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Multi-Generational Effects of Δ9-Tetrahydrocannabinol Exposure on Gene Expression in Liver Tissue

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MULTI-GENERATIONAL EFFECTS OF Δ9-TETRAHYDROCANNABINOL EXPOSURE ON GENE EXPRESSION IN LIVER TISSUE

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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

KAYLA LOVITT: MULTI-GENERATIONAL EFFECTS OF Δ9-TETRAHYDROCANNABINOL EXPOSURE ON GENE EXPRESSION IN LIVER TISSUE

Cannabis is the most commonly used, cultivated, and trafficked illicit drug worldwide. Increased availability and acceptance of cannabis and cannabinoid-containing products provide the necessity for understanding how these substances influence aging. In this study, zebrafish (*Danio rerio*) were exposed to concentrations of Δ^9-tetrahydrocannabinol (THC) (0.08, 0.4, 2 µM) during embryonic-larval development, the effects on aging were measured 30 months later and in the offspring of the exposed fish (F1 generation. We observed results indicating a biphasic and hormetic effect. Treatment with the lowest concentration of THC significantly increased egg production, while higher concentrations resulted in impaired fecundity. Treatment with the lowest dose of THC also significantly reduced wet weight, the incidence of kyphosis, and the expression of several senescence and inflammatory markers (*p16*, *tnfα*, *il-1β*, *il-6, pparα,* and *pparγ*) in the liver, but not at higher doses. Within the F1 generation, many of these changes were not observed, such as the changes in gene expression in genes related to cell senescence and inflammation. However, the reduction in fecundity due to THC exposure was adversely impacted in the F1 generation, because offspring whose parents received a high dose of THC were completely unable to reproduce. Together, our results demonstrate that a developmental exposure to THC can cause significant effects on longevity and health span of zebrafish in a dose-dependent biphasic manner.

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LIST OF ABBREVIATIONS

CBD	Cannabidiol
cDNA	Complementary Deoxyribonucleic Acid
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
DevAge	The Developmental Theory of Aging
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
F0	Parent Generation
F1	First Filial Generation
G1	Growth 1
hpf	Hours Post Fertilization
HPO	Hypothalamic-Pituitary-Ovarian
Il	Interleukin
mRNA	Messenger RNA
NIDA	National Institute of Drug Abuse
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
ppar	Peroxisome Proliferator Activated Receptor
ppm	Parts Per Million
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
RT-qPCR	Reverse Transcriptase Quantitative Polymerase Chain Reaction
S	Synthesis
SE	Standard Error
THC	Δ^9-Tetrahydrocannabinol
tnf∝	Tumor Necrosis Factor alpha
µM	Micromolar

1. INTRODUCTION

Aging is a consequence of an organism's development, and is accompanied with adverse effects that lead to age-related diseases. The Developmental Theory of Aging (DevAge) defends that aging is a result of development, and that both aging and development are regulated by the same genetic mechanisms and processes (Dilman, 1971). Aging is accepted as a universal and irreversible process that gradually promotes physiological cellular decay. The same molecular mechanisms that can benefit young organisms can also be deleterious over long periods of time. For example, oxygen is necessary for proper tissue function, but long-term exposure leads to oxidation of proteins, lipids, and nucleic acids, which is correlated with aging-associated diseases, including cancer (Leroi et al., 2005). When an organism exactly begins "aging" has not yet been pinpointed. However, several aging-associated pathologies are thought to result from genetic predisposition combined with specific environmental factors that become evident during an organism's life span (de Magalhães and Church, 2005). Furthermore, environmental changes during pregnancy can lead to differential developmental outcomes by altering the epigenome via changes in methylation and acetylation of DNA and histones, which ultimately modifies gene expression patterns (Langley-Evans, 2006). These alterations can significantly impact fetal growth and tissue

development. There are several hallmarks of aging, both beneficial and harmful. One of the most notable of these hallmarks is inflammation, which has been demonstrated to be intimately correlated with aging diseases such as Parkinson's, Alzheimer's, sarcopenia, osteoporosis, and atherosclerosis (Franceschi et al., 2000). Throughout our lives, inflammation is a helpful resource in our immune system's response to foreign pathogens. The inflammaging theory proposes that chronic inflammation that one experiences over the course of its lifespan as a result of constant exposure to various antigens results in reduced efficiency of the immune system (immunosenescence), which promotes the development of aging-associated and autoimmune diseases (Franceschi et al., 2000).

1.1 Zebrafish as an Aging Model

In recent years, zebrafish have proved useful as a model organism when determining age-related changes in biology and behavior (Beis and Agalou, 2020). On average, zebrafish live about three years. As they age, they exhibit cellular and molecular changes similar to those seen in humans (Adams and Kafaligonul, 2018). Age-related mitochondrial dysfunction and oculopathy (Wang et al., 2019), nervous system aging and disease (Van houcke et al., 2015), age-related changes in DNA methylation (Shimoda et al., 2014), oncology (Barriuso et al., 2015), Peutz-Jeghers syndrome (van der Velden and Haramis, 2011), telomere attrition (Wagner et al., 2017), osteoarthritis (Hayes et al., 2013), osteoporosis (Zhang et al., 2018), immune system and endocrine decline have all been described in zebrafish (Arslan-Ergul and Adams, 2014; Beis and Agalou, 2020; Carneiro et al., 2016). Zebrafish also have a highly conserved endocannabinoid system,

further presenting them as an ideal model for observing the effects of cannabinoids and aging (Oltrabella et al., 2017). In addition to these reasons, we utilize zebrafish (*Danio rerio*) as a model for this study due to their extra-uterine and rapid development, high fecundity, low culture costs, and availability of genetic resources.

1.2 THC and the Effects of THC on Aging

The *Cannabis sativa* plant has been utilized for its medicinal and recreational purposes, dating back to 12,000 years ago (Andre et al., 2016). Cannabinoids, found in cannabis, are the lipophilic ligands that bind to corresponding cell-surface receptors. There are three subdivisions of cannabinoids: phytocannabinoids, endocannabinoids, and synthetic cannabinoids. This study exclusively focused on phytocannabinoids, which are cannabinoids derived directly from the cannabis plant. Although there are over 100 natural phytocannabinoid compounds found in cannabis (Pertwee, 2006), one of the most common and abundant is Δ^9-tetrahydrocannabinol (THC).

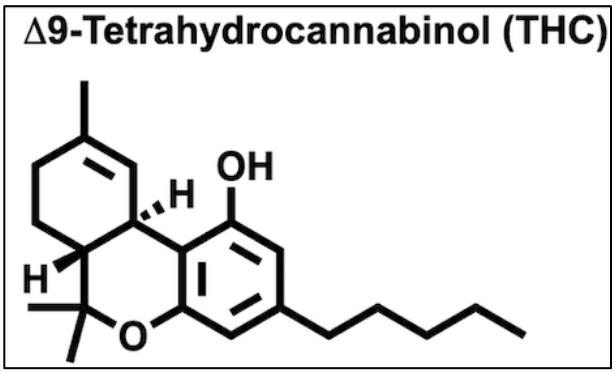

Figure 1: Structure of Δ^9-tetrahydrocannabinol (THC)

THC is the psychoactive component of cannabis. THC can cross the placenta if consumed during pregnancy (Hurd et al., 2005; Hutchings et al., 1989), and alter the cannabinoid signaling pathways in a developing fetus. Because of the wide range of dose concentrations available, potentially confounding effects from additional cannabinoids found in cannabis, as well as exposure to other drugs, alcohol, or environmental factors, it has proved very difficult to clearly establish the effects of THC on human development. Moreover, THC studies in humans have focused on the short-term consequences, leaving the effects on aging largely still in question. However, the DevAge (Barker, 2007; Feltes et al., 2015) suggests that early-life stressors can instigate long-term changes in health. These early-life stressors, whether environmental and/or chemical (reviewed in: Haugen et al., 2015), can either increase the risk of disease (Richardson et al., 2006; Zawia and Basha, 2005), or conversely, or can increase resilience with respect to age-related dysfunction (Calabrese and Mattson, 2017; Gidday, 2015; Hodges and Ashpole, 2019). Evidence suggests that this increase in resilience might be due to the fact that preconditioning with low THC doses protects against neuronal damages (Assaf et al., 2011; Fishbein-Kaminietsky et al., 2014; Hodges and Ashpole, 2019). However, the resilient effects are acute, and evidence has yet to prove that they are sustained through the months following THC exposure. In animal models, when cannabinoids are introduced in advanced age, they provide protection against neurodegenerative disease. This protection is attributed to the anti-inflammatory effects that THC might contribute (Ramírez et al., 2005). Even more interesting is the fact that while high doses of THC can cause memory deficits (Varvel et al., 2001), low doses can slow or even halt the

progression of Alzheimer's disease by reducing amyloid beta (Cao et al., 2014), as well as restoring cognitive function in old mice (Bilkei-Gorzo et al., 2017).

1.3 Gene Expression

One of the many ways gene expression is controlled is though regulatory DNA sequences. Regulatory sequences determine which genes are expressed by specific cells under certain circumstances. Most cells in multicellular organisms are capable of altering their patterns of gene expression in response to extracellular cues, such as hormones. Gene expression has many points of regulation in the processes of transcription and translation, as DNA is converted to RNA and, eventually, protein. A cell can control the types and levels of proteins it makes in several ways. Transcriptional control will determine when and how often a given gene is transcribed. RNA processing control will determine how the RNA transcript is spliced. RNA localization control determines which completed mRNAs are exported from the nucleus into the cytosol. Translational control involves selecting which mRNAs in the cytoplasm are actually translated at ribosomes (Alberts et al., 2002). These are just a few of the regulatory mechanisms of gene expression that control up- or down-regulation of certain genes in various environmental conditions. Through our experiment, we evaluated when the particular genes of interest were up- or down-regulated in response to different doses of THC.

1.4 Genes Studied

1.4.1 *p16*

p16, also known as $p16^{INK4a}$, cyclin-dependent kinase inhibitor 2A, CDKN2A, or multiple tumor suppressor 1, is a protein that slows cell division. To accomplish this, it slows the progression of the cell cycle from the G1 phase to the S phase, thereby acting as a tumor suppressor. *p16* is encoded by the CDKN2A gene. As organisms age, *p16* expression increases in order to reduce the proliferation of stem cells (Krishnamurthy et al., 2006). Reduced cell division protects against cancer, but as a trade-off, increases the risks associated with cellular senescence. Assessing *p16* levels is a potential measure of how quickly the body's tissues are aging at a molecular level.

1.4.2 *tnf*∝

Tumor necrosis factor alpha (*tnf*∝) is a cell signaling protein involved in systemic inflammation. *tnf*∝ is produced mainly by activated macrophages, but also lymphocytes, NK cells, neutrophils, mast cells, eosinophils, and neurons. *tnf*∝ is a member of the TNF superfamily, consisting of transmembrane proteins with a homologous TNF domain. The primary role of *tnf*∝ is to regulate immune cells (Locksley et al., 2001). As an endogenous pyrogen, it has the capability to induce fever, apoptosis, cachexia (muscle loss), and inflammation. It also works to inhibit tumorigenesis and viral replication, and to respond to sepsis via il-1β – and Il-6-producing cells. An irregularity in TNF production is implicated in a variety of human diseases including Alzheimer's disease, cancer, major depression, psoriasis, and inflammatory bowel disease (Schmid-Hempel, 2013).

1.4.3 *il-1β*

The Interleukin-1 family is a group of cytokines (cell-signaling molecules) that is ultimately responsible for the production of inflammation and the promotion of fever. As a pyrogen, *il-1β* affects the activity of the hypothalamus, the thermoregulatory center, which leads to a rise in body temperature (fever), along with vasodilation and hypotension (Matzinger, 2012). Activated macrophages, neutrophils, epithelial cells, and endothelial cells produce *il-1β*, and it then binds to the *il-1β* receptor. It plays a central role in the regulation of immune responses, including helping activate *tnf∝*, which, as mentioned previously, also promotes inflammation and fever. One major role of *il-1β* is to increase the expression of adhesion factors on endothelial cells to enable transmigration of immunocompetent cells to sites of infection (Contassot et al., 2012).

1.4.4 *il-6*

Il-6 is an interleukin that acts as both a pro-inflammatory cytokine and an anti-inflammatory myokine (cytokine released by muscle cells in response to muscular contractions). The anti-inflammatory effect of Il-6 is mediated through its inhibitory effects on *tnf∝* and *il-1β* (Brandt and Pedersen, 2010). It is capable of crossing the blood-brain barrier and changing the body's temperature set point to mediate the fever response (Banks et al., 1994). Il-6 can be secreted by macrophages in response to specific pathogen molecules (PAMPs), which can bind to pattern recognition receptors or Toll-like receptors to induce intracellular signaling cascades that give rise to inflammatory cytokine production. Il-6 also has an important role in a number of diseases. It stimulates the inflammatory and autoimmune processes in diabetes, atherosclerosis, depression,

Alzheimer's disease, erythematosus, Beçhets disease, and rheumatoid arthritis (Swardfager et al., 2010).

1.4.5 *ppar∝, pparγ*

Peroxisome proliferator-activated receptors (PPARs) are ligand-modulated transcriptional factors that belong to the nuclear hormone receptor superfamily responsible for peroxisome proliferation. Peroxisomes are primarily involved in lipid metabolism and the conversion of reactive oxygen species (such as hydrogen peroxide) into safer molecules like water and oxygen. Cannabinoids, such as THC, activate and target different isoforms of the PPARs. Activation of all the variants of the PPAR genes mediates some of the analgesic, neuroprotective, and anti-inflammatory effects of some cannabinoids. The isoform *ppar∝* (peroxisome proliferator activated receptor alpha) is a transcription factor along with a major regulator of lipid metabolism in the liver. It is necessary for ketogenesis as a key adaptive response to prolonged fasting (Kersten et al., 1999). Activation of *ppar∝* promotes uptake, utilization, and catabolism of fatty acids by upregulation of genes involved in these processes. *ppar∝* is primarily activated via ligand binding, and is essential for peroxisome proliferation. *ppar∝* may also be a site of action for certain anticonvulsants (Puligheddu et al., 2013). *pparγ* regulates fatty acid storage and glucose metabolism. It also decreases the inflammatory response of many cardiovascular cells, primarily endothelial cells. Hypofunctionality of *pparγ* in glial cells could be present in some neurodegenerative diseases, eventually participating in pathological mechanisms through peroxisomal damage (Giampietro et al., 2019). THC naturally binds to *pparγ*, and the activation of *pparγ* may be responsible for inhibiting

the growth of cultured human breast, gastric, lung, prostate, and other cancer cell lines. It is predicted that THC will act as an agonist of *pparγ*, which will then lead to growth inhibition, apoptosis, and differentiation of potentially harmful cells.

1.4.6 *18s*

When analyzing quantitative PCR of gene expression, normalization to appropriate, stably-expressed reference genes is essential for accurate quantitative measurement.18s ribosomal RNA is a control used in polymerase chain reaction (PCR) analyses because of its invariant expression in tissues, cells, and experimental treatments (Valente et al., 2009).

1.5 Sex-Specific Effects

Previous research has shown sex-specific effects of THC on aging (Austad, 2019), and therefore we assessed potential differences in our study as well. THC alters the hypothalamic-pituitary-ovarian (HPO) axis functionality. Similarly, hormones produced by the HPO axis also affect the physiological and behavioral effects of THC (Brents, 2016). Estradiol and progesterone are two hormones that play a larger role in modulating THC's effects and are also two major hormones produced in female organisms. Because of this, female rats displayed greater tolerance to THC than males (Marusich et al., 2015; Wakley et al., 2014). This tolerance also was exhibited in response to other cannabinoids, such as WIN55,212, where males demonstrated behavioral and neuronal deficits and females were unaffected (Bara et al., 2018). With this evidence, we suspected that developmental exposure to THC would cause sex-

specific alterations, with high concentrations of THC causing negative outcomes in male fish, while females would exhibit more tolerance.

1.6 Research Objectives

The goal of this study was to determine whether embryo-larval exposure to THC results in later-life, sex-specific changes in the expression of genes related to senescence and inflammation in the liver. To accomplish this, embryo-larval fish that had been developmentally-exposed to THC or control were enrolled into the study at 12 months of age, and the effects of aging were assessed at 30 months of age. THC was hypothesized to worsen the age-related increase in senescence and inflammation. To test multi-generational effects, aged F1 (whose parents were exposed to the highest THC dose) were also cultured and assessed at 30 months of age. Analysis of the change in gene expression will help determine the long-term consequences of embryonic exposure to THC.

2. MATERIALS AND METHODS

2.1 Zebrafish Care

Standard Tg(fli1:egfp) zebrafish were acquired from the Zebrafish International Resource Center in Eugene, Oregon. All experiments were conducted under the approved University of Mississippi IACUC protocol. Fish were maintained in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) with zebrafish water (pH 7.0—7.5, 760 μS Instant Ocean, Cincinnati, OH) in a 25-28°C climate, with a 14:10 light-dark cycle. Zebrafish were fed twice a day with Gemma Micro food (Gemma Micro 300, Skretting Nutreo Company, Westbrook, ME). Sexually mature and healthy fish without any signs of deformities or disease were selected as breeders.

For egg collection, the Tg(fli1:egfp) zebrafish were transferred to breeding tanks, with a 1:1 ratio of males to females, for one night prior to collection day. When the lights turn on, fish lay their eggs. The eggs were collected an hour later. To prevent fish from feeding on their own eggs, a protective grate is in place at the bottom of the tank. All eggs that fell through this protective grate were collected by pouring water from the breeding tanks through a small sieve. Cleaned eggs were then transferred to a petri dish, and kept in embryo water (pH 7.5, 60 ppm Instant ocean, 14:10 light-dark cycle) in a 28°C incubator until exposure began at 6 hours post fertilization (hpf).

2.2 Acute Exposure

Before I joined the lab, fertilized embryos were transferred to scintillation vials with 10 embryos/vial and exposed to sub-lethal concentrations (Carty et al., 2018), 0.08, 0.4, 2.0 μM (0.024, 0.12, 0.6 mg/L) THC, or 0.05% DMSO control water at a 0.6:1 (mL water:fish) ratio from 6-96 hpf. Vials were lightly covered and placed in a 28°C incubator. Each day, unfertilized/dead eggs or debris were removed from the vials using a pipette. All THC used throughout these developmental exposures was supplied by the NIDA Drug Supply Program.

Following the completion of the exposure, fish were transferred to tanks and raised in clean water until 12 months of age. At 12 months of age, F0 (exposed) or F1 (parents exposed) fish were entered into the aging study and observed until 30 months of age. A young cohort (7 months old) was also maintained in the same room, under the same conditions, to act as a young control.

At 30 months of age, fish were euthanized with MS-222 (300 mg/L) buffered with sodium bicarbonate (600 mg/L) and their liver was removed, flash frozen, and placed in a -80°C freezer until ready for RNA extraction.

2.3 RT-qPCR

In order to determine changes in gene expression due to exposure to THC, mRNA expression of *p16*, *tnf∝*, *il-1b*, *il-6*, *ppar∝*, and *pparγ* was measured in the liver using qPCR. Liver tissue was extracted and homogenized in TRIzol (Invitrogen #A33251; Waltham Massachusetts). Using manufacturer's recommended protocol, RNA was then isolated using an RNase Free DNase set (Qiagen #79254; Walencia California) and

RNeasy mini kit (Qiagen #74004). The isolated RNA was then quantified and evaluated for purity on a NanoDrop 2000 (Thermo Fisher, Waltham, MA). A ratio of 1.9-2.1 is generally accepted as "pure" RNA (Thermo Scientific, 2009). The RNA (250 ng) was then reverse transcribed to 10 ng/μL cDNA following manufacturer's protocol (Invitrogen kit #4304134). The abundance of each gene's expression was normalized to the *18s* reference gene expression. RT-qPCR was performed using an Applied Biosystems 7200 real-time cycler with SYBR Green detection chemistry (Applied Biosystems #4309155) following manufacturer's protocol, in order to quantify the gene expression for each gene. The following parameters were used for RT-qPCR: 10 min at 95°C, then 40 cycles of 95°C for 15 sec and 60°C for 1 min, followed by 95°C for 15 sec, 60°C for 1 min, and 95°C for 15 sec dissociation curve. Technical duplicates were performed and resulting data was evaluated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) and graphed using the average log(2)ΔΔCt ± standard error of the mean.

2.4 Statistical Analysis

Gene expression data were analyzed with the $2^{-\Delta\Delta C_T}$ method detailed in (Livak and Schmittgen, 2001). This is a method of relative quantification that relates the PCR signal of the target transcript in a treatment group to that of another sample in an untreated control. In this experiment, threshold values were averaged across each plate to account for variability among plates, and the C_T values were normalized to the *18s* reference gene. This represents the ΔC_T. The $\Delta\Delta C_T$ is given by assessing the difference between the target/dosed groups to the control/untreated group (aged fish). Data has been

presented as the fold change (log2) in comparison to the control group of aged fish. Using the calculated ΔC_T values, two tests were run. To compare the effects of THC, differences in varying doses of THC versus aged controls were assessed using a one-way ANOVA followed by a Dunnett's post-hoc test. To compare aged control versus young control, a t-test was used. Graphing and statistical analysis was conducted using Sigmaplot 14.0 software.

2.5 Previous Data

Data previously published and reviewed from our lab will be referenced throughout this paper. This data refers to the changes seen in phenotype or reproduction in the zebrafish. The methods for these measurements can be found in our previous publication (Pandelides et al., 2020).

3. Results

3.1 Senescence and Inflammation in Aged Fish Developmentally-Exposed to THC

Using the young and aged control groups for comparison, changes in the expression of inflammatory and senescence related genes were assessed and quantified in the F0 generation. Early-life exposure to THC in the F0 generation did not significantly alter *p16* expression in either male or female zebrafish (Table 1). Both male and female zebrafish exposed to the lowest concentration of THC (0.08 μM) showed significant down-regulation in *tnfα* (Figure 2a-b). The effects of THC on *tnfα*, *il-1β, il-6, pparα* and *pparγ* gene expression were hormetic and did not follow a typical dose response model. Exposure to all concentrations of THC (0.08, 0.4, 2.0 μM) resulted in significant down-regulation of *il-1β* in F0 females, but not males (Figure 3a-b). Developmental exposure to all concentrations of THC also resulted in significant up-regulation of *il-6* expression in F0 males, but not females (Figure 4a-b). Developmental exposure to THC also led to up-regulation of *pparα* and *pparγ* in F0 males (Figure 5a-b, 5 a). However, in F0 females, *pparγ* was down-regulated in a dose-dependent manner, with significant reductions in expression seen in the 2 μM THC treatment group (Figure 6b).

3.2 Fecundity Following THC Exposure

Eggs and sperm were quantified, along with survival of fertilized eggs, to determine the late-life fecundity of exposed zebrafish (Pandelides et al., 2020). Embryonic exposure to 0.08 and 0.4 μM THC did not significantly affect sperm

production. Measurements for 2 μM THC were not conducted due to a lack of extra male fish. Embryonic exposure to THC resulted in a hormetic response with increased egg production in response to the lower THC developmental exposure concentrations. In fact, females exposed to 0.08 μM THC produced 493% more eggs than the aged controls (Pandelides et al., 2020). These offspring had similar 96 hpf survival to both young and aged control fish. However, while fish exposed to 2 μM THC produced a similar number of eggs as aged control fish, only 16% of these survived by 96 hpf, as compared with the 62% survival seen in control fish. Exposure to the intermediate 0.4 μM THC resulted in a non-significant increase in egg production with a non-significant decrease in survival (Pandelides et al., 2020).

3.3 Phenotypic Changes in Aged Fish Exposed to THC

For the exposed males, there was no significant change in body mass or length compared to control groups. Aged control females are typically significantly longer and heavier than the young controls. However, developmental exposure to the lowest (0.08 μM) concentration resulted in female zebrafish that weighed significantly less than aged control females. The prevalence of kyphosis, or spinal curvature, was also assessed. It was determined that embryonic-exposure to THC did not significantly alter the age-related increase in kyphosis observed in males and females, although there was a visual trend of reduced severity correlating with decreasing THC concentrations (Pandelides et al., 2020).

3.4 Multi-Generational Effects of THC Exposure

For the F1 generation, there were no significant instances of up- or down-regulation in gene expression, as compared to the control group. (Table 2)

Table 1: Male and female F0 (mean ± SE) THC exposed (μM) *p16* gene expression (log(2)ΔΔCt) relative to the aged controls, n=6. Exposure groups were normalized to 18s and the ΔCT values were assessed for significance by one-way ANOVA with Dunnett's post-hoc test ($p \leq 0.05$), or by t-test for aged compared to young ($p \leq 0.05$).

	Male	Female
Young Control	-0.76 ± 0.61	-1.13 ± 0.29
Aged Control	0 ± 0.4	0 ± 0.43
0.08 THC	-0.04 ± 0.04	0.50 ± 0.24
0.4 THC	0.92 ± 0.26	0.31 ± 0.08
2 THC	1.05 ± 0.12	0.01 ± 0.26

No significant difference between THC and Aged control ($p>0.05$)

Table 2: Male and female F1 (mean ± SE) gene expression (log(2)ΔΔCt) of fish (parents exposed to 2 µM THC) relative to the aged controls, n=6. Exposure groups were normalized to 18s and the ΔCT values were assessed for significance by t-test ($p \leq 0.05$).

Males	***p16* ± SE**	***tnfα* ± *SE***	***il-1β* ± *SE***	***il-6* ± SE**	***pparα* ± *SE***	***parγ* ± SE**
Aged Control	0 ± 0.59	0 ± 0.9	0 ± 0.43	0 ± 0.16	0 ± 0.09	0 ± 0.19
2 µM THC	0.4 ± 0.82	0.89 ± 0.85	-0.45 ± 0.58	-0.62 ± 0.62	-0.52 ± 0.59	-0.64 ± 0.52

Females	***p16* ± SE**	***tnfα* ± *SE***	***il-1β* ± *SE***	***il-6* ± SE**	***pparα* ± *SE***	***parγ* ± SE**
Aged Control	0 ± 50	0 ± 0.57	0 ± 0.52	0 ± 0.26	0 ± 0.48	0 ± 0.18
2 µM THC	-0.51 ± 0.55	0.13 ± 0.77	-0.86 ± 1.27	-0.02 ± 0.56	-0.92 ± 0.88	-1.13 ± 0.70

No significant difference between THC and Aged control ($p > 0.05$)

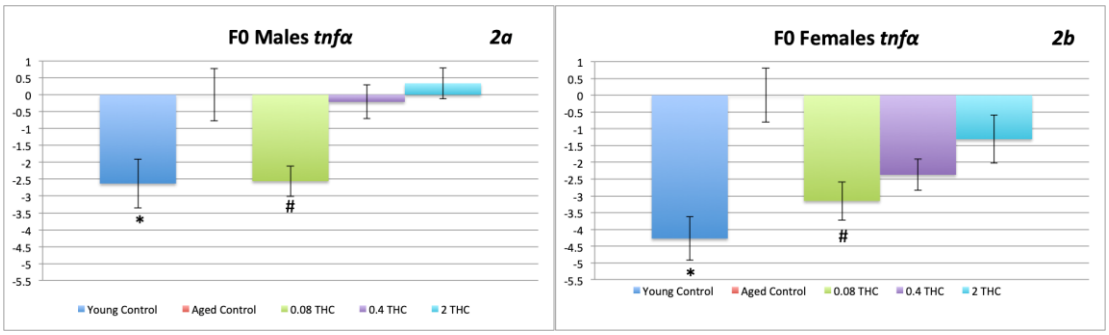

Figure 2: F0 Male & Female *tnf*∝ Expression After Developmental (6-96 hpf) THC Exposure. Both male and female F0 zebrafish experienced downregulation of *tnf*∝ expression after exposure to THC. Significant downregulation of *tnf*∝ gene expression was noted after developmental exposure to 0.08 µM THC compared to aged control. Young control consists of a 7-month old cohort kept in the same room, system, and conditions as the aged fish. Aged control refers to fish enrolled at the study at 12 months of age, and assessed for effects at 30 months (2.5 years) of age.
**p ≤ 0.05 t-test, Aged control vs Young control*
#p ≤ 0.05 ANOVA, Dunnett's post hoc vs Aged control

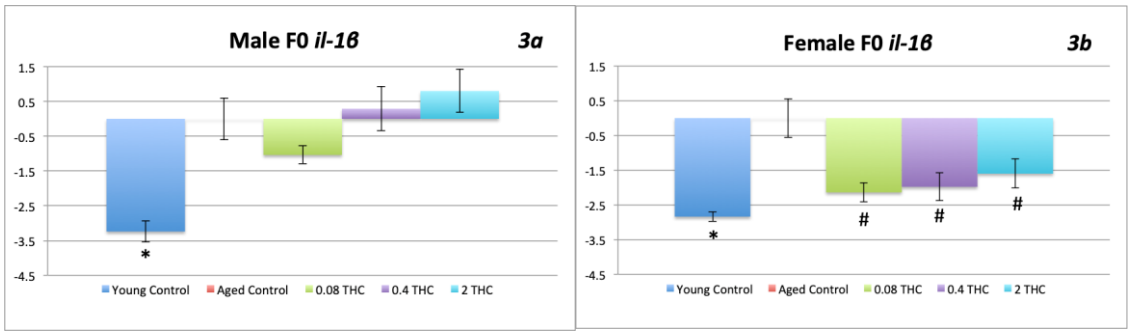

Figure 3: F0 Male & Female *il-1β* Expression After Developmental (6-96 hpf) THC Exposure. Female F0 zebrafish exhibited significant downregulation in *il-1β* gene expression after developmental exposure to any of the 3 doses of THC (0.08, 0.4, 2.0 µM) compared to aged control. Males did not exhibit any of these effects. Young control consists of a 7-month old cohort kept in the same room, system, and conditions as the aged fish. Aged control refers to fish enrolled at the study at 12 months of age, and assessed for effects at 30 months (2.5 years) of age.
**p ≤ 0.05 t-test, Aged control vs Young control*
#p ≤ 0.05 ANOVA, Dunnett's post hoc vs Aged control

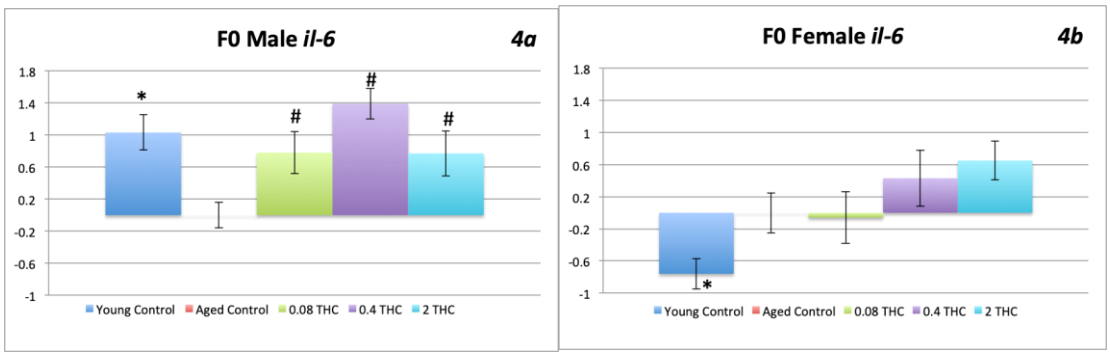

Figure 4: F0 Male & Female *il-6* Expression After Developmental (6-96 hpf) THC Exposure. Male F0 zebrafish experienced significant upregulation in *il-6* gene expression after developmental exposure to any of the 3 doses of THC (0.08, 0.4, 2.0 μM) compared to aged control. Females did not exhibit these effects. Young Control consists of a 7-month old cohort kept in the same room, system, and conditions as the aged fish. Aged Control refers to fish enrolled at the study at 12 months of age, and assessed for effects at 30 months (2.5 years) of age.
**p ≤ 0.05 t-test, Aged control vs Young control*
#p ≤ 0.05 ANOVA, Dunnett's post hoc vs Aged control

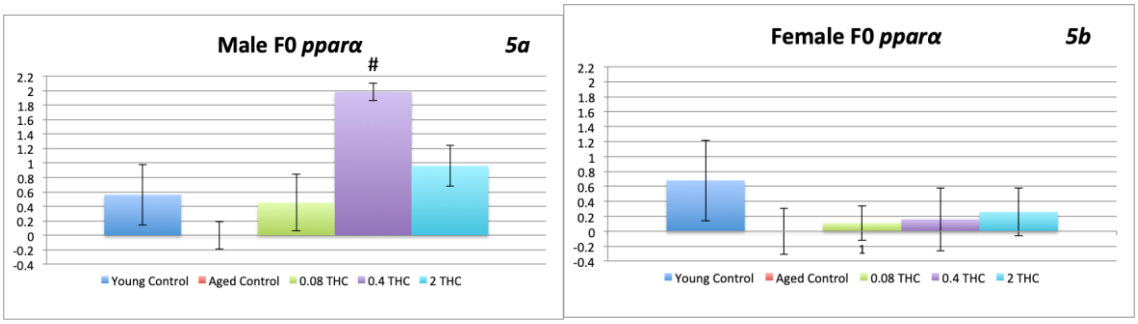

Figure 5: F0 Male & Female *pparα* Expression After Developmental (6-96 hpf) THC Exposure. Male F0 zebrafish exhibited upregulation in *pparα* gene expression after exposure to THC. Significant upregulation of *pparα* gene expression was noted after developmental exposure to 0.4 μM THC compared to aged control. Females did not exhibit these effects. Young Control consists of a 7-month old cohort kept in the same room, system, and conditions as the aged fish. Aged Control refers to fish enrolled at the study at 12 months of age, and assessed for effects at 30 months (2.5 years) of age.
**No significant difference*
#p ≤ 0.05 ANOVA, Dunnett's post hoc vs Aged control

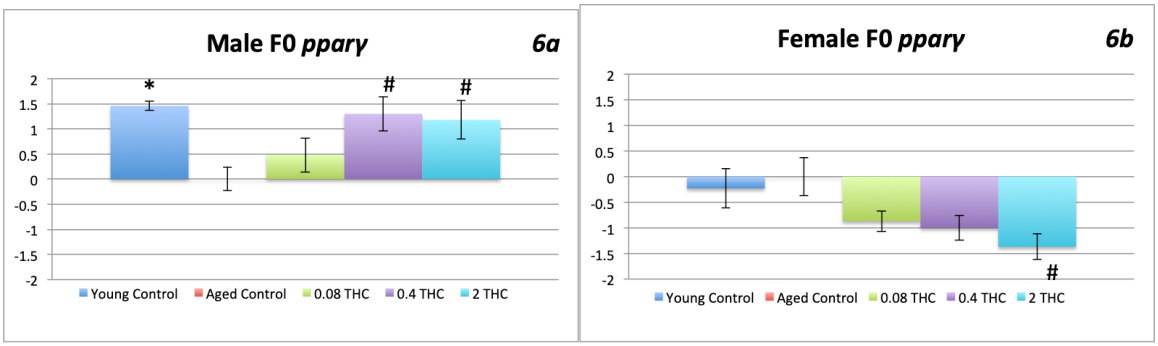

Figure 6: F0 Male & Female *pparγ* Expression After Developmental (6-96 hpf) THC Exposure. Male F0 zebrafish exhibited significant upregulation in *pparγ* gene expression after developmental exposure to THC (0.4 and 2 µM) compared to aged control. Female F0 zebrafish exhibited significant downregulation in *pparγ* gene expression after developmental exposure to THC (2 µM) compared to aged control. Young Control consists of a 7-month old cohort kept in the same room, system, and conditions as the aged fish. Aged Control refers to fish enrolled in the study at 12 months of age, and assessed for effects at 30 months (2.5 years) of age.
**p ≤ 0.05 t-test, Aged control vs Young control*
#p ≤ 0.05 ANOVA, Dunnett's post hoc vs Aged control

4. Discussion

It is well-understood that early-life exposure to certain chemicals—naturally or externally introduced—can increase the risks for certain diseases later in life (Richardson et al., 2006; Zawia and Basha, 2005). By exposing zebrafish embryos to THC, we were able to investigate the role it plays in growth, reproduction, cell senescence and inflammation.

4.1 Senescence and Inflammation in Aged F0 Fish Exposed to THC

p16 acts as both a tumor suppressor as well as contributor to cell senescence by slowing cell division. Early life exposure to THC did not significantly alter the expression of *p16* in aged male or female zebrafish. This result might indicate that treatment with THC, will not have the same effects on *p16* as it does on other genes that mark cell senescence. Furthermore, as *p16* is known to slow cell division (Krishnamurthy et al., 2006), it might be important to note that, based on our study, treatment with THC will not hinder the tumor-suppressing effects of *p16.*

Aging, as seen in our aged control models as well as other animal models, is accompanied by increased inflammation. Developmental exposure to THC reduced the expression of these genes associated with inflammation, which may have contributed to the increased lifespan we observed. This finding correlates with the well-known idea that *tnfα* and *il-1β* activation tend to be complimentary to one another (Contassot et al., 2012). A reduction in the expression of *tnfα* and *il-1β* may protect from

neuroinflammation-damage, as seen in mice (Fishbein-Kaminietsky et al., 2014). However, *tnfα,* and one of its activators *il-1β,* play a role in inhibiting tumorigenesis. Reduction in gene expression of *tnfα* and *il-1β* could make an organism more susceptible to developing tumors.

However, there was an observed difference in the expression of *il-6* that was distinct from that of *tnfα* and *il-1β*. In aged male fish (control), *il-6* was significantly decreased compared to young control males. This same decrease was not observed in the THC-exposed males, as they displayed increased *il-6* expression when exposed to any of the three THC concentration, compared to the male aged controls. Female fish did not experience the same significant increase in *il*-6 expression after developmental exposure to THC. These results suggest that *il-6* plays a role in sex-specific regulation. Furthermore, as *il-6* is also known to inhibit *tnfα* and *il-1β*, the increase in its expression could play a role in the correlated decrease in *tnfα* and *il-1β* expression.

Overall, our results suggest that embryonic exposure to THC can alter the expression of pro- and anti-inflammatory genes in aged fish. This alteration leads to a reduction in aging-related inflammation throughout the life cycle, and could be a potential factor in the observed increased lifespan.

The effects of developmental THC exposure on *tnfα* and *il-1β* gene expression were biphasic/hormetic, where most down-regulation was observed at the lowest dose given (0.08 µM THC). Low doses of THC protect mice from neuroinflammation-induced damage (Fishbein-Kaminietsky et al., 2014), and our study shows persistent effects 30 months following developmental exposure. These data suggest that exposure during early developmental periods can induce persistent alterations to gene expression.

4.2 Fecundity Following THC Exposure

We observed a decreased fecundity at the highest dose of THC tested. The reduction in fecundity observed at the high dose of THC in this current study supports previous studies that demonstrate maternal exposure to THC reduces the fecundity of male mice offspring 60-80 days later (Dalterio et al., 1984). While we were unable to test sperm concentration due to a shortage of male adult fish at the highest dose, it is possible that sperm concentration/quality played a role in the poor fecundity observed; as treatment with THC at the other two concentrations reduced sperm count in a dose dependent manner. Moreover, other animal models have demonstrated the adverse effects of THC on sperm (Morgan et al., 2012). Furthermore, altered endocannabinoid signaling is linked to infertility in human sperm (Lewis et al., 2012), and the strongest evidence for the negative effects of cannabis on humans is its impacts on semen parameters such as reduced concentration (Reviewed in: Payne et al., 2019).

4.3 Sex-Specific Effects

We suspected that, because of the functionality of the HPO axis and inequality of estradiol/progesterone levels between male and females, the embryonic exposure to THC would cause sex-specific alterations, ultimately with high concentrations of THC causing negative outcomes in male zebrafish. Because the negative effects of THC on sperm is not CB1 mediated (Morgan et al., 2012), the effect of THC on reproduction is likely mediated through another receptor/mechanism. THC treatment significantly reduces ATP levels in sperm (Morgan et al., 2012). Thus, the reproductive effects could be a result of altered metabolism or mitochondrial function.

Also, we did observe sex-specific changes in the expression of both *pparα* and *pparγ*. Males had increased expression of *pparα* and *pparγ*, while females had a concentration-dependent decrease in expression of *pparγ*. PPARγ regulates adipogenesis (Berger and Moller, 2002; Bouaboula et al., 2005; Gerhold et al., 2002), and lifelong alteration of energy metabolism in developmentally-exposed females could have contributed to the reduced weight observed. As sexual maturation is dependent in zebrafish (Parichy et al., 2009), it is possible the developmentally exposed female fish simply matured later than the aged controls, resulting in lower egg production at a younger age and higher egg production later.

4.4 Multi-Generational Effects of THC Exposure

The majority of the effects observed in the parental F0 generation following THC exposure were not observed in the F1 generation. There were no significant differences in up- or down-regulation of gene expression, suggesting that the influence of THC on aging and inflammation was not cross-generational. However, if the exposure had been extended over a longer period of time, or at a different stage of development, the results might have differed. In addition, we only observed the F1 generation given the highest (2 μM) concentration of THC, whereas we initially only observed effects from the lowest dose given.

4.5 Implications of Study

This study demonstrates that a single embryonic exposure to THC can cause significant effects on the outcome of zebrafish. When measured 30 months prior to

exposure, early-life exposure to THC had significant impacts on the expression of genes related to cell senescence and inflammation.

The exposure to F0 fish during early development caused biphasic effects on fecundity and the expression of genes such as *tnfα* and *il-1β* in aged zebrafish. High dose exposure resulted in reproductive toxicity, significantly reducing the survival and reproduction of F1 progeny. Low dose exposure resulted in reduced expression of genes related to senescence and inflammation, and increased survival. Exposure to THC also caused a concentration-dependent reduction in *pparγ* expression in female zebrafish, and increase in *pparγ* expression in male zebrafish. This study suggests that *in utero* exposure to chemicals will reflect the ideas of the DevAge theory. Due to the biphasic nature of THC, it is difficult to determine how the doses given in this experiment compare to what would be administered to a developing human fetus. Therefore, it remains clear that achieving desirable effects would require extremely controlled dosage with very specific concentrations. Because of this, future research considering long-term and multigenerational impact of low doses of THC merit further study.

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