track fish velocity, freezing duration, and time spent in each respective zone. The bucket was recorded from above using a

color GigE camera. Twenty-four fish per treatment were tested for immature fish (3 and 11 wpf), and 12 males and 12 females (n=12/sex/treatment) were tested for adult fish (24 wpf).

2.5 Statistical Analysis

All data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. Statistical analysis was performed following the LPR assay on the total distance traveled during the acclimation, dark, and light phases separately. An ANOVA was performed to determine respective differences between the total distance traveled of fish during each phase of the assay separately (ANOVA, SNK posthoc, $p \leq 0.05$). An ANOVA on ranks was conducted for all data that was unable to meet the requirements of the parametric tests previously stated. SigmaPlot 14.0 software was used for graphical and statistical analysis.

OFT collected behavioral data across fish lifespan, and measured total distance traveled, freezing duration, and time spent in periphery. For immature fish, a one-way ANOVA was performed with treatment as the sole factor (ANOVA, SNK posthoc, $p \leq 0.05$). For sexually mature adult fish, a two-way ANOVA was performed with both sex and treatment as factors. (SNK posthoc, $p \leq 0.05$.)

III. RESULTS

3.1 Influence of Multi-Generational THC Exposure on Zebrafish Behavior in Larval Photometer Response Assay at 120 hpf

The LPR assay was performed at 120 hpf in the F1 offspring. During the acclimation phase of the LPR, fish whose parents were developmentally exposed to the two highest concentrations of THC (0.4 and 1 μM) were significantly hypoactive compared to the control group. In contrast, during the dark phase of the LPR, significant ($p \leq 0.05$) hyperactivity was observed in the F1 zebrafish following F0 exposure to 0.4 and 1.0 μM THC (Figure 7).

A.

B.

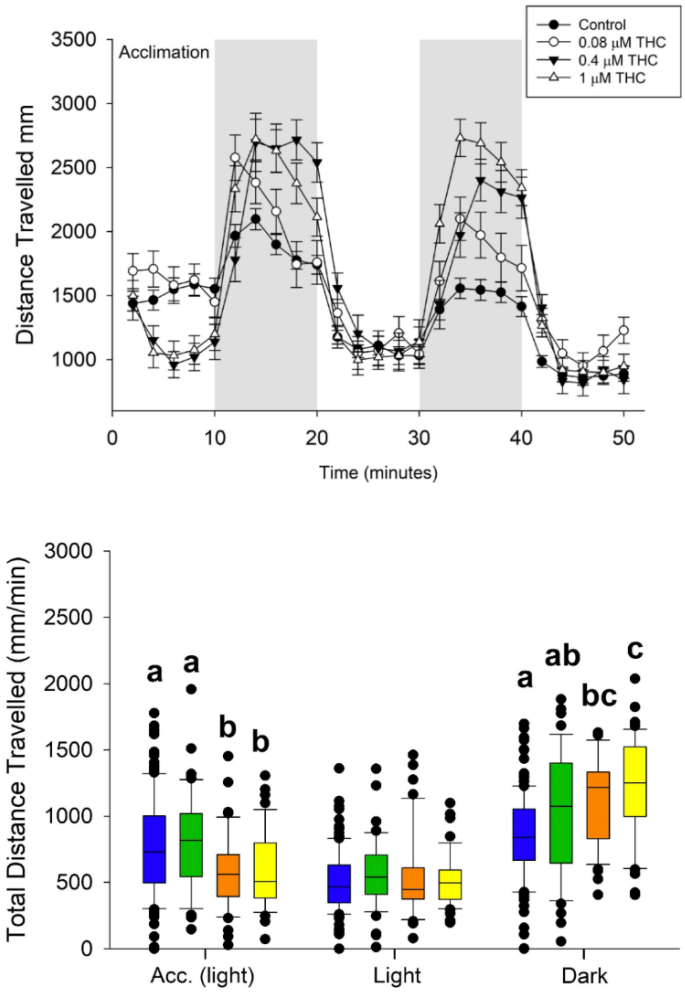


Figure 7: Larval Behavior. LPR assay results; A) Distance traveled (mm) by treatment groups across duration of 50-minute assay, B) Total distance traveled per minute (mm/min) during each respective light/dark phase of LPR assay. Results indicate significant ($p \leq 0.05$) hypoactivity during the acclimation phase and hyperactivity in the dark phase in F1 following F0 exposure to 0.4 and 1.0 µM THC compared to controls. Letters not in common indicate a significant difference between treatments. (ANOVA, SNK post-hoc, $p \leq 0.05$, n = 50 fish per treatment).

3.2 Influence of Multi-Generational THC Exposure on Zebrafish Behavior in Open Field Tests at 3, 11, and 24 wpf

OFT measured fish velocity, freezing duration, and time spent in the periphery at 3, 11, and 24 wpf. At 3 wpf, F1 velocity, freezing duration, and time spent in the periphery were not significantly altered compared to controls (Figure 8A-C). There was a significant ($p \leq 0.05$) decrease in time spent in the periphery in the F1 zebrafish bred from F0 fish exposed to 0.08 compared to 1.0 μM THC (Figure 8C). At 11 wpf, velocity and time spent in the periphery were not significantly altered compared to controls (Figure 9A, 9C). Freezing duration was significantly ($p \leq 0.05$) increased following parental 0.4 μM THC exposure compared to 0.08 μM THC but not controls (Figure 9B).

Adult male and female fish were assessed at 24 wpf. In males, there was a significant ($p \leq 0.05$) increase in velocity in fish whose parents were exposed to 0.08 and 1.0 μM THC, compared to controls. However, velocity in females was not statistically significant across all treatment groups (Figure 10A). Effects of THC exposure on thigmotaxis did not persist to adulthood, as time spent in the periphery was not statistically different for male or female fish (Figure 10C). Female F1 fish from the F0 exposed to 0.08 μM THC exhibited significantly lower freezing duration at 24 wpf compared to female fish exposed to 0.4 μM THC (Figure 10B).

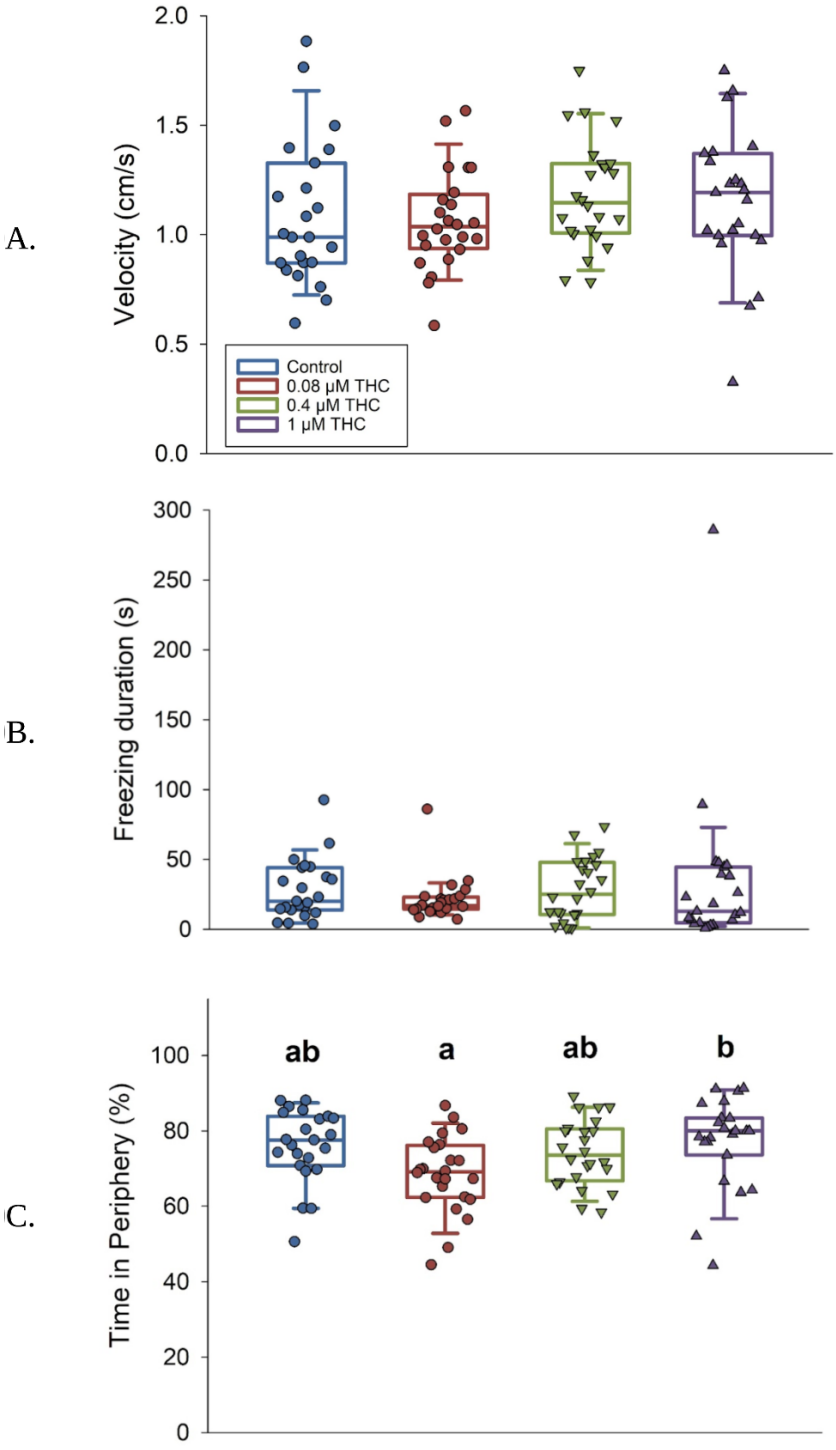


Figure 8: Open Field Behavior of F1 3 wpf zebrafish. A) velocity (cm/s), B) freezing duration (s), C) time spent in the periphery of the arena (%) (n=24). OFT results indicate dose-dependent increased thigmotaxis in F1 fish multi-generationally exposed to 1.0 µM THC compared to those exposed to 0.08 µM THC. Letters not in common indicate a significant difference (ANOVA, SNK post-hoc, $p \leq 0.05$).

A.

B.

C.

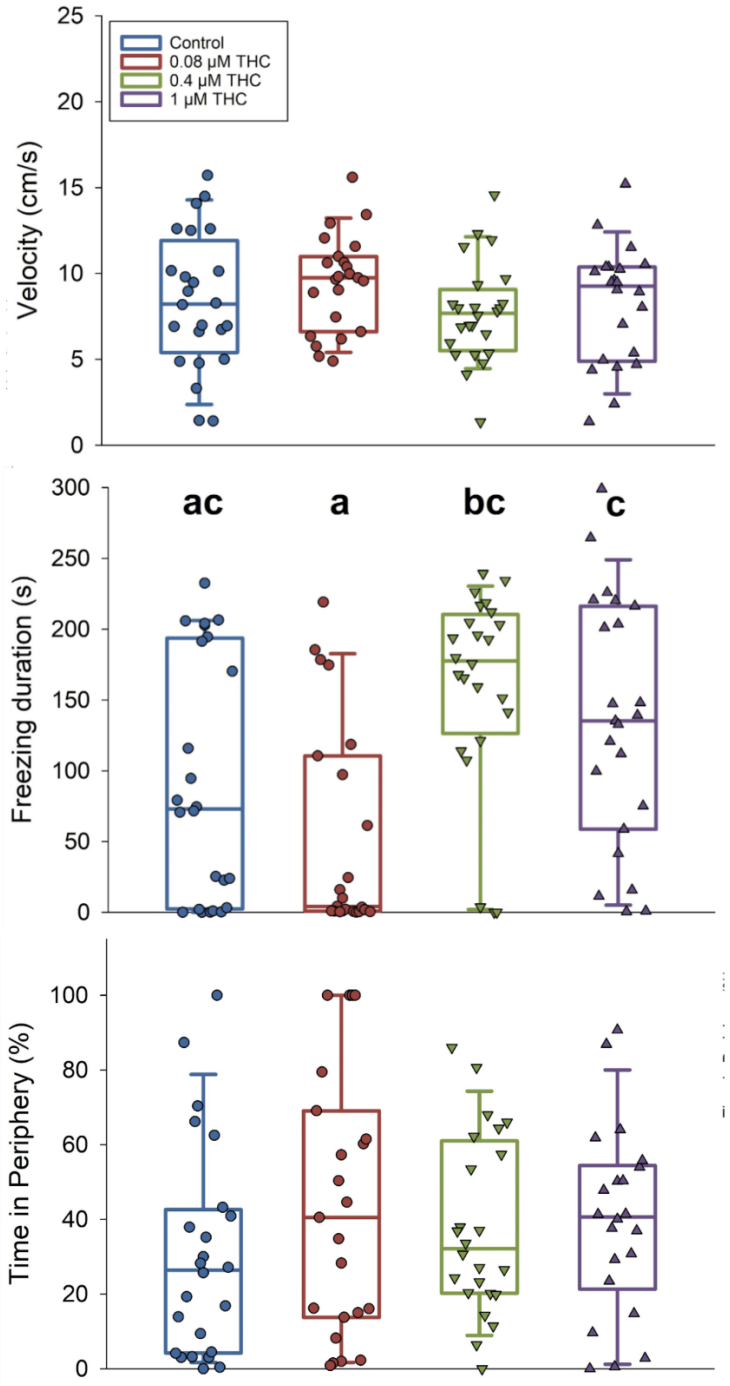


Figure 9: Open Field Behavior of F1 (11 wpf) Zebrafish. A) velocity (cm/s), B) freezing duration (s), C) time spent in the periphery of the arena (%) (n=24). Results indicate no significant effects of THC exposure on thigmotaxis and hyperactivity compared to controls, but show increased freezing duration in fish multigenerationally exposed to 1.0 µM THC compared to those exposed to 0.08 µM THC. Letters not in common indicate a significant difference (ANOVA, SNK post-hoc, $p \leq 0.05$).

A.

B.

C.

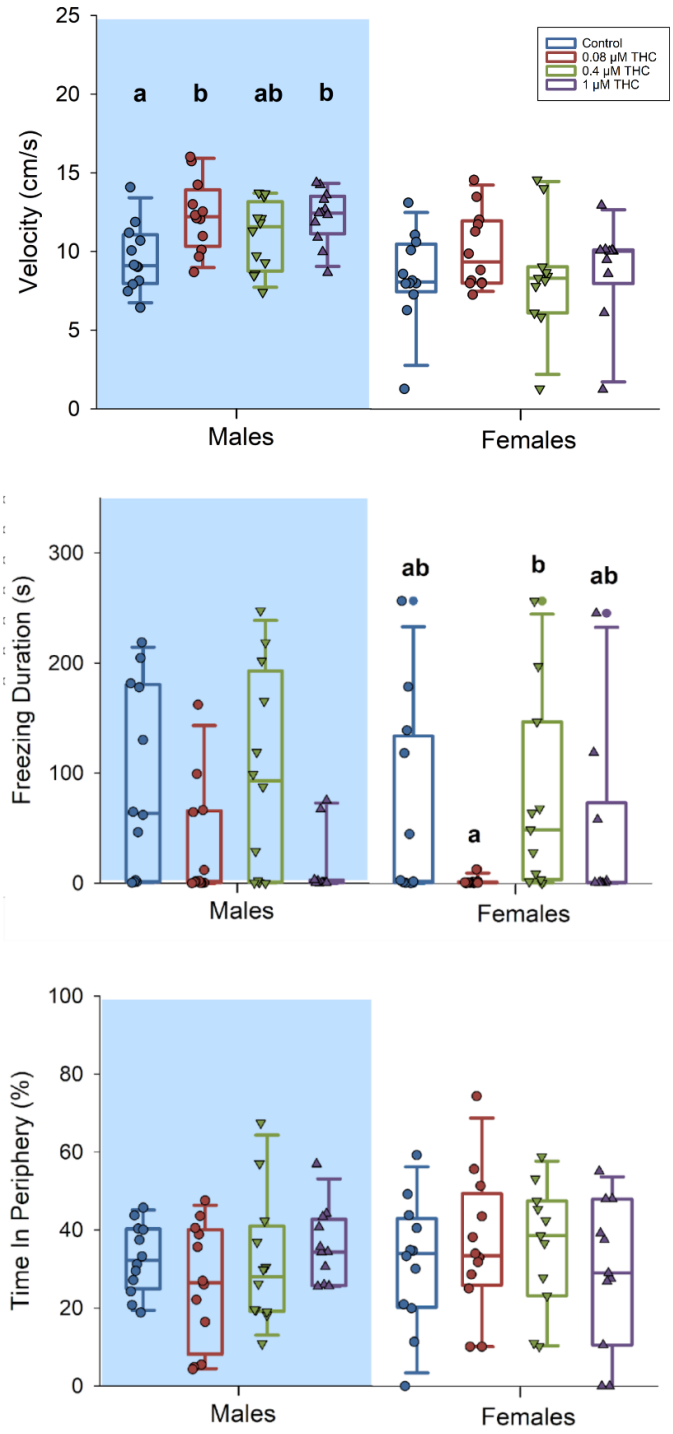


Figure 10: Open Field Behavior of F1 Male and Female Adult (24 wpf) Zebrafish. A) velocity (cm/s), B) freezing duration (s), C) time spent in the periphery of the arena (%) (n=12/sex/treatment). For velocity, in males only, 0.08 and 1 µM parental THC exposure cause significant hyperactivity. THC's thigmotactic effects did not persist into F1 adulthood. Letters not in common indicate a significant difference (ANOVA, SNK, post-hoc, $p \leq 0.05$).

IV. DISCUSSION

4.1 Multigenerational THC Toxicity

Given the importance of the ECB system in regulating various crucial processes during brain development, studies which investigate the effects of exogenous THC exposure during development are of the utmost importance. Following an F0 early developmental exposure to THC, studies have confirmed behavioral abnormalities in rodents and zebrafish at low and high doses (Jackson, 2022; Navarro et al., 1994; Richendrfer et al., 2012; Rubio et al., 1995; Schneider, 2009). In rats, direct prenatal exposure to THC had effects on locomotor activity characterized by hyperactivity which persisted from early postnatal stages to adulthood, in addition to the presence of sex-dependent effects at full maturity (Schneider, 2009). Adult hyperactivity, alteration in locomotor habituation, and exploratory behavior have been consistently observed in female rats, whereas male rats displayed marginally less consistent locomotive alteration at all ages tested (Navarro et al., 1994; Rubio et al., 1995). The existence of sex-dependent effects in previous literature in addition to the suspected epigenetic effects of exogenous cannabinoid exposure raises the need for further research into the multigenerational effects of an F0 exposure to THC on F1 offspring in order to determine if prenatal THC exposure impairs offspring neurobehavior. In F0 zebrafish, whose offspring were used in this study, exposure to THC during critical periods of development caused substantial behavioral effects that persisted across lifespan (Jackson, 2022).

There is limited literature available which investigates the multigenerational effects of exogenous cannabinoid exposure; however, the results of those published tell a congruent story. These previous studies have confirmed the effect of parental THC exposure on the neurodevelopment of the subsequent generation via epigenetic action on striatal gene expression

and reward-related behavior. Further, they discuss the finding that the epigenetic effects of THC exposure become more pronounced in the dorsal striatum throughout lifetime, as opposed to during adolescence where genetic modifications were more evident in the ventral striatum, with the total number of modifications increasing throughout lifetime (Szutorisz et al., 2016). In terms of behavioral findings in the Szutorisz study, female adult rats actually exhibited significantly decreased locomotive behavior compared to males, showing that statistically significant increased activity did not persist throughout lifetime in females. Both male and female F1 adults were found to have impairment in dorsal striatal long-term depression as a result of functional synaptic change of neuroplasticity, although it was apparent that parental THC history did not induce a gross impairment of motor behavior in males or females, aside from that of adult females which showed decreased locomotor activity. These findings have particular relevance in the context of the onset of many neuropsychiatric disorders which are characterized by age-dependent vulnerability (Jutras-Aswad et al., 2009; Smith et al., 2020; Szutorisz et al., 2016; Vaz et al., 2019). Although the current study did not examine the neurophysiological changes incurred by F1 zebrafish throughout lifetime, its aim is consistent with that of the Szutorisz study in assessing the zebrafish's anxiety-like behaviors as a measure of the effects of the epigenetic action of THC across multiple generations.

In F1 zebrafish, we found lingering effects of F0 developmental exposure to THC which contributed to alteration in F1 locomotive activity, most notably characterized by early-life hyperactivity in the dark phase of the LPR assay. These results are consistent with previous studies which have identified increased levels of anxiety-characterizing or addiction-related behaviors in offspring not directly exposed to THC due to epigenetic effects of exogenous cannabinoids (Szutorisz and Hurd, 2016). The results of our lifetime assays found the most

substantial hyperactivity in the 120 hpf LPR assay, with most anxiety characterizing behaviors indistinguishable from controls as fish developed to adulthood. Additionally, at 3 wpf 1 μM THC exposure significantly increased time in periphery compared to 0.08 μM THC. Previous studies have provided evidence of THC and other cannabinoids' biphasic dose-dependent effects on adult zebrafish behavior (Akhtar, 2013; Lovitt, 2020; Pandelides et al., 2020). The nuance of THC's biphasic effect has not been thoroughly researched thus far, as the role of possible epigenetic processes such as DNA methylation in the transmission of the effects of cannabinoids from parent to offspring requires further studies to assess.

4.2 Analysis of Larval Behavioral Assay

Unless zebrafish are prompted by touch, they may begin to swim as early as 48-72 hpf. Young zebrafish lack many sensory abilities until they are further developed, but it has been noted that after 96 hpf, fish exhibit different swimming behavior in response to light or dark stimuli, with increased movement being associated with dark exposure (Saint-Amant and Drapeau, 1998). As discussed, the LPR assay is utilized to analyze this behavior in response to cyclical light and dark stimuli over the course of a 50-minute period in 120 hpf fish. This assay is particularly useful and was selected to measure F1 zebrafish behavior due to its utility in previous studies which have found photomotor responses to be a viable measure of toxicity in model organisms (Rihel and Schier, 2012; Shen et al., 2020; Yang et al., 2018). Heightened activity of larval zebrafish in response to dark conditions is likely an evolutionary response, driven by the urge to seek light conditions to maximize avoidance of predators and acquire food (Burgess and Granato, 2007). The results of this study are consistent with the existing literature confirming increased activity during dark conditions, as all treatment groups demonstrated higher activity in the dark phase of the assay regardless of exposure concentration (Figure 7).

However, F1 zebrafish from parents that were exposed to 0.4 and 1.0 μM THC exhibited significant hyperactivity in the dark phase compared to controls – fish exposed to 0.08 μM THC did not exhibit activity statistically significant from controls. In the previous study examining F0 zebrafish, hyperactivity in dark conditions was also observed via LPR assay exclusively in fish exposed to 0.4 μM THC (Jackson, 2022). F0 fish exposed to 1.0 μM THC did not exhibit statistically significant activity in the dark phase compared to controls.

Our findings confirm the presence of multi-generational implications following early developmental THC exposure, given the sustained dose-dependent hyperactivity across both generations at 120 hpf. These results are congruent with those of other studies which have confirmed multi-generational effects of cannabinoids on behavior of subsequent generations (Carty et al., 2019; Szutorisz et al., 2014), although no studies to date report specifically increased levels of dose-dependent hyperactivity at the earliest stages of offspring development. Our findings insinuate that the effects of an F0 early life THC exposure may be exacerbated in F1 offspring, as hyperactivity was observed in offspring following parental exposure to both 0.4 and 1.0 μM THC, whereas only the parents exposed to 0.4 μM THC showed hyperactivity themselves at 120 hpf.

For future work, my committee provided suggestions to re-analyze the LPR results. Here we pooled the two dark phases together and the two light phases together to determine significance, but an individual fish could behave differently in dark phase one compared to dark phase two as it appears for the controls in Figure 8. In the future, a two-way repeated measures ANOVA could be used for both F0 (Jackson 2022) and F1 analyses to account for repeated measurements over the course of 50 minutes with treatment and time as the factors.

4.3 Analysis of Lifetime Behavioral Assay

Open field tests were conducted across zebrafish lifespan to determine the persistence of multigenerational effects of THC exposure on fish anxiety. Anxiety in fish is characterized by thigmotaxis, which is exhibited by fish swimming close to the periphery wall of an arena (Baiamonte et al., 2016; Nielsen et al., 2018). Similar to larval sensitivity to dark stimuli, thigmotaxis is indicative of stress, likely a product of evolution where escape or protection is desirable in a dangerous situation. An additional measure of anxiety in fish is freezing duration, or the time fish spent non-moving during OFT, which is a fear response mechanism (Egan et al., 2009). Analyzing F1 zebrafish behavior throughout lifetime using these metrics of measurement allows for insight into how human exposure to THC may influence the progression of neuropsychiatric disease in subsequent generations, as well as further investigate the importance of the ECB system in regulating neurodevelopmental processes. In humans, developmental exposure to THC results in causative long-term effects related to focus, reward and goal-oriented behaviors, and further can be associated with the development of a variety of psychiatric disorders, including obsessive-compulsive disorder (OCD) and attention deficit hyperactivity disorder (ADHD). However, the effects of THC on the development of neuropsychiatric disease across generations have been limited in study and inconclusive in results thus far, emphasizing the urgency in studies of this nature.

The thigmotaxis paradigm, based upon the statistical analysis of fish preference to swim near the arena wall, provides a measure of anxiety-driven behavior in model organisms (Licitra et al., 2022). In another study, F1 zebrafish exposed through the germ line to low doses of THC ($\leq$ 0.6 mg/L) showed no significant behavioral changes as adults, but demonstrated significant attrition of thigmotaxis in F1s (Carty et al., 2019). In the present study at the juvenile stage (3

wpf) and the onset of sexual maturity (11 wpf), there was no significant thigmotaxis across any treatment groups compared to controls (Figures 9, 10). In parent F0 zebrafish, thigmotaxis declined throughout lifetime as well as was observed in their offspring, but at a less substantial rate; during the juvenile (3 wpf) stage, significant thigmotaxis was observed at the two highest concentrations of THC, and at F0 onset of sexual maturity (11 wpf) thigmotaxis was only significant in the highest concentration of THC. By adulthood (24 wpf), thigmotaxis was no longer statistically significant across treatment groups (Jackson, 2022). The effects of multi-generational THC exposure on thigmotaxis did not persist to adulthood in F1 fish, whereas it persisted more strongly to adulthood in F0 fish who were directly exposed to THC during development.

Other measurements, such as freezing duration and fish velocity, can be used in OFT to identify anxiety-characterizing behaviors and further assess thigmotaxis following germline exposure to THC. Sex-dependent thigmotaxis was observed in male adult zebrafish whose parents were exposed to 0.08 or 1.0 μM THC via significantly increased velocity compared to controls, but no significant effects on locomotor behavior were observed in adult F1 female zebrafish across any measures of anxiety explored in this study (Figure 10A). In addition, female F1 fish whose parents were exposed to 0.08 μM THC exhibited significantly lower freezing duration at 24 wpf compared to F1 fish exposed to the next highest exposure group at 0.4 μM THC. Results from parent F0 zebrafish do not indicate any apparent trends in freezing duration across treatment or sex.

The most significant trend observed in our research is an early-life, dose-dependent hyperactivity in F1 zebrafish following an F0 developmental exposure to THC. These findings confirmed our hypothesis that F1 behavior will exhibit dose-dependent behavioral alteration as a

result of THC exposure to F0 zebrafish. While research on exogenous cannabinoid interference with the germline is still ongoing, these results provide further evidence for the belief that THC exposure during development not only influences the behaviors of an individual throughout their own lifetime, but influences the neurodevelopment and behavior of subsequent generations.

4.4 Conclusion

This study found that exposure to THC during early development causes multi-generational effects in zebrafish that influence the behavior of subsequent generations who did not themselves have a direct exposure. These results provide novel information beyond previous studies which have been limited in examining the multi-generational effects of an early-life exposure. Further, the results of this study indicate a need for additional cannabis-based studies to be conducted assessing the multi-generational impacts beyond just the subsequent generations, but to grandchildren and beyond, to further shed light on and assess the breadth of THC's impact on neurodevelopment.

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