Spring 5-9-2024

Early Developmental Exposure to Δ9-Tetrahydrocannabinol Causes Latent Behavioral, Growth, and Bioenergetic Effects in Danio Rerio

Astra Hahm

EARLY DEVELOPMENTAL EXPOSURE TO Δ9-TETRAHYDROCANNABINOL CAUSES LATENT BEHAVIORAL, GROWTH, AND BIOENERGETIC EFFECTS IN *DANIO RERIO*

By

Astra S. Hahm

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

Oxford, MS

May 2023

Approved By

_____

Advisor: Professor Kristine Willett

_____

Reader: Professor Joshua Bloomekatz

_____

Reader: Professor Courtney Roper

ACKNOWLEDGEMENTS

I would like to extend my sincere gratitude to Dr. Willett for her mentorship and guidance throughout this process. You have supported me tremendously throughout this process with your leadership and encouragement, which have been crucial in my growth in college. I would also like to thank Cammi Thornton for her flexibility and understanding. Thank you for patiently guiding me through this project despite several setbacks. I would also like to thank the Sally McDonnell Barksdale Honors College for allowing me the opportunity to pursue my own research, allowing me to further my education and pursue my future ambitions. I would like to extend my thanks to Dr. Josh Bloomekatz and Dr. Courtney Roper as well for aiding my thesis. Finally, I would like to thank my parents and friends for their love, understanding, and encouragement with all that I aim to do. Your support has been a tremendous help throughout this process.

ABSTRACT

ASTRA HAHM: Early developmental exposure to Δ9-tetrahydrocannabinol causes latent behavioral, growth, and bioenergetic effects in *Danio rerio*

(Under the direction of Dr. Kristine Willett)

Δ9-tetrahydrocannabinol (Δ9-THC) is used during pregnancy to mitigate symptoms such as nausea, pain, and insomnia. It is established that Δ9-THC exposure early in life causes adverse effects in children's development including behavioral disorders. Our goal was to determine the latent and generational impacts on growth, behavior, and mitochondrial bioenergetics following exposure to Δ9-THC. Wildtype (5D) zebrafish were exposed to 0 (0.05% DMSO), 0.08, 0.4, or 1 μM Δ9-THC from 6-96 hours post-fertilization (hpf), then subsequently raised in clean water. At 6 months pf (mpf), fish from all treatments were bred to obtain the F1 generation. Both F0 and F1 zebrafish were raised and mortality, growth, and behavior were assessed at 120 hpf, and 3, 11, and 24 weeks pf (wpf). At 13-15 mpf, brains were harvested, mitochondria isolated, and oxygen consumption rate (OCR) was measured with the Seahorse Mito Stress Test. In the F0 generation, Δ9-THC (0.4 μM) caused significant hyperactivity throughout life. At 13-15 mpf, Δ9-THC (0.4 and 1 μM) significantly increased brain mitochondrial OCR in both male and female F0. The parental exposure to Δ9-THC also caused significant hyperactivity in the F1 generation at 120 hpf and 24 wpf, but not at 3 and 11 wpf. OCR was not significantly different in the F1 generation in either sex. Growth, behavior, and bioenergetics were persistently impacted in the F0 generation 15 mpf after early developmental exposure, highlighting the need for further research to determine implications of human fetal Δ9-THC exposure. This research was supported by COBRE-NPN P30 GM 122733.

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

LIST OF ABBREVIATIONS

Δ9-THC	Δ9-tetrahydrocannabinol
hpf	Hours post fertilization
wpf	Weeks post fertilization
mpf	Months post fertilization
OCR	Oxygen consumption rate
DMSO	Dimethylsulfoxide
MS-222	Tricaine Methanesulfonate
CB1	Cannabinoid Receptor 1
CB2	Cannabinoid Receptor 2

1. INTRODUCTION

1.1 Δ9-Tetrahydrocannabinol

Δ9-Tetrahydrocannabinol (Δ9-THC, **Figure 1**) is a cannabinoid product extracted from the *Cannabis sativa* plant (Ng et al, 2023). Δ9-THC binds to cannabinoid receptor 1 (CB1) to induce psychoactive effects (Cooper & Haney, 2009), which can trigger the release of dopamine in the mesolimbic dopamine system (Tanda et al, 1997). Though not as physiologically addictive as other drugs of abuse, such as opioids, Δ9-THC has psychologically addictive properties and is the third most commonly used drug after alcohol and tobacco (Zehra et al, 2018).

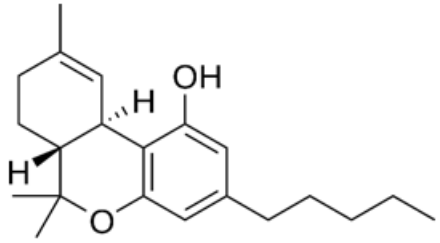

Figure 1: Structure of Δ9-Tetrahydrocannabinol

In the United States, drugs are classified into five different categories, or schedules, depending on the drug's potential for abuse and acceptable medical use. Schedule I drugs have a high potential for abuse and no current medical usage, which cannabis products fall under (DEA, 2018). Thus, while states can choose to legalize cannabis products, cannabis products are federally illegal, though there is increasing public pressure to make the usage of cannabis products federally legal (Fasinu et al, 2016). In Mississippi, the recreational use of cannabis products is illegal. However, despite not having any federally approved medical usage, state-licensed medical cannabis dispensaries can dispense cannabis products to registered patients with prescriptions (Mississippi, 2023).

Despite being federally illegal, the usage of cannabis products has been increasing throughout the years. The daily usage of cannabis products increased from 8% in 2017 to

11% in 2022, and the total reported usage of cannabis products from adults between 35 and 50 increased from 25% in 2021 to 28% in 2022 (NIDA, 2023). Currently, cannabis products are the most frequently used federally illegal drugs.

Pregnant people are among those that use cannabis products. Though the number may be higher, 4.2% of women have self-reported that they use cannabis products during pregnancy because of pain, nausea, stress, and anxiety (Ko et al, 2017). Anxiety is a common mental disorder in humans, with around 31.1% of adults facing anxiety at some point in their lives (Harvard, 2007). The prevalence of anxiety in adolescents is a similar 31.9% (Kessler et al, 2005). While many associate cannabis use with decreased anxiety, cannabis use may also increase anxiety, among other adverse psychiatric effects like mania or paranoia in humans (Keung et al, 2023).

Currently, no amount of cannabis product usage has been deemed safe during pregnancy; in fact, the US Food and Drug Administration has issued guidance against using THC or cannabidiol (CBD) products while pregnant (FDA, 2019). Δ9-THC can pass through the placental barriers and may cause risks such as low birth weight, stillbirth, babies born before 37 weeks of gestation, and long-term developmental issues that could affect memory, behavior, and learning (Ryan et al, 2018). Additionally, little research has been done to determine whether exposure specifically to Δ9-THC causes multigenerational effects.

1.2 Brain Mitochondria

One way to assess the adverse effects of early developmental exposure to Δ9-THC is through behavior and growth which has been implicated following prenatal exposure to Δ9-THC (Ryan et al, 2018). Mitochondria are one of the key players in behavior and growth as

they contribute to physiological processes through their role in energetics (Daniels et al, 2020).

Mitochondria are membrane-bound organelles that play an important role in a cell by producing the majority of necessary chemical energy for biological processes (National Human, 2023). Thus, organs that need a lot of energy, like the brain, are highly influenced by mitochondria as energy is created and stored through oxidative phosphorylation (Rango et al, 2018). Mitochondrial metabolism may also impact growth of an organism, and this metabolism may vary from organism to organism (Quéméneur et al, 2022). Moreover, impaired mitochondrial function has a relationship with neurodegenerative diseases (Reddy, 2009).

The mitochondria's production of chemical energy for biological processes, as well as its connection to growth and neurodegenerative diseases, are not the only reasons to study mitochondria. The mitochondria, particularly in the brain, are known to have their own CB1 receptors that regulate the production of energy and cellular respiration (Bénard et al, 2012). The CB1 receptors on mitochondria can be directly impacted by cannabis usage, which lead to impaired mitochondrial function by decreasing mitochondrial potential (Malheiro et al, 2023).

1.3 Zebrafish Model

No animal model can perfectly replicate humans, but zebrafish are still excellent model organisms due to having 70% similar genes with humans as well as having a functional organ system that is observable, unlike bacteria or yeast (Howe et al, 2013). Zebrafish also have a fully sequenced genome and can be susceptible to genetic manipulation, which can be useful

in biomedical research (Tsegay et al, 2019). Moreover, zebrafish are good for multigenerational studies due to their rapid life cycle, high fecundity, and small size as adults, which allows for easier maintenance and more affordable culture costs (Gutiérrez-Lovera et al, 2017).

Zebrafish are also useful in early developmental and toxicology studies due to their large clutch size, rapid development of embryos, and transparency of embryos which allows visualization (Sant & Timme-Laragy, 2018). In addition, both CB1 and cannabinoid receptor 2 (CB2) receptors are found in zebrafish, making their endocannabinoid pathways similar to those found in humans (Krug & Clark, 2015).

Chemical exposures can induce neurological changes in fish and a viable endpoint to detect neurological impairments is by zebrafish activity, such as locomotor activity (Tierney, 2011). Defects in locomotion can be detected through spontaneous swimming, an established behavioral characteristic in zebrafish. Spontaneous swimming can be observed through open field tests (Fitzgerald et al, 2021). Another established behavioral characteristic of zebrafish is thigmotaxis, where the zebrafish swimming in the periphery, rather than the center, can be used to assess anxiety-like behavior in zebrafish (Schnörr, 2012).

1.4 Study Goals

Due to the rising usage of cannabis products, as well as the known effects of prenatal cannabinoid exposure, we wanted to assess if early cannabinoid exposure would lead to negative latent and multigenerational impacts on behavior, growth, and mitochondrial bioenergetics.

The two main goals of this study were to:

1. Assess how early developmental F0 Δ9-THC exposure would latently impact their behavior, growth, and mitochondrial bioenergetics.
2. Evaluate the multigenerational impacts of Δ9-THC by assessing the effects of Δ9-THC on behavior, growth, and mitochondrial bioenergetics in the F1 generation, which were not directly exposed.

Our hypotheses were:

1. Lower birth weight due to THC exposure will lead to lower weight throughout life.
2. THC exposure will cause anxiety-like behavior (i.e. hyperactivity) leading to higher oxygen consumption rate (OCR).

2. MATERIALS AND METHODS

2.1 Zebrafish Husbandry

Prior to my arrival in the lab, wild-type (5D) zebrafish were obtained and raised according to approved IACUC procedures. The zebrafish were maintained in an Aquatic Habitat ZF0601 Zebrafish Stand-Alone system at 25-28°C in water suitable for zebrafish (pH 7.0-7.5, 60 parts per million (ppm) Instant Ocean, Cincinnati, OH). Every day, the zebrafish were fed Gemma 300 micro food (Skretting USA, UT) in the morning and in the afternoon. At ~6 months post fertilization (mpf), fish that did not have deformities or indications of disease were chosen for breeding.

Male and female fish, in a ratio of 1:1, were placed in breeding tanks with dividers an afternoon before egg collection. Zebrafish breed when the lights are on, so the next morning, the lights were turned on and the dividers were removed to allow breeding. An hour later,

zebrafish eggs located at the bottom of the breeding tanks were collected using a sieve. These eggs were moved to a petri dish to be cleaned and counted, where they were then raised in embryo water (pH 7.5, 60 ppm Instant Ocean, 14:10 light dark cycle) and stored in a 28°C incubator until ready for exposure.

2.2 Exposure

Prior to my involvement in the lab, the parental (F0) generation were placed in scintillation vials with 10 eggs per vial, 3 vials per treatment (**Figure 2**). The eggs were exposed to either control (0.05% dimethylsulfoxide [DMSO]), 0.08, 0.4, or 1 μM of Δ9-tetrahydrocannabinol (Δ9-THC) from 6-96 hours post fertilization (hpf), then subsequently raised in non-exposed water. The F0 generation was bred at 6 mpf to obtain the F1 generation and raised to 15 mpf. The F1 generation was not exposed to Δ9-THC. The F1 generation at 13-15 mpf was the focus of my research.

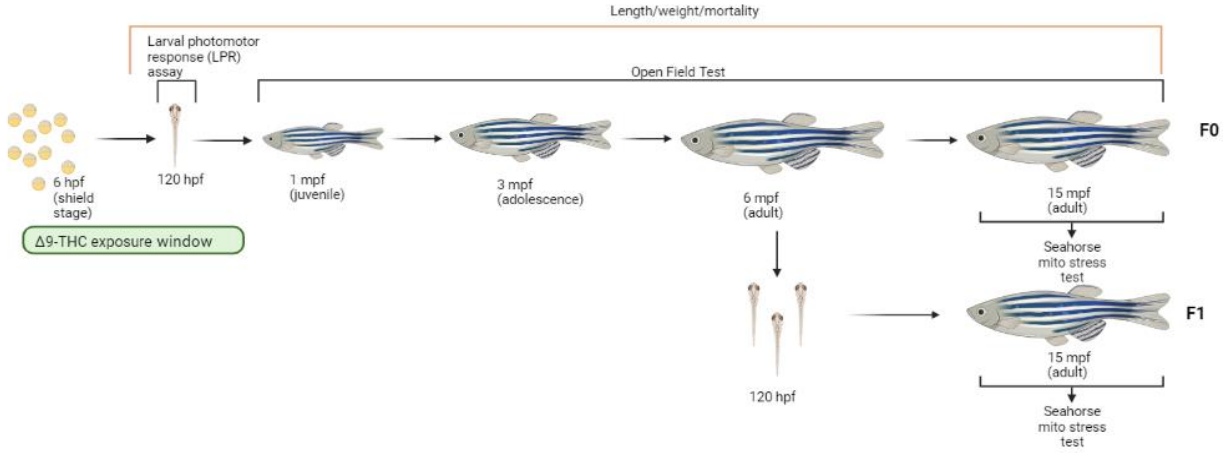

Figure 2: Zebrafish Exposure Timeline. From 6-96 hpf, zebrafish were exposed to control (0.05% dimethylsulfoxide [DMSO]), 0.08, 0.4, or 1 μM of Δ9-THC and then transferred to non-exposed embryo water. At 120 hpf, the larval photomotor assay (LPR) assay was used to measure behavior. Behavior was measured with the open field test from 1 to 15 mpf. At 6 mpf, F0 were bred to obtain the F1 generation. The F1 generation was not exposed to Δ9-THC. The LPR and open field test were used to measure behavior in F1 generation. Length, weight, and mortality were periodically measured throughout.

2.3 Behavior Analysis

After the exposure, behavior was assessed periodically (120 hpf, 3, 11, and 24 wpf) for both the F0 and F1 generation prior to my arrival in the lab. The final assessment for behavior of the F1 generation was observed at 13-14 mpf. Twenty-four fish from each exposure group (12 for each sex) were acclimated to a darkened behavioral testing room (27-28°C) for 20 minutes in preparation for the open field behavioral assessment. Following acclimation, one fish was transferred to a water-filled bucket (diameter of 23 cm and depth of 25 cm; **Figure 3**) at a time. For 5 minutes, the fish were allowed to swim and explore while being tracked by the Noldus Ethovision 14 software through an overhead camera. For the

open field behavioral assessment, the testing area was lit to 9 Lux. After the testing, the fish were removed from the open field area and placed in a holding container to be euthanized.

The open field arena was split into two areas for analysis. One was the periphery, which constituted of the outer 50% of the area, and the center, which constituted of the inner 50% of the arena. Velocity, time in periphery, and freezing duration were calculated by Ethovision. Ethovision calculated velocity as the speed the fish would travel in 5 minutes by tracking the center of the fish. Time in periphery was calculated as how much time the fish spent in the outer 50% of the area and freezing duration was calculated as how often the fish remained still instead of swimming. Ethovision tracks were cleaned manually to negate potential inconsistencies that occurred due to water movements or shadows. The F0 behavior was measured similarly prior to my involvement in the lab.

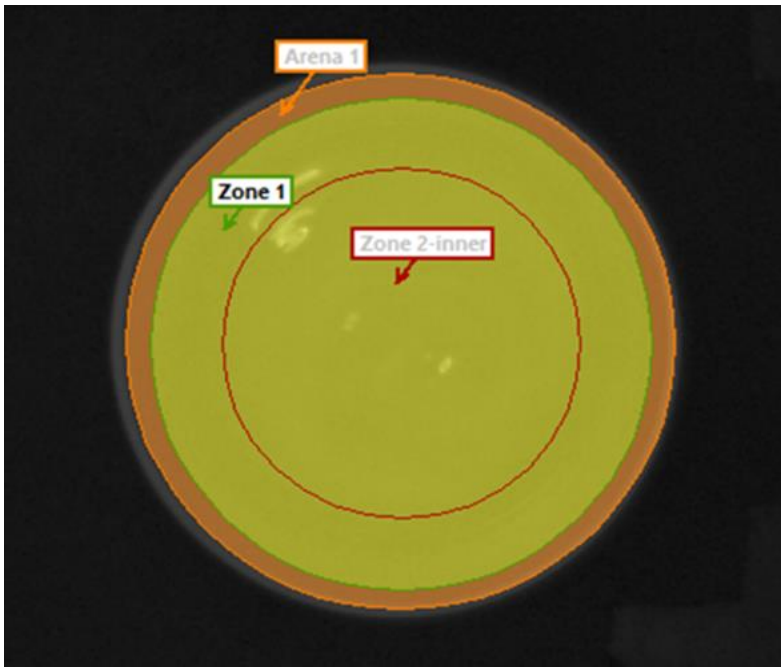

Figure 3: Open Field Test Arenas. The bucket was split into two zones, zone 1 (outer zone; periphery) and zone 2 (inner zone; center), to observe time spent in periphery (zone 1) during behavioral analysis.

2.4 Growth

Prior to my involvement, the length and weight of the F0 and F1 generation were measured throughout their life from 120 hpf to 13-15 mpf by being anesthetized in order to track growth. A ruler was used to measure length and a balance was used to measure weight. At 13-15 mpf, fish were euthanized with Tricaine Methanesulfonate (MS-222) and measured for length and weight. The condition factor was calculated by using the equation $\frac{Weight}{Length^3} x10^5$ in order to account for the variability in weight and length due to different conditions such as food intake and amount of daily movement.

2.5 Seahorse Instrument

At 13-15 mpf, fish were euthanized with MS-222 and brains were obtained. Brain mitochondria were extracted by homogenizing 3-5 brains per pool in 1.6 mL of Extraction Buffer with bovine serum albumin (BSA) and protease inhibitors, before being transferred to microcentrifuge tubes. The samples were centrifuged at 4°C at 700 x g for 10 minutes, then supernatant was extracted and moved to another microcentrifuge tube while the pellet was discarded (**Figure 4**). This step occurred twice. Then, the samples were centrifuged once at 4°C at 10,000 x g for 15 minutes. The supernatant was discarded while the pellet was kept. Using 1 mL of extraction buffer without protease inhibitors, the pellet was resuspended. The pellet was centrifuged at 4°C at 10,000 x g for 15 minutes. The supernatant was discarded and the pellet was resuspended with 150 µl of extraction buffer. By using the NanoDrop 2000 Spectrophotometer, protein was quantified. Following quantification, 50-150 µg of

protein was added to a 96-well plate and centrifuged at 2000 rpm at 4°C to attach the mitochondria to the plates.

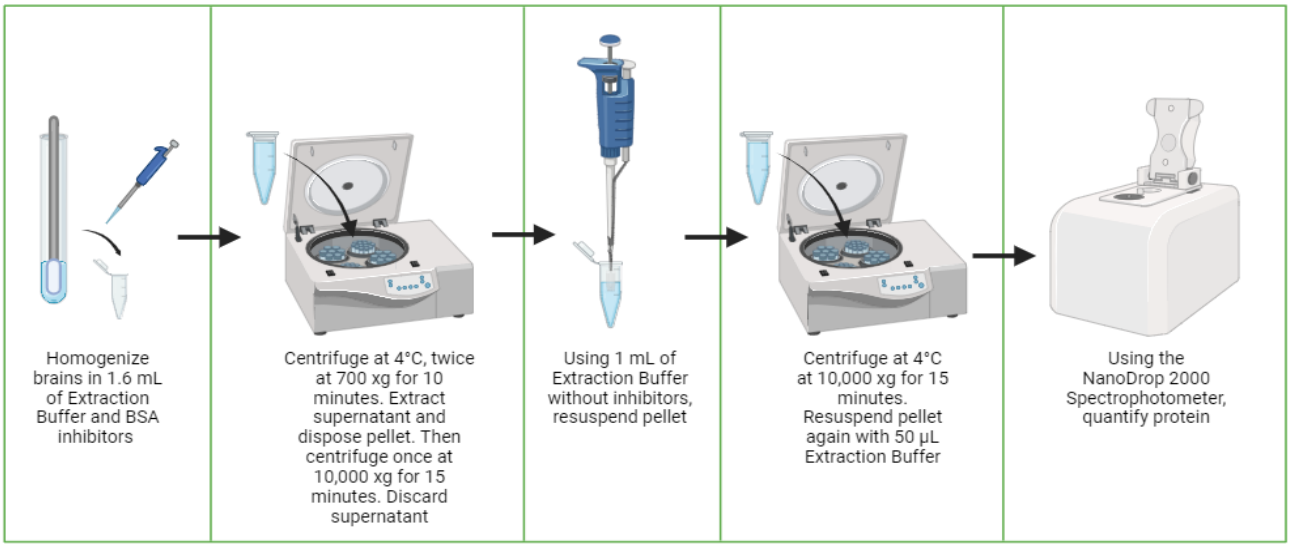

Figure 4: Preparation of Mitochondria for Seahorse Instrument. Visualization of the mitochondria isolation procedure.

Meanwhile, in order to test the various complexes of the electron transport chain, oligomycin, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), and rotenone/antimycin A were prepared and loaded into the sensor cartridge for final concentrations of 1.5, 1, and 0.5 µM, respectively.

The 96 well plate was placed into the Seahorse instrument along with oligomycin, FCCP, and rotenone/antimycin A. The Seahorse instrument measured oxygen consumption rate (OCR) three times over the course of 18 minutes at the baseline, and following the addition of oligomycin, FCCP, and rotenone/antimycin A. When oligomycin is added, it inhibits adenosine triphosphate (ATP) synthase, which reduces electron flow and oxygen consumption, which prevents ATP production in the mitochondria. Adding FCCP causes an

increase of oxygen consumption due to the electron flow being uninhibited. When rotenone and antimycin A are added together, the electron transport chain is completely shut down (**Figure 5**).

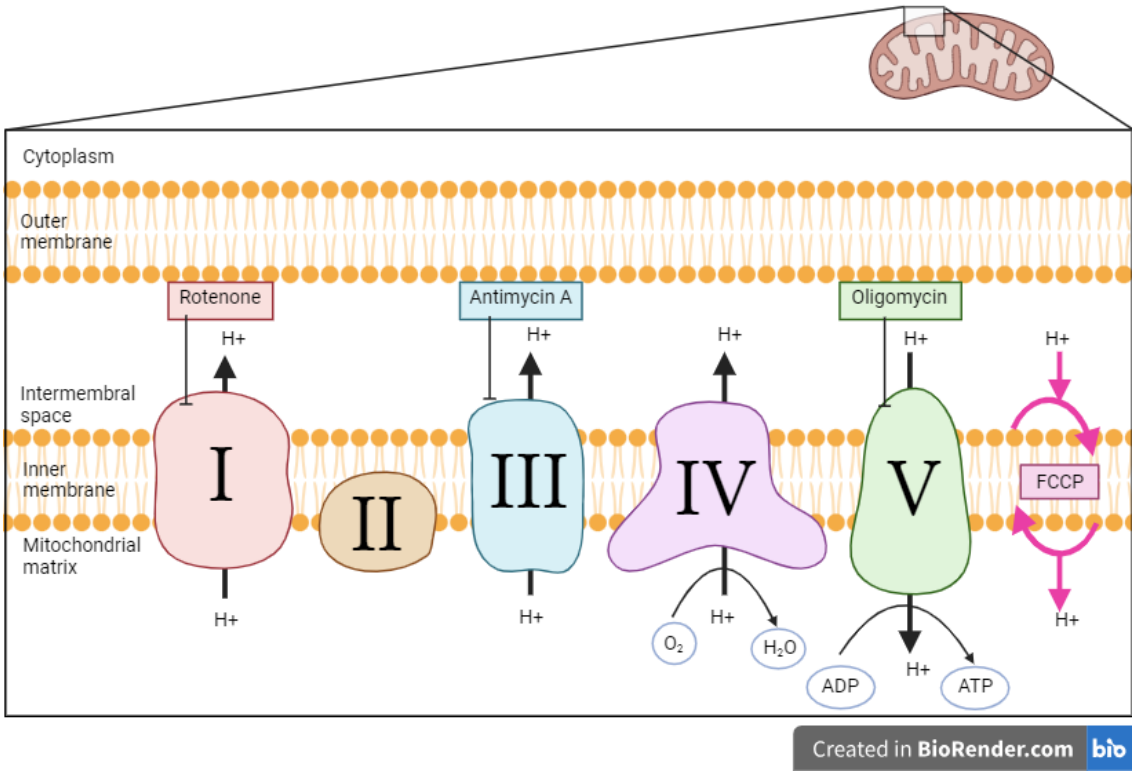

Figure 5: Electron Transport Chain inside the Mitochondria. Oligomycin was added first, which inhibits complex V (ATP synthase), reducing electron flow and oxygen consumption to prevent ATP production. FCCP was added next, increasing oxygen consumption by collapsing the proton gradient and allowing uninhibited flow of electrons. Afterwards, rotenone and antimycin A were added together. Rotenone inhibits complex I and antimycin A inhibits complex III, which shuts down the electron transport chain, leading to no OCR.

2.6 Statistics

Sigma Plot 14.0 software was used to analyze data. The behavior and growth data were presented with box and whisker plots. Raw OCR data was presented as a line graph, while

the averaged data was analyzed and presented as a box and whisker plot. For all the data analysis, male and female fish were analyzed separately. To test statistical significance, two-way ANOVA was utilized to compare both sex and treatment groups. The data was statistically significant if $p<0.05$.

3. RESULTS

At 13-14 mpf, the behavior, growth, and OCR for the F1 zebrafish exposed to either control (0.05% dimethylsulfoxide [DMSO]), 0.08, 0.4, or 1 μM of Δ9-THC were measured. Velocity (cm/s), time in periphery (%), and freezing duration (s) were collected for behavior (**Figure 6**). Velocity was measured as an increase in activity and could be correlated to an increase in OCR. Time in periphery and freezing duration were measured as an indicator of anxiety-like behavior in zebrafish through thigmotaxis, also known as staying in the periphery for too long rather than exploring the inner area. Thigmotaxis can be visualized with a heat map. As seen in Figure 6 for F1 adults, neither treatment nor sex had a significant difference in velocity and freezing duration. For time in periphery, there was no sex related difference, but the fish exposed to 1 μM Δ9-THC stayed in the periphery longer for both male and female fish compared to controls.

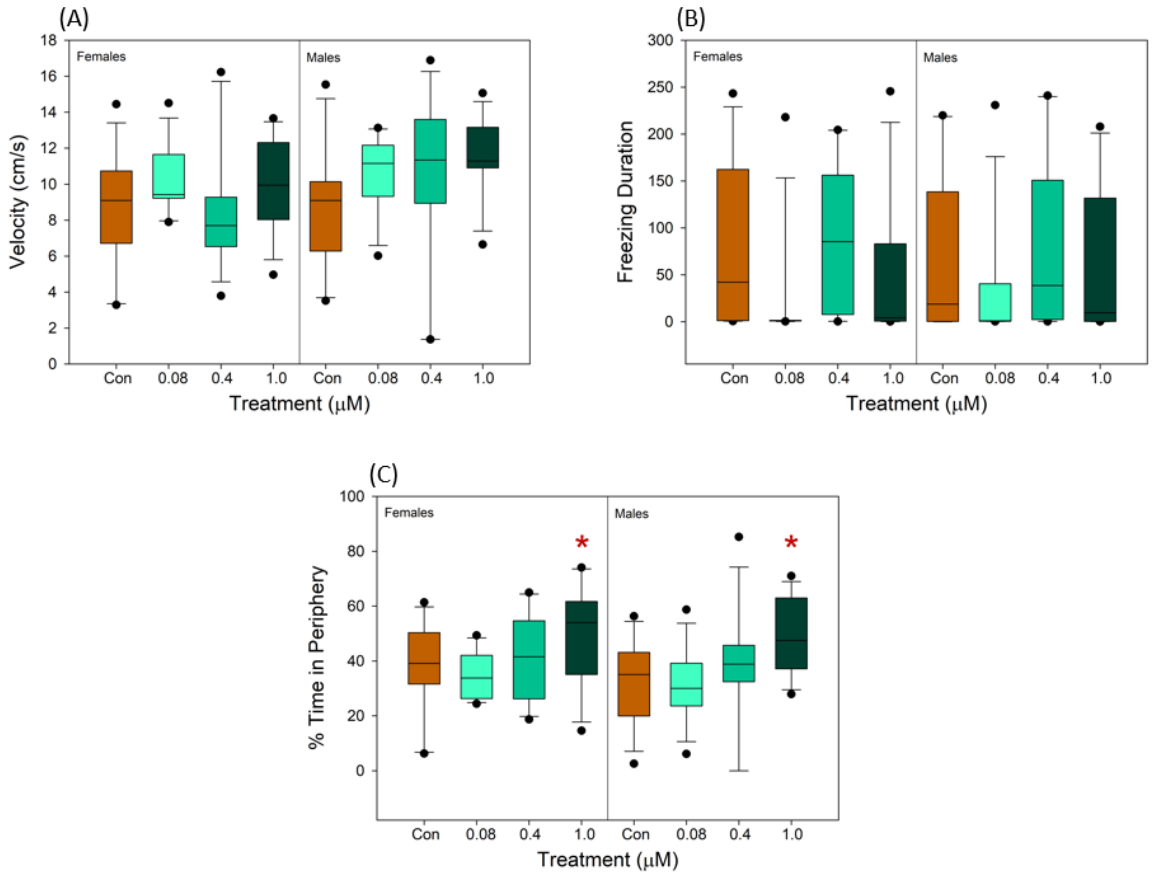

Figure 6: 5D F1 Open Field Behavior at 13-14 mpf. Noldus Ethovision 14.0 software was used to record and analyze zebrafish behavior (velocity (A), freezing duration (B), and time spent in periphery (C) during the 5-minute period). Data was analyzed with 2-way ANOVA to determine statistical significance among treatment groups (Δ9-THC) as well as sex differences. The (*) symbol above the bars indicates statistical significance from the control (Dunnett's post-hoc; $p \leq 0.05$; n = 12 per treatment per sex).

Growth was assessed by collecting the length (cm) and weight (g) of the zebrafish. Then, the length and weight were put into the formula $\frac{Weight}{Length^3} \; x \; 10^5$ to calculate condition factor. As seen on **Figure 7**, there was no statistical significance among treatment groups, but there was statistical significance between sex. Male zebrafish were smaller than female zebrafish regardless of treatment.

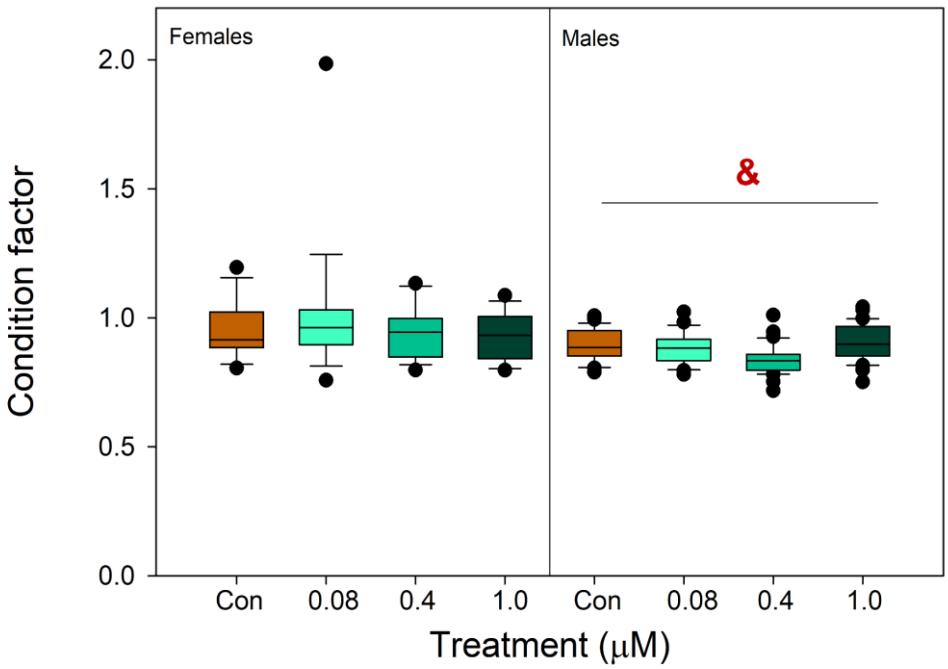

Figure 7: 5D F1 condition factor. Length and weight were measured using a ruler and a balance, respectively. For each fish, the condition factor was calculated (n = 23/25/sex). Data was analyzed with 2-way ANOVA and Student-Newman-Keuls post hoc test ($p \leq 0.05$) per treatment group (Δ9-THC) and sex to determine statistical significance. The & indicates statistical significance between sexes.

OCR (pmol/min) was measured to analyze brain mitochondria activity. **Figure 8** showcases an example of the raw data of OCR. Three measurements were taken for basal respiration and after each of the biological modifiers were added.

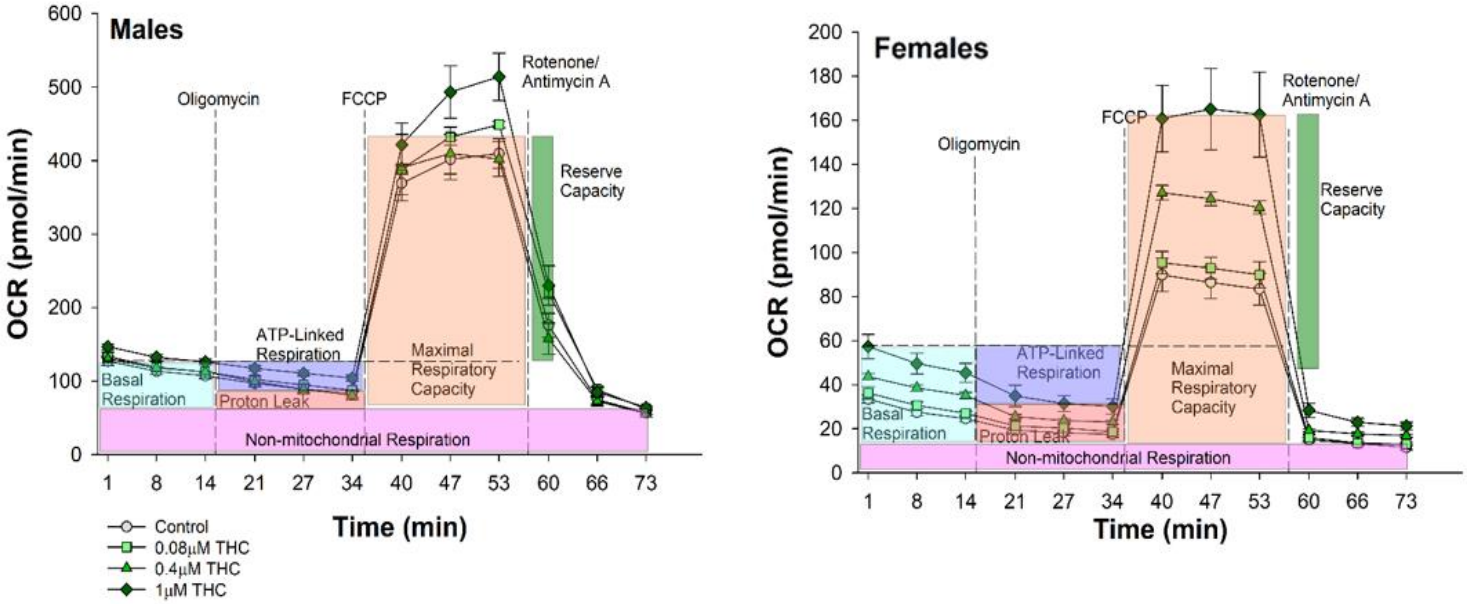

Figure 8: 5D F1 Oxygen Consumption Rate.
OCR was measured by utilizing the Seahorse Mito Stress Test using the Agilent Seahorse XFe96 Analyzer. Three measurements were taken for each condition over the course of 18 minutes. Basal respiration is the normal respiration of the zebrafish. After the addition of oligomycin, the OCR decreased due to inhibition in electron flow. The addition of FCCP led to an increase in OCR due to free flow of electrons. Rotenone/antimycin A shut down the electron transport chain completely, leading to no OCR. There were no statistical differences between sex or treatment group.

To better analyze the OCR data and run statistics on it, the three measurements per treatment were averaged. As seen in **Figure 9**, there was no significant difference present between the variables.

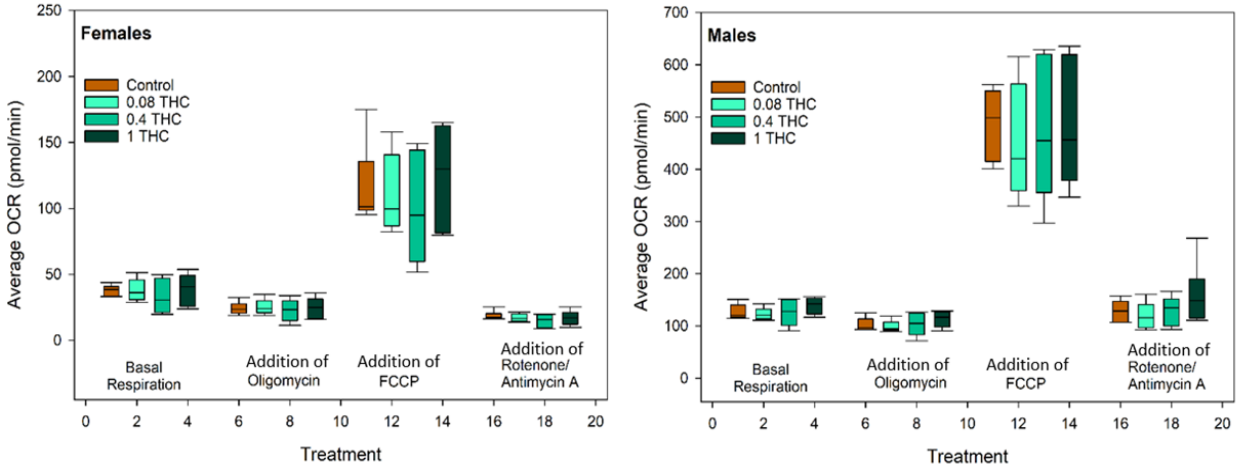

Figure 9: 5D F1 Average Oxygen Consumption Rate
The 3 measurements per condition (n = 4-8/sex) were averaged per treatment and analyzed with a one-way ANOVA ($p \leq 0.05$) to compare treatment groups per sex. There were no statistical differences for either sex.

4. DISCUSSION

4.1 Prenatal THC Exposure Toxicity

The increasing usage of cannabis products, especially by people of reproductive age, is a public health concern as cannabis products have increased in potency, which can be dangerous to reproductive health and fetal outcomes (Lo et al, 2022). Moreover, the abuse of cannabis products during pregnancy is associated with defects in neural development in children that were prenatally exposed (Wu et al, 2011). The adverse effects of cannabis during pregnancy exposure, even for a brief period, are important to consider before usage. For example, the Generation R Study led by El Marroun studied around 7,452 pregnant women who reported their substance use. Ultrasounds were used to determine fetal growth during early, mid-, and late pregnancy. After birth, birth weight was observed. The

Generation R Study observed that growth was more restricted with maternal cannabis use, even in comparison to tobacco use (El Marroun et al, 2009).

Moreover, the Maternal Health Practices and Child Development Study (MHPCD), a longitudinal cohort study of prenatal marijuana exposure, interviewed 1360 women in their fourth gestational month. From the 1360 women, two study cohorts were chosen and they were interviewed again at the seventh prenatal month. These women and their children were evaluated based on multiple factors such as psychological, social, and environmental factors at birth, 8 and 18 months, and 3, 6, 10, 14, 16, and 22 years postpartum. Prenatal cannabis exposure led to an increase in depressive symptoms and attention deficits, leading to an increase in delinquent behavior in ninth graders (Day et al, 2011).

The adverse effects of prenatal cannabis exposure, as portrayed by the Generation R Study and MHPCD Study, showcases the importance of studying the endocannabinoid system, which impacts many biological functions such as anxiety, reproduction, and growth (Skaper & Di Marzo, 2012). In particular, we were interested in the latent and multigenerational effects prenatal cannabis exposure could potentially lead to, especially in relation to behavior, growth, and brain mitochondria.

4.2 Analysis of Behavioral Assay

For this experiment, zebrafish were chosen as a model organism to study the latent and multigenerational impacts of prenatal exposure to cannabinoids as zebrafish are acknowledged as viable model organism for toxicity studies involving compounds such as cannabinoids and nano plastics (Sarmah et al, 2020; Bhagat et al, 2020). One way to assess toxicity in zebrafish is through open field tests, which measure parameters such as velocity, freezing duration, and time in periphery. Zebrafish were placed in a bucket split into two

different zones (center and periphery) to determine the time spent in periphery. The preference for swimming along the periphery rather than the center is called thigmotaxis, and can showcase anxiety-like behavior in zebrafish (Richendrfer et al, 2012).

A previous study done by our laboratory showcases that F0 generation had an increase in hyperactivity in larval zebrafish exposed to 0.4 μM THC. At 3 and 11 wpf, hyperactivity and thigmotaxis were increased in zebrafish exposed to 0.08 and 1 μM THC. Adult zebrafish at 6 mpf had increased velocity in fish exposed to 0.4 and 1 μM THC, as well as behavioral differences between male and female zebrafish (Jackson, 2020). Furthermore, in the F1 generation, at 120 hpf, zebrafish that were offspring of F0 zebrafish exposed 0.4 and 1 μM THC had an increase in hyperactivity. At 11 wpf, freezing duration was increased for F1 zebrafish following parental 0.4 μM THC exposure. At 24 wpf, there were not any significant differences (Cripe, 2023).

Our results showcase that at 13 to 15 mpf, there were no significant differences in the F1 generation, unlike the F0 generation. The F0 generation were only exposed once and the F1 generation were not directly exposed to THC, yet both had increased hyperactivity at early ages. These results showcase that early parental exposure to THC may influence behavior and neural development throughout multiple generations.

The impact of prenatal exposure to THC in humans is particularly important because while the F0 generation zebrafish were only exposed once, humans are typically repeat users of cannabis. In fact, 3 in 10 users of cannabis products have cannabis use disorder, or cannabis addiction (Hasin et al, 2015). People who use cannabis before they are eighteen are more likely to develop cannabis use disorder as well (Lopez-Quintero et al, 2011). Moreover, even if a child does not smoke cannabis, their family may, which could lead to second-hand

marijuana smoking. Second-hand marijuana smoking can lead to cannabis also getting into a non-smoker's system (Holitzki et al, 2017), so exposing children to cannabis can be dangerous, even if it is not prenatal exposure. While the F1 generation did not have significant behavioral differences in adulthood, they were hyperactive early on despite never being exposed to THC directly. Likewise, some children may face adverse effects due to second-hand marijuana smoke despite not using cannabis themselves.

4.3 Analysis of Growth and Brain Mitochondria

Another aspect of determining the impact of cannabinoids is to observe the brain mitochondria. Brain mitochondria are vital and influence brain function and brain cognition, which in turn influences behavior and growth (Picard & McEwen, 2013). Furthermore, increasing mitochondrial metabolism leads to faster maturation of neurons, whereas inhibition leads to a decrease in neuron development in humans and mice (Iwata et al, 2023).

A previous study done by our laboratory measured OCR in the F0 generation, where exposure to 1 μM Δ9-THC led to an increase in OCR for both male and female zebrafish at 13-15 mpf (**Figure 10**). For female zebrafish, 0.4 μM Δ9-THC exposure led to an increase in OCR for basal respiration, after FCCP addition, and after rotenone/antimycin A addition. There was no statistical significance in the F1 generation at 13-15 mpf (**Figure 9**).

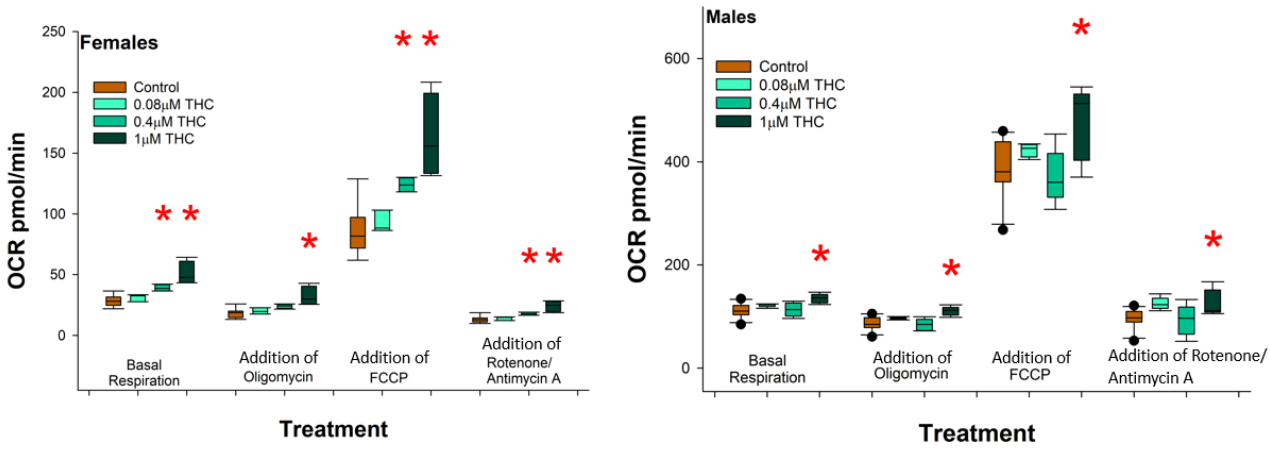

Figure 10: 5D F0 Average Oxygen Consumption Rate. The 3 measurements per condition were averaged per treatment (n = 4-8/sex) and analyzed with a one-way ANOVA and Dunn's post hoc ($p \leq 0.05$) to compare treatment groups per sex.

Mitochondria can produce energy through aerobic cellular respiration, which is composed of glycolysis, the citric acid (Krebs) cycle, and oxidative phosphorylation involving the electron transport chain (Ahmed et al, 2022). Glycolysis outside the mitochondria may increase as a compensatory response due to some dysfunction, which in turn impacts the oxidative phosphorylation inside the mitochondria and OCR (Plitzko & Loesgen, 2018). As seen in **Figure 10**, there is an increase in OCR at higher THC dosage, suggesting a compensatory response due to dysfunction.

Dysfunction in mitochondria may lead to dysfunctional production of ATP, as well as affecting production and secretion of the human growth hormone (Boal et al, 2019). However, when looking at the growth data for F0 zebrafish at 24 wpf, there is no statistical significance in those that were exposed to 0.4 or 1 μM Δ9-THC, which would have been expected due to the increase in OCR. Instead, statistically significant differences in growth can be observed due to 0.08 μM Δ9-THC exposure in both sexes (**Figure 11**).

In the F1 generation, there were no statistical differences in condition factor at 13-15 mpf (**Figure 7**), which could be expected due to there being no statistically significant differences in OCR (**Figure 9**). However, prior to my arrival in the lab, the condition factor for the F1 generation was calculated and zebrafish whose parents were exposed to THC had statistical differences in their condition factor, regardless of concentration (**Figure 11**).

Both F0 and F1 generations had statistically significant differences in condition factor between sexes, which is common for zebrafish due to the female's bigger abdomen (Kossack, 2019).

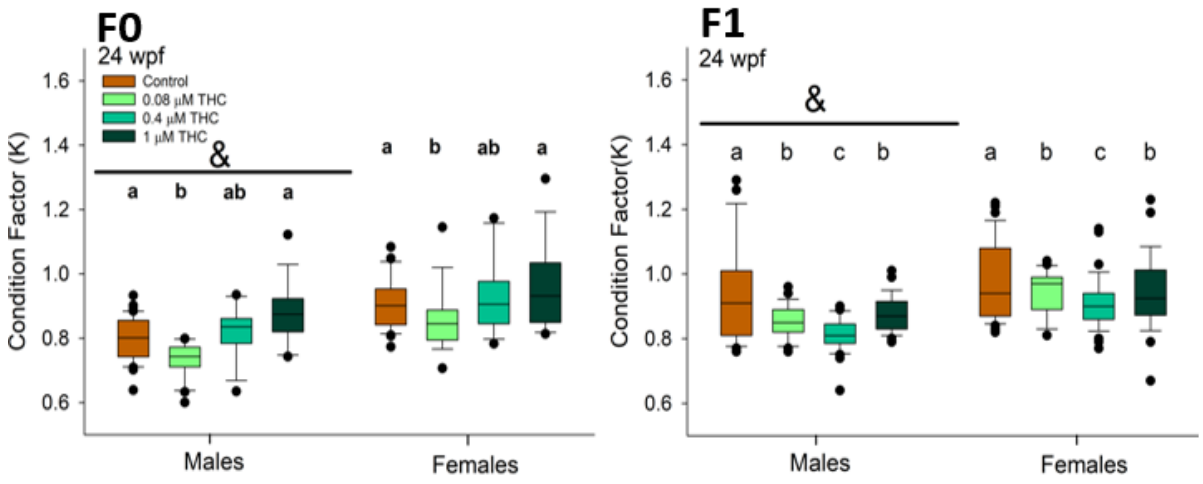

Figure 11: 5D F0 and F1 Condition Factor. Condition factor at 24 wpf (n=23-25/sex) was significantly decreased following 0.08 µM Δ9-THC exposure in F0 fish of both sexes. In the F1 generation, sex and parental dosage influenced condition factors. A statistical analysis was conducted with the 2-way ANOVA and the Newman-Keuls post hoc test ($p \leq 0.05$). Letters that are not in common indicate significant differences between doses and the "&" indicates sex differences.

The F0 generation, who were directly exposed to THC, showed significant differences in behavior, condition factor, and OCR. The F1 generation, which were not exposed to THC,

showed significant differences early on, but outgrew those differences as they aged. In humans, children who are born with a low birth weight usually have normal outcomes, though they have higher rates of illnesses and neurodevelopmental problems (Hack, 1995). Regular usage of cannabis during pregnancy can lead to low birth weight in humans (Hatch & Bracken, 1986; National Academics, 2017). While children with lower birth weight typically have normal expected outcomes, prenatal exposure to cannabis impacts more than just birth weight as it may be associated with higher risk of physiological and psychological consequences such as an increased risk of cognitive impairment (Nashed et al, 2021).

4.4 Conclusion

This study found that a single exposure to THC during early development has multi-generational impacts in zebrafish, even if the exposure was not direct. Initially, we hypothesized that lower birth weight due to THC exposure would lead to lower birth weight throughout life. We also hypothesized that THC exposure will cause anxiety-like behavior, such as hyperactivity, leading to higher OCR.

At 24 wpf, there were statistically significant differences in behavior and growth in the F1, but the F1 generation outgrew these differences by 13-15 mpf, contrary to the hypotheses. OCR could not be measured at larval stages due to the lack of information on how to extract larval brain mitochondria to measure OCR. Streamlining the process for larval brain mitochondrial extraction may assist in the understanding of the latent and multigenerational effects of early developmental cannabinoid exposure as OCR can be a measure of mitochondrial dysfunction.

Furthermore, exposing the parental generation to THC throughout their life rather than at one point during early development may better replicate the repeated usage of cannabis products in humans. Exposing the F1 generation to THC during early development and comparing those results to the F0 generation may also give insight into multigenerational effects of cannabis, especially in regards to whether or not the F1 generation will be more susceptible to cannabis products due to exposure of the F0 generation.

LIST OF REFERENCES

Ahmad M, Wolberg A, Kahwaji CI. Biochemistry, Electron Transport Chain. [Updated 2022 Sep 5]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK526105/

Bénard G, Massa F, Puente N, Lourenço J, Bellocchio L, Soria-Gómez E, Matias I, Delamarre A, Metna-Laurent M, Cannich A, Hebert-Chatelain E, Mulle C, Ortega-Gutiérrez S, Martín-Fontecha M, Klugmann M, Guggenhuber S, Lutz B, Gertsch J, Chaouloff F, López-Rodríguez ML, Grandes P, Rossignol R, Marsicano G. Mitochondrial CB_1 receptors regulate neuronal energy metabolism. Nat Neurosci. 2012 Mar 4;15(4):558-64. doi: 10.1038/nn.3053. PMID: 22388959.

Bhagat J, Zang L, Nishimura N, Shimada Y. Zebrafish: an emerging model to study microplastic and nanoplastic toxicity. Sci Total Environ. 2020;728:138707.

Boal RL, Ng YS, Pickett SJ, Schaefer AM, Feeney C, Bright A, Taylor RW, Turnbull DM, Gorman GS, Cheetham T, McFarland R. Height as a Clinical Biomarker of Disease Burden in Adult Mitochondrial Disease. J Clin Endocrinol Metab. 2019 Jun 1;104(6):2057-2066. doi: 10.1210/jc.2018-00957. PMID: 30423112; PMCID: PMC6469958.

Cooper ZD, Haney M. Actions of delta-9-tetrahydrocannabinol in cannabis: relation to use, abuse, dependence. Int Rev Psychiatry. 2009 Apr;21(2):104-12. doi: 10.1080/09540260902782752. PMID: 19367504; PMCID: PMC2731700.

Cripe, Jenna, "Early Life Δ9-Tetrahydrocannabinol Exposure of F0 Zebrafish Causes Hyperactivity in F1 Offspring" (2023). Honors Theses. 2901. https://egrove.olemiss.edu/hon_thesis/2901

Daniels TE, Olsen EM, Tyrka AR. Stress and Psychiatric Disorders: The Role of Mitochondria. Annu Rev Clin Psychol. 2020 May 7;16:165-186. doi: 10.1146/annurev-clinpsy-082719-104030. Epub 2020 Feb 24. PMID: 32092280; PMCID: PMC8007172.

Day NL, Leech SL, Goldschmidt L. The effects of prenatal marijuana exposure on delinquent behaviors are mediated by measures of neurocognitive functioning. Neurotoxicol Teratol. 2011 Jan-Feb;33(1):129-36. doi: 10.1016/j.ntt.2010.07.006. PMID: 21256427; PMCID: PMC3052937.

DEA. 10 July, 2018. Drug Scheduling. Retrieved from https://www.dea.gov/drug-information/drug-scheduling on 24 October, 2023

El Marroun H, Tiemeier H, Steegers EA, Jaddoe VW, Hofman A, Verhulst FC, van den Brink W, Huizink AC. Intrauterine cannabis exposure affects fetal growth trajectories: the Generation R Study. J Am Acad Child Adolesc Psychiatry. 2009 Dec;48(12):1173-81. doi: 10.1097/CHI.0b013e3181bfa8ee. PMID: 19858757.

FDA. (2019, October 16). What you should know about using CBD when pregnant or breastfeeding. U.S. Food and Drug Administration. https://www.fda.gov/consumers/consumer-updates/what-you-should-know-about-using-cannabis-including-cbd-when-pregnant-or-breastfeeding#:~:text=Pregnant%20or%20Breastfeeding-,What%20You%20Should%20Know%20About%20Using%20Cannabis%2C%20Including%20CBD%2C%20When,during%20pregnancy%20or%20while%20breastfeeding.

Fasinu PS, Phillips S, ElSohly MA, Walker LA. Current Status and Prospects for Cannabidiol Preparations as New Therapeutic Agents. Pharmacotherapy. 2016 Jul;36(7):781-96. doi: 10.1002/phar.1780. PMID: 27285147.

Fitzgerald JA, Könemann S, Krümpelmann L, Županič A, Vom Berg C. Approaches to Test the Neurotoxicity of Environmental Contaminants in the Zebrafish Model: From Behavior to Molecular Mechanisms. Environ Toxicol Chem. 2021 Apr;40(4):989-1006. doi: 10.1002/etc.4951. Epub 2021 Feb 1. PMID: 33270929.

Gutiérrez-Lovera C, Vázquez-Ríos AJ, Guerra-Varela J, Sánchez L, de la Fuente M. The Potential of Zebrafish as a Model Organism for Improving the Translation of Genetic Anticancer Nanomedicines. Genes (Basel). 2017 Nov 28;8(12):349. doi: 10.3390/genes8120349. PMID: 29182542; PMCID: PMC5748667.

Hack M, Klein NK, Taylor HG. Long-term developmental outcomes of low birth weight infants. Future Child. 1995 Spring;5(1):176-96. PMID: 7543353.

Harvard Medical School, 2007. National Comorbidity Survey (NCS). (2023, November 2). Retrieved from https://www.hcp.med.harvard.edu/ncs/index.php . Data Table 1: Lifetime prevalence DSM-IV/WMH-CIDI disorders by sex and cohort .

Hasin DS, Saha TD, Kerridge BT, Goldstein RB, Chou SP, Zhang H, Jung J, Pickering RP, Ruan WJ, Smith SM, Huang B, Grant BF. Prevalence of Marijuana Use Disorders in the United States Between 2001-2002 and 2012-2013. JAMA Psychiatry. 2015 Dec;72(12):1235-42. doi: 10.1001/jamapsychiatry.2015.1858. PMID: 26502112; PMCID: PMC5037576.

Hatch EE, Bracken MB. Effect of marijuana use in pregnancy on fetal growth. Am J Epidemiol. 1986 Dec;124(6):986-93. doi: 10.1093/oxfordjournals.aje.a114488. PMID: 3776981.

Holitzki H, Dowsett LE, Spackman E, Noseworthy T, Clement F. Health effects of exposure to second- and third-hand marijuana smoke: a systematic review. CMAJ Open. 2017 Nov 24;5(4):E814-E822. doi: 10.9778/cmajo.20170112. PMID: 29192095; PMCID: PMC5741419.

Howe K, Clark MD, Torroja CF, et al. The zebrafish reference genome sequence and its relationship to the human genome. Nature. 2013 Apr;496(7446):498-503. DOI: 10.1038/nature12111. PMID: 23594743; PMCID: PMC3703927.

Iwata R, Casimir P, Erkol E, Boubakar L, Planque M, Gallego López IM, Ditkowska M, Gaspariunaite V, Beckers S, Remans D, Vints K, Vandekeere A, Poovathingal S, Bird M, Vlaeminck I, Creemers E, Wierda K, Corthout N, Vermeersch P, Carpentier S, Davie K, Mazzone M, Gounko NV, Aerts S, Ghesquière B, Fendt SM, Vanderhaeghen P. Mitochondria metabolism sets the species-specific tempo of neuronal development. Science. 2023 Feb 10;379(6632):eabn4705. doi: 10.1126/science.abn4705. Epub 2023 Feb 10. PMID: 36705539.

Jackson, Victoria, "Early Developmental Exposure to Δ9-Tetrahydrocannabinol Causes Hyperactivity In Larval Zebrafish That Persists Into Adulthood" (2022). Honors Theses. 2540. https://egrove.olemiss.edu/hon_thesis/2540

Kessler RC, Chiu WT, Demler O, Merikangas KR, Walters EE. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Arch Gen Psychiatry. 2005 Jun;62(6):617-27. PMID: 15939839

Keung MY, Leach E, Kreuser K, Emmerich BW, Ilko S, Singh M, Sapp T, Barnes M, Ouellette L, Jones JS. Cannabis-Induced Anxiety Disorder in the Emergency Department. Cureus. 2023 Apr 26;15(4):e38158. doi: 10.7759/cureus.38158. PMID: 37252542; PMCID: PMC10218760.

Ko JY, Coy KC, Haight SC, et al. Characteristics of Marijuana Use During Pregnancy — Eight States, Pregnancy Risk Assessment Monitoring System, 2017. MMWR Morb Mortal Wkly Rep 2020;69:1058–1063. DOI: http://dx.doi.org/10.15585/mmwr.mm6932a2external icon.

Kossack ME, Draper BW. Genetic regulation of sex determination and maintenance in zebrafish (Danio rerio). Curr Top Dev Biol. 2019;134:119-149. doi: 10.1016/bs.ctdb.2019.02.004. Epub 2019 Mar 21. PMID: 30999973; PMCID: PMC6894417.

Krug RG 2nd, Clark KJ. Elucidating cannabinoid biology in zebrafish (Danio rerio). Gene. 2015 Oct 10;570(2):168-79. doi: 10.1016/j.gene.2015.07.036. Epub 2015 Jul 17. PMID: 26192460; PMCID: PMC4943030.

Lo JO, Hedges JC, Girardi G. Impact of cannabinoids on pregnancy, reproductive health, and offspring outcomes. Am J Obstet Gynecol. 2022 Oct;227(4):571-581. doi: 10.1016/j.ajog.2022.05.056. Epub 2022 May 31. PMID: 35662548; PMCID: PMC9530020.

Lopez-Quintero C, Pérez de los Cobos J, Hasin DS, Okuda M, Wang S, Grant BF, Blanco C. Probability and predictors of transition from first use to dependence on nicotine, alcohol, cannabis, and cocaine: results of the National Epidemiologic Survey on Alcohol and Related Conditions (NESARC). Drug Alcohol Depend. 2011 May

1;115(1-2):120-30. doi: 10.1016/j.drugalcdep.2010.11.004. Epub 2010 Dec 8. PMID: 21145178; PMCID: PMC3069146.

Malheiro RF, Carmo H, Carvalho F. Cannabinoid-mediated targeting of mitochondria on the modulation of mitochondrial function and dynamics. Pharmacological Research. Volume 187, 2023, 106603, ISSN 1043-6618. doi: 10.1016/j.phrs.2022.106603.

Mississippi marijuana laws 2023. Mississippi Cannabis Information Portal. (n.d.). https://mississippistatecannabis.org/laws

Nashed MG, Hardy DB, Laviolette SR. Prenatal Cannabinoid Exposure: Emerging Evidence of Physiological and Neuropsychiatric Abnormalities. Front Psychiatry. 2021 Jan 14;11:624275. doi: 10.3389/fpsyt.2020.624275. PMID: 33519564; PMCID: PMC7841012.

National Academies of Sciences, Engineering, and Medicine; Health and Medicine Division; Board on Population Health and Public Health Practice; Committee on the Health Effects of Marijuana: An Evidence Review and Research Agenda. The Health Effects of Cannabis and Cannabinoids: The Current State of Evidence and Recommendations for Research. Washington (DC): National Academies Press (US); 2017 Jan 12. 10, Prenatal, Perinatal, and Neonatal Exposure to Cannabis. Available from: https://www.ncbi.nlm.nih.gov/books/NBK425751/

National Human Genome Research Institute . (2023, October 24). Mitochondria. Genome.gov. https://www.genome.gov/genetics-glossary/Mitochondria

NIDA. 2023, August 17. Marijuana and hallucinogen use, binge drinking reached historic highs among adults 35 to 50. Retrieved from https://nida.nih.gov/news-events/news-

releases/2023/08/marijuana-and-hallucinogen-use-binge-drinking-reached-historic-highs-among-adults-35-to-50 on 2023, October 25

Ng T, Gupta V, Keshock MC. Tetrahydrocannabinol (THC) [Updated 2023 May 2]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing; 2023 Jan-. Available from: https://www.ncbi.nlm.nih.gov/books/NBK563174/

Picard M, McEwen BS. Mitochondria impact brain function and cognition. Proc Natl Acad Sci U S A. 2014 Jan 7;111(1):7-8. doi: 10.1073/pnas.1321881111. Epub 2013 Dec 23. PMID: 24367081; PMCID: PMC3890847.

Plitzko B, Loesgen S. Measurement of Oxygen Consumption Rate (OCR) and Extracellular Acidification Rate (ECAR) in Culture Cells for Assessment of the Energy Metabolism. Bio Protoc. 2018 May 20;8(10):e2850. doi: 10.21769/BioProtoc.2850. PMID: 34285967; PMCID: PMC8275291.

Quéméneur, JB., Danion, M., Cabon, J. et al. The relationships between growth rate and mitochondrial metabolism varies over time. Sci Rep 12, 16066 (2022). https://doi.org/10.1038/s41598-022-20428-9

Rango M, Bresolin N. Brain Mitochondria, Aging, and Parkinson's Disease. Genes (Basel). 2018 May 11;9(5):250. doi: 10.3390/genes9050250. PMID: 29751692; PMCID: PMC5977190.

Reddy PH. Role of mitochondria in neurodegenerative diseases: mitochondria as a therapeutic target in Alzheimer's disease. CNS Spectr. 2009 Aug;14(8 Suppl 7):8-13; discussion 16-8. doi: 10.1017/s1092852900024901. PMID: 19890241; PMCID: PMC3056539.

Richendrfer H, Pelkowski SD, Colwill RM, Creton R. On the edge: pharmacological evidence for anxiety-related behavior in zebrafish larvae. Behav Brain Res. 2012 Mar 1;228(1):99-106. doi: 10.1016/j.bbr.2011.11.041. Epub 2011 Dec 6. PMID: 22155488; PMCID: PMC3264763.

Ryan, S. A., Ammerman, S. D., O’Connor, M. E., Gonzalez, L., Patrick, S. W., Quigley, J., Walker, L. R., Meek, J. Y., Johnston, M., Stellwagen, L., Thomas, J., & Ware, J. (2018, September 1). Marijuana use during pregnancy and breastfeeding: Implications for neonatal and childhood outcomes. American Academy of Pediatrics. https://publications.aap.org/pediatrics/article/142/3/e20181889/38625/Marijuana-Use-During-Pregnancy-and-Breastfeeding?autologincheck=redirected

Sant KE, Timme-Laragy AR. Zebrafish as a Model for Toxicological Perturbation of Yolk and Nutrition in the Early Embryo. Curr Environ Health Rep. 2018 Mar;5(1):125-133. doi: 10.1007/s40572-018-0183-2. PMID: 29417450; PMCID: PMC5876134.

Sarmah S, Sales Cadena MR, Cadena PG, Marrs JA. Marijuana and opioid use during pregnancy: using zebrafish to gain understanding of congenital anomalies caused by drug exposure during development. Biomedicines. 2020;8(8):279

Schnörr SJ, Steenbergen PJ, Richardson MK, Champagne DL. Measuring thigmotaxis in larval zebrafish. Behav Brain Res. 2012 Mar 17;228(2):367-74. doi: 10.1016/j.bbr.2011.12.016. Epub 2011 Dec 19. PMID: 22197677.

Skaper SD, Di Marzo V. Endocannabinoids in nervous system health and disease: the big picture in a nutshell. Philos Trans R Soc Lond B Biol Sci. 2012 Dec

5;367(1607):3193-200. doi: 10.1098/rstb.2012.0313. PMID: 23108539; PMCID: PMC3481537.

Tanda G, Pontieri FE, Di Chiara G. Cannabinoid and heroin activation of mesolimbic dopamine transmission by a common mu1 opioid receptor mechanism. Science. 1997 Jun 27;276(5321):2048-50. doi: 10.1126/science.276.5321.2048. PMID: 9197269.

Tierney, KB. Behavioural assessments of neurotoxic effects and neurodegeneration in zebrafish. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease, Volume 1812, Issue 3, 2011, Pages 381-389, ISSN 0925-4439, https://doi.org/10.1016/j.bbadis.2010.10.011.

Tsegay Teame, Zhen Zhang, Chao Ran, Hongling Zhang, Yalin Yang, Qianwen Ding, Minxu Xie, Chenchen Gao, Yongan Ye, Ming Duan, Zhigang Zhou, The use of zebrafish (Danio rerio) as biomedical models, Animal Frontiers, Volume 9, Issue 3, July 2019, Pages 68–77, https://doi.org/10.1093/af/vfz020

Wu CS, Jew CP, Lu HC. Lasting impacts of prenatal cannabis exposure and the role of endogenous cannabinoids in the developing brain. Future Neurol. 2011 Jul 1;6(4):459-480. doi: 10.2217/fnl.11.27. PMID: 22229018; PMCID: PMC3252200.

Zehra A, Burns J, Liu CK, Manza P, Wiers CE, Volkow ND, Wang GJ. Cannabis Addiction and the Brain: a Review. J Neuroimmune Pharmacol. 2018 Dec;13(4):438-452. doi: 10.1007/s11481-018-9782-9. Epub 2018 Mar 19. PMID: 29556883; PMCID: PMC6223748.