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Deducing the Reproductive, Behavioral, and Learning Effects of Developmental Cannabinoid Exposure in Zebrafish

Marisa L. Kutchma
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Deducing the Reproductive, Behavioral, and Learning Effects of Developmental Cannabinoid Exposure in Zebrafish

By:
Marisa L. Kutchma

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
April 24, 2019

Approved by

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Thank you to my reader, Dr. Nicole Ashpole, for guiding my research in the right direction and always being available to chat when the fish decide to be obstinate. Thank you to my other reader, Dr. Kristopher Harrell, for your time and effort spent towards this project.

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ABSTRACT

With the spreading legalization of marijuana, it is important to investigate the effects of two of its active ingredients—Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD). THC and CBD differ in that THC exerts psychoactive effects, while CBD does not. Thus, CBD is renowned for its analgesic effects in treating a variety of ailments, for example, childhood drug-resistant epilepsy. In addition, there is an increase in reports of prenatal CBD usage. As it is increasingly used, research has fallen far behind the proliferation of CBD and more needs to be done, particularly in the developmental realm. This study utilizes a developmental origins of health and disease (DOHaD) multigenerational paradigm after an embryo-larval exposure of F0 zebrafish to several low concentrations of THC (0.024, 0.12, 0.6 mg/L; 0.08, 0.4, 2 µM) and CBD (0.006, 0.03, 0.15 mg/L; 0.02, 0.1, 0.5 µM). Three primary tests were conducted: a reproductive assessment, an adult behavioral Open Field Test, and a learning and memory T-maze test. In terms of reproduction, fecundity was significantly reduced in several exposed F0 groups, but not in F1 groups. In the Open Field Test, there were no significant findings in the F0 fish, but there were significantly altered behaviors measured in the F1 fish whose parents were exposed to the highest concentration of THC. The T-maze is an ongoing experiment and has not produced any significant outcomes in relation to learning and memory. The results of this experiment reveal the need for increased investigation into the lifelong and multigenerational effects of developmental THC and CBD exposure.
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<tr>
<td>CB1-R</td>
<td>Cannabinoid Receptor 1</td>
</tr>
<tr>
<td>CB2-R</td>
<td>Cannabinoid Receptor 2</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DOHaD</td>
<td>Developmental origins of health and disease</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>dpf</td>
<td>Days post fertilization</td>
</tr>
<tr>
<td>ECS</td>
<td>Endocannabinoid System</td>
</tr>
<tr>
<td>GCMS</td>
<td>Gas Chromatography/Mass Spectrometry</td>
</tr>
<tr>
<td>hpf</td>
<td>Hours post fertilization</td>
</tr>
<tr>
<td>hpt</td>
<td>Hours post treatment</td>
</tr>
<tr>
<td>IACUC</td>
<td>Institutional Animal Care and Use Committee</td>
</tr>
<tr>
<td>Mpf</td>
<td>Months post Fertilization</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>THC</td>
<td>$\Delta^9$-tetrahydrocannabinol</td>
</tr>
<tr>
<td>ZFIN</td>
<td>Zebrafish International Resource Center</td>
</tr>
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</table>
1. INTRODUCTION

1.1 Marijuana

*Cannabis sativa* is the flowering plant responsible for many strains of marijuana. Marijuana is known to simultaneously be a hallucinogen, a stimulant, and a depressant (Murray, 1986). These unique properties led to its place in medical history, beginning in 2737 B.C. in Asia (Earleywine, 2002). Its use has persisted into the modern day as a widespread drug with many uses. Since the progressive legalization of medical and recreational marijuana, it has become a prominent concern in public health. As such, its toxicity is being questioned in order to assess possible consequences of the drug. Research is being conducted in order to elucidate the possible dangers of cannabis as it continues to become more commonplace in society. Recreationally, marijuana is known for its calming and psychoactive effects on users. Additionally, marijuana is known to adjust appetite, reduce seizure activity, and treat nausea (Elikkottil *et al.*, 2009). In terms of medical uses, the drug is favored for its numerous analgesic effects when treating patients who suffer from chronic pain or are undergoing cancer treatments. Marijuana has been found to have positive effects for consumers. These effects can be attributed in part to the most common phytocannabinoids—$\Delta^9$-tetrahydrocannabinol (THC) and cannabidiol (CBD).

1.1a $\Delta^9$-tetrahydrocannabinol (THC)

THC is the most well known active ingredient in marijuana. THC is typically what is thought of in conjunction with marijuana and is the compound responsible for the
psychoactive properties of marijuana. THC chiefly exerts its effects through cannabinoid receptor 1 (CB1-R) in the brain (Howlett, 2002). The CB1 receptor is primarily expressed in the central nervous system (CNS), particularly at presynaptic terminals (Howlett, 2002). This receptor is a G-protein coupled receptor, which is involved in inhibiting adenylyl cyclase. In addition to CB1-R, there is also a cannabinoid receptor 2 (CB2-R), which is found to function primarily in the immune system. This receptor interacts with endocannabinoids within the body (Lu & Mackie, 2016). Besides THC, there are several endocannabinoids that originate from the body that interact with CB1-R. CB1-R is known for its action in pain relief, anti-emesis, and appetite modulation (Lupica et al., 2004). On the other hand, it has also been discovered that it functions in hallucinations, mood, and memory. Research has shown that CB1-R antagonists like SR141716 block the effects of this pathway (Howlett, 2002). The psychoactive property of THC detracts from its more positive medical benefits such as analgesia. The schedule 1 drug classification of marijuana has caused researchers to hesitate in freely using the drug for medical purposes. Overall, in addition to pain relief, THC has been previously used to treat glaucoma, asthma, and hypertension (Akhtar et al., 2016). Impaired cognitive development could be a major long-term effect of marijuana use during development. This outcome appears more prevalent as the age of exposure decreases (NIDA, 2018). Studies have even shown that long-term use can increase one’s risk for schizophrenia when an individual is at genetic risk for the disorder as well (NIDA, 2018). THC is known to have developmental toxicological effects, so this study examined the developmental origins of health and disease (DOHaD) (Wadhwa et al., 2009).
1.1b Cannabidiol (CBD)

CBD, another active ingredient in marijuana, has recently become a hot topic in modern day science. Its increasing popularity is due to the fact that it does not have the same psychoactive properties that THC possesses, due to a single structural difference between the two cannabinoids, as shown in Figure 1. For example, CBD is used instead of THC in order to treat a variety of disorders such as drug-resistant epilepsy in children (Rosenberg et al., 2017). CBD functions through a pathway that is distinct from the CB1 receptor that THC can act through because it has minimal affinity to CB1-R. This is what allows CBD to evade the psychoactive effects that THC possesses.

Research shows that like THC, CBD utilizes multiple pathways in the body (Szaflarski & Martina Bebin, 2014). There is a reward pathway in the brain that is especially affected by cannabinoids and other drugs. This pathway consists of three structures: the Nucleus Accumbens, the Ventral Tegmental Area, and the Medial Forebrain Bundle. These form the biological basis for addiction in the brain (NIDA, 2016). To add to this, endocannabinoids are cannabinoids that are naturally occurring in the body (Mechoulam & Parker, 2013). There is an endocannabinoid system (ECS), through which endocannabinoids and the active ingredients in marijuana cause effects. Since CBD is just now becoming popular, there is a lag in information available on the possible negative consequences of this cannabinoid. As marijuana legalization has become more commonplace, so has the use of CBD. Scientists are utilizing it in order to treat depression, chronic pain, and cancer (Halford, 2018). It is especially crucial to understand CBD’s possible side effects because it is being used to treat children in addition to adults. Cannabinoids are responsible for DOHaD effects on reproduction and
behavior (Carty et al., 2019; Hanson & Gluckman, 2014; Wadhwa et al., 2009), but marijuana is a schedule I drug. Thus, this restrictive scheduling has limited research to explore expanded effects of CBD and THC exposure or the underlying mechanisms associated with toxic effects. We hypothesize that THC will have greater developmental toxicology than CBD.

![Figure 1: The structural differences between Δ-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) structures](image)

1.2 Zebrafish as a model organism

Zebrafish (*Danio rerio*) is a vertebrate model organism frequently employed in many different sectors of research. This tropical freshwater fish comes from the carp family and originated in the region of Southeastern Asia. This fish is able to survive in a diverse array of conditions and is hardy even in the harshest of circumstances. Thus, they
can be found in freshwater bodies of water or in small puddles. These fish have a high fecundity and reproduce relatively quickly. Thus, it is feasible to create genetically modified lines of zebrafish with ease. The development of a zebrafish in one day can be equivalent to that of a human in one month (Wellcome Genome Campus, 2014). To add to this, their transparent eggs are ideal for studying development because one can see the entire process when using microscopy. Because the genome of the zebrafish is similar to that of humans, it is an excellent model organism for genetic analysis. Zebrafish and humans share 70% of their genes, and 84% of human disease genes have corresponding zebrafish genes. The entire zebrafish genome was sequenced in 2013 (Wellcome Genome Campus, 2014). Another advantage is that the fish has a clear dichotomy of male and female individuals. Females tend to have a deeper pink tint and are larger, especially when carrying eggs. Males are more slender and possess more neutral colors. Additionally, zebrafish are diurnal and have a clear sleep-waking schedule that corresponds to light-dark periods in their environment. They tend to be social animals and are used in models to study behavior and learning in addition to genetics and reproduction (Norton and Bally-Cuif, 2010). Notably, the ECS has been highly conserved in both zebrafish and mammals (Krug & Clark, 2015). This quality is rare in model organisms and, thus, makes the zebrafish an ideal choice for this research.

1.3 Reproduction

Rapid reproduction rates and high fecundity characterize the breeding process of zebrafish. Breeding zebrafish depends on many intricate elements, including visual, olfactory, and social factors. Light-dark routines and feeding schedules are also
imperative to the spawning process (Nadiadka & Clark, 2012). After a male fertilizes a female’s eggs, the female deposits the eggs, and they fall to the bottom of the container. The egg is considered a zygote from 0-0.75 hours post fertilization (hpf). Next, cleavage occurs until 2.25 hours and then it is considered a blastula until 5.25 hours. This is when a series of rapid mitotic divisions occurs. Gastrulation occurs until 10.33 hours and segmentation then lasts until 24 hours. During this period, tissue layers and the body form develop. The pharyngula period occurs until 48 hpf, during which body systems mature. The zebrafish hatch at 48-72 hpf. Finally, they are considered larvae until 30 days post fertilization (dpf) (Hill, 2019). The early stages of development are pictured in Figure 2.

In contrast to mammals’ dimorphic gametes that determine biological sex, sex determination in zebrafish is partially determined by the environment in which it resides in addition to genetic factors. This complex process involves temperature, hormones, and oxygen composition (Hoo et al., 2016).
Figure 2: The stages of zebrafish development in the hours post fertilization (hpf).
1.4 Behavior

In recent years, researchers have been testing the behavior of zebrafish (Kalueff & Stewart, 2012). A focus of this thesis was behavioral effects following CBD and THC exposures. For the purpose of this study, behavior can be defined as how an organism interacts with its environment (Orger & de Polavieja, 2017). Analysis of behavior is done using video tracking equipment. This provides a systematic quantitative model in order to study the zebrafish with minimal handling. Common behavioral tests include the mirror image test, light/dark preference test, and tank diving test (Kalueff & Stewart, 2012). In this study, an Open Field Test was utilized in order to assess zebrafish anxiety-like behavior. Anxiety can be measured in an Open Field Test by assessing the location of the fish during the trial. Zebrafish experiencing stress will typically swim on the outer edges of an environment. Additionally, they will exhibit ‘freezing behavior’, in which they tend to stay in one place as if frozen (Norton and Bally-Cuif, 2010). This is an evolutionary mechanism in response to predation. If the fish stays still, it is less likely to be found and become prey. Overall locomotion was also assessed in conjunction with the anxiety behaviors. Open Field Tests have been used previously to study zebrafish that have been exposed to drugs such as LSD (Grossman et al., 2010).

1.6 Learning

Using zebrafish as a model for learning is a more recent trend, and there are few learning publications compared to its use in anxiety research. That said, zebrafish can develop associative memory in relation to visual stimuli (Kim et al., 2017) in addition to showing directional and color preference (Bault et al., 2015). The number of studies
focused on this aspect at present is still growing rapidly and additional research in zebrafish learning is called for, as learning assays are still being improved. For example, a device called a T-maze can be used to quantify learning in a variety of animals, including zebrafish. This task is objectively simple, but the learning and memory variables that are measured from it are relatively complex (Braida, et al., 2014). The T-maze is an ideal example of testing learning because of its straightforward parameters that are easy to assess. Developmental exposure to other compounds such as bisphenol A (BPA) have had differential effects on zebrafish learning and memory exhibited in a T-maze (Saili et al., 2011). Many learning assessments like the T-maze are also used in conjunction with behavioral tests, like the Open Field Test in this study. Other learning tests used with zebrafish include: the rotating escape test, bite test, novel tank test, and the place preference test (Kalueff & Stewart, 2012).

The T-maze protocol assesses learning by measuring the time and distance that an individual organism covers in order to make it to a determined target zone. This ‘positive reservoir’ is learned through training. Thus, this study utilizes operant conditioning by using positive reinforcement in order to produce learning. However, many different papers use a variety of types of T-mazes. These typically differ in dimension, reward, and visual effects (Bault et al., 2015). In this thesis, various versions of T-mazes were utilized in an attempt to identify a protocol that successfully evoked learning in the control zebrafish.
11.7 Study Goals

- Test the results of a developmental zebrafish exposure to various concentrations of THC and CBD
- Assess the reproductive outcomes of zebrafish exposed to cannabinoids
- Discover the persistent behavioral modifications that occur in developmentally exposed zebrafish
- Explore the learning and memory abilities of control zebrafish compared to that of developmentally exposed zebrafish
2. METHODS AND MATERIALS

2.1 Zebrafish care

Tg(fli1:egfp) zebrafish were obtained from the Zebrafish International Resource Center (ZFIN, Eugene Oregon). Adult zebrafish were kept in a controlled environment that consisted of a flow-through system (Aquatic Habitats, Apopka, Florida) at a pH of 7.5-8, dissolved oxygen of 7.2-7.8 mg/L, conductivity of 730–770 µS, and a water temperature of 27-29°C. Zebrafish were fed twice a day, once in the morning and once in the afternoon with Gemma Micro 300 (Skretting Nutreco Company, Westbrook, Maine) and were kept on a diurnal light-dark schedule of 14 light hours and 10 dark hours. Fish were kept under approved Institutional Animal Care and Use Committee (IACUC) protocols for culture and exposure. For breeding, healthy adults were placed into breeding tanks overnight. Three breeding tanks from 2-3 separate spawning events were used to obtain embryos for the exposures described below (Carty et al., 2019). Following one hour of light, eggs were collected in a sieve, unfertilized and dead eggs were removed, and developing embryos kept in petri dishes with egg water (60 ppm Instant Ocean (Instant Ocean, Cincinnati, Ohio), pH of 7.4-7.7) for sorting.

2.1a Exposure

Embryos were sorted into scintillation vials (n=5 per exposure group per time point). Each vial had 15-30 fish depending on the time point because as the fish age, fewer fish per replicate pool are needed for gene expression analysis. Embryos (F0) were exposed to THC (0.024, 0.12, 0.6 mg/L; 0.08, 0.4, 2 µM), CBD (0.006, 0.03, 0.15 mg/L;
0.02, 0.1, 0.5 µM), or 0.05% DMSO from 6 to 96 hpf with 0.6:1 mL water:fish (Carty et al., 2018). Only F0 fish were exposed but not the subsequent F1 generation as shown in Figure 3. THC and CBD used in this study were acquired from the NIDA Drug Supply Program. Following the F0 developmental exposure, fish were raised under normal culture conditions to assess reproductive fitness and behavior and learning assays.

**Figure 3:** The exposure paradigm utilized in this study.
2.2 Reproductive evaluation

As mentioned, only F0 zebrafish were exposed to THC and CBD. However, the breeding protocol was kept the same for both F0 and F1 fish. At 6 months and 11 months post fertilization (mpf), respectively, F0 and F1 fish were placed in 750 mL (Aquatic Biosystems) tanks with 4 fish total (2 males and 2 females). The foursome was kept in these static tanks at 28°C in the conditions listed above. Fish were allowed a week to adjust to the new housing conditions, and the water was changed two times per week in addition to when the fish spawned. After the acclimation period, fertilized eggs were collected from the bottom of the tanks for three days in a row. This method was followed in order to guarantee that eggs came from both females (Reed and Jennings, 2011). Spawning tanks were cleaned each time eggs were collected, and the embryos were placed in clean embryo water (60 ppm Instant Ocean; pH 7.5-7.8), where they would be examined for fertility, deaths, defects, and hatching. Embryos were checked every 24 hr until 96 hpf. The number of eggs produced per tank was recorded. Because the exposure concentrations were low, there was no significant incidence of malformations found in the embryos and larvae. Subsequently, the F1 zebrafish larvae were raised and also assessed for reproductive fitness (Carty, et al., 2019).

2.3 Behavior

The Open Field Test was conducted using the F0 and F1 zebrafish. The fish were housed between trials within the same type of tanks as used in the reproductive tests (Aquatic Biosystems). The F0 fish were 18 months old and F1 fish were 12 months old at the time of the Open Field Test. F0 groups included: 0.024, 0.12, and 0.6 mg/L THC;
0.006, 0.03, and 0.15 mg/L CBD; and control (0.05% DMSO). There were n=7-10 per sex per concentration. F1 groups included: 0.6 mg/L THC; 0.03 mg/L CBD; and control (0.05% DMSO). These groups had \(n = 8–10\) per sex per treatment. Open Field Tests were done on different cohorts of naïve individual fish to avoid employing the test battery effect. Advanced video equipment and trained observers analyzed the zebrafish behavior. In particular, the video equipment utilized was EthoVision XT 13 (Noldus Information Technology, Netherlands) with a color GigE camera. The Open Field Test is a well-established model that is used to test the behavior of a number of model organisms. This test has been specifically modeled after one used for rodents (Christmas & Maxwell, 1970). Open Field Testing focuses on anxiety behaviors and locomotor activity (Stewart et al., 2014). In this particular study, the Open Field Test accounted for freezing behaviors, locomotion, and thigmotaxis. In general, thigmotaxis is the preference of the periphery of an environment over the central region (Nielsen et al., 2018). Freezing is a quantitative measure of anxiety in the fish. In order to perform the test, we filled a white bucket (21 cm diameter, 24 cm height) to 12 cm with zebrafish water as shown in Figure 4. Utilizing the video equipment, two zones were created: a center circle (13 cm diameter) and a periphery (remaining outer 4 cm). To avoid diurnal variation, the same concentration groups of fish were not recorded at the same times. To start the test, a single fish was placed in the middle of the center zone and was recorded with EthoVision for 6 minutes. Variables measured included: distance traveled, velocity, time in center vs. time in periphery, center visits, periphery visits, freezing frequency, and freezing duration (Carty, et al., 2019).
Figure 4: A view of the Open Field Test setup (A) and EthoVision tracking (B).

The test required the use of a bucket with the following dimensions: 21 cm diameter, 24 cm height, and filled with water to 12 cm high. The bucket was divided into two zones: Center (13 cm inner yellow circle) and Periphery (4 cm outer pink circle). The blue arrow is pointing to a fish in the bucket during an Open Field Test trial.
2.4 Learning

To assess learning and memory of the F0 zebrafish, a T-maze was employed. T-mazes are well established for learning and memory tests in rodents (Schaefers and Winter, 2011). The apparatus is a transparent Plexiglas maze in a ‘T’ shape that contained: a starting area, a starting arm, a positive reservoir, and a neutral reservoir (Freeman et al., 2015). An individual fish was placed in the starting area, where it was kept in the enclosed area by a gate. After, the gate was raised using a pulley system operated by a trained observer. The fish was then allowed to leave and explore. Once the fish left the area, the gate was closed by the observer. The goal was for the fish to find the positive reservoir and remain in that area for a minimum of 20 seconds (Braida et al., 2014). The positive reservoir contained a positive stimulus for the fish, which was varied with each version during method optimization. The fish would then repeat this trial for a designated set of days to test memory and learning abilities of the fish. The amount of days differed for the different versions of the T-maze set-up. Variables measured were the total path length before reaching the positive reservoir and the time it took to reach the positive reservoir. Each run was recorded using EthoVision as described above in the Open Field Test section. Directional preference was eliminated due to placing the maze in a West-East direction (Freeman, et al., 2015). The temperature and lighting of the environment in which the maze was placed were kept constant and ideal for testing memory. The T-maze was partitioned from the rest of the room with a white sheet that removed external light interference. The only light present during the testing came from the lighting equipment to properly record the trials. The light was placed at an ideal location so that dark and light areas were non-existent (Facciol et al., 2017). The sides of
the maze were covered with white paper so that the fish could not view anything outside of the maze. The top of the maze was not covered so that the recording equipment could capture the trials. The room temperature was kept at a constant 26.7°C. Water was changed between days of trials.

To begin a trial, a fish was placed in the starting area, which was contained by the sliding gate. After 5 minutes of acclimation, a trained observer would manually lift the gate and gently encourage the fish to leave using a blue fish net. The tests could last up to 10 minutes, depending on the version of the T-maze being used. If the fish did not reach the positive reservoir, the trained observer would gently guide it to the area and then leave it for 1 minute before the fish was removed from the maze. Several variations of the T-maze protocol were employed in this study to optimize assessments of learning and memory in zebrafish as described below.

2.4a T-maze version I

The first T-maze had the dimensions pictured in Figure 5. The maze included: a long arm (45.72 cm), two short arms (30.48 cm), and two large reservoirs (22.86 square cm, 5.08 cm deeper than rest of maze). These dimensions were taken from Darland and Dowling (2001). In this T-maze, the positive reservoir contained blue and green rocks, marbles, and fake plants. We considered reaching the positive reservoir and remaining in it for 20 seconds as an effective positive stimulus. The neutral zone was left empty (Braida, et al., 2014). To prevent procedural novelty anxiety, three days of habituation trials were conducted in version I. The fish were placed in the T-maze in groups of 16 (day 1), 8 (day 2), and 4 (day 3) before the video recording began. They were given an
hour each day to freely explore the maze in these groups. Then, there were four consecutive days of normal testing trials during which each fish was tested once (Braela, et al., 2014). The fish were left in the maze for 10 minutes regardless of when or if they made it to the positive reservoir. After performing a full phase of the 7-day combined habituation and testing protocol on 48 fish from the exposed and control groups, these dimensions were deemed unproductive because the fish did not show statistically significant signs of learning, so we sought out new dimensions to test.

Figure 5: Version I of the T-maze, including the positive reservoir with rocks, marbles, and fake plants.
2.4b T-maze versions II and III

The second version of the Plexiglas T-maze had the following dimensions: a starting area (30 cm x 10 cm), a long arm (50 cm x 10 cm), two short arms (20 cm x 10 cm), and positive and neutral reservoirs (20 cm x 10 cm) (Freeman et al., 2015). The entire maze was 10 cm deep and was filled to a level of 7 cm with zebrafish water. The fish were placed in the maze once a day for three days in a row (Saili et al., 2011). The fish were not tested on the fourth day and then the same procedure was performed on a fifth day. Fish were removed from the maze once they reached positive reservoir and stayed for 20 seconds. At first, the positive reservoir contained the same protocol from version I, but two further trials with 6 fish each showed that the smaller dimensions caused the rocks, marbles, and plants to be aversive stimuli for the fish because the water was too shallow. Thus, a food ring, as shown in Figure 6, was used to present food and quarantine it in the positive reservoir. A small amount of food that the fish were fed daily was placed in the food ring to act as positive reinforcement. During training, the experimental fish were fed only when they located the food reward in the maze (Jia et al., 2014). They were not fed in the evening or the morning prior to testing. The fish were housed in boxes of 1 female and 1 male and the experiment was double blinded. Each fish was tested consistently at the same time of day throughout the entire trial. There were 8 F0 fish tested for the following four groups: control, 0.6mg/L THC, 0.12 mg/L THC, and 0.15 mg/L CBD. However, fish were removed from the tests when they failed to make it to the positive reservoir within 10 minutes both of the first two days. The different versions of the T-maze are compared in Table 1.
**Figure 6:** Version III of the T-maze, containing: the starting area (bottom arm), a positive reservoir with food (left arm), and a neutral reservoir (right arm). It consists of a real time picture of a maze trial with the yellow arrow pointing to an adult zebrafish and the green arrow pointing to the food ring.
Table 1: The different versions of T-maze trials utilized.

\(^a\)The large T-maze to the fish was comparable in length to the size of 2/3 football field for an adult human (Darland and Dowling, 2001).

\(^b\)The small T-maze to the fish is comparable to 20 yards of a football field for an adult human (Freeman et al., 2015).

<table>
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<th>Trial</th>
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<td>Large(^{a,2})</td>
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<td>2</td>
<td>Small(^{b,3})</td>
<td>Rocks/plants</td>
</tr>
<tr>
<td>3</td>
<td>Small(^{b,3})</td>
<td>Food ring</td>
</tr>
</tbody>
</table>
2.5 Statistical Analysis

The reproductive success assessment as well as F1 survival were analyzed with a one-way ANOVA ($p \leq 0.05$) for all of the treatment groups regarding: average number of eggs per tank, percent fertilized, percent survival at 24 hpf, percent hatched at 48 hpf, percent hatched at 72 hpf, and percent survival at 96 hpf. The Open Field Test was analyzed with a two-way ANOVA ($p \leq 0.05$) followed by a one-way ANOVA ($p \leq 0.05$) for each treatment group. A t-test was used in order to analyze the F1 THC 0.6 mg/L group. The T-maze data was analyzed using a one-way ANOVA for each treatment group separated for latency and path length followed by a t-test ($p > 0.05$). GraphPad Prism 5.0 software and StatPlus was utilized in order to analyze each of the tests.
3. RESULTS

3.1 Reproductive Outcomes

Total offspring, survival, fertilization, and hatching rates were measured from F0 fish at 6 mpf and F1 fish at 11 mpf. (Table 2). The F0 fish exposed to 0.15 mg/L CBD, 0.024 mg/L THC, and 0.12 mg/L THC produced significantly fewer eggs per tank when compared to the solvent control (p ≤ 0.05). However, F0 fertilization, survival, and hatching rates were not significantly changed in the exposed fish. F1 fecundity, fertilization, survival, and hatching rates were not significantly affected following parental exposure to THC or CBD (p > 0.05) (Carty et al., 2019).
### Table 2: Reproductive assessment on F0 and F1 fish (Carty et al., 2019).

<table>
<thead>
<tr>
<th>Group</th>
<th>Nominal water concentration (mg/L) at 5 hpf (tanks)</th>
<th>Average # eggs per tank</th>
<th>% Fertilized</th>
<th>% Survival at 24 hpf</th>
<th>% Hatched at 48 hpf</th>
<th>% Hatched at 72 hpf</th>
<th>% Survival at 96 hpf</th>
</tr>
</thead>
<tbody>
<tr>
<td>F0 Control 0.05% DMSO</td>
<td>198 ± 24</td>
<td>98.2 ± 1.4</td>
<td>78.9 ± 7.5</td>
<td>20.9 ± 8.9</td>
<td>96.5 ± 2.4</td>
<td>74.1 ± 8.9</td>
<td></td>
</tr>
<tr>
<td>F0 THC 0.024 (3)</td>
<td>64 ± 19*</td>
<td>100.0 ± 0.0</td>
<td>90.4 ± 4.9</td>
<td>18.4 ± 15.4</td>
<td>95.7 ± 2.3</td>
<td>89.8 ± 4.8</td>
<td></td>
</tr>
<tr>
<td>F0 THC 0.12 (3)</td>
<td>61 ± 34*</td>
<td>99.2 ± 0.6</td>
<td>61.3 ± 24.0</td>
<td>40.7 ± 2.0</td>
<td>100.0 ± 0.0</td>
<td>60.4 ± 26.0</td>
<td></td>
</tr>
<tr>
<td>F0 THC 0.6 (5)</td>
<td>110 ± 26</td>
<td>99.3 ± 0.3</td>
<td>91.7 ± 1.6</td>
<td>25.3 ± 8.3</td>
<td>99.2 ± 0.8</td>
<td>89.4 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>F0 CBD 0.006 (3)</td>
<td>192 ± 33</td>
<td>95.8 ± 2.2</td>
<td>85.3 ± 5.9</td>
<td>24.4 ± 3.9</td>
<td>99.0 ± 0.3</td>
<td>77.6 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>F0 CBD 0.03 (3)</td>
<td>113 ± 19</td>
<td>98.9 ± 0.5</td>
<td>87.5 ± 5.4</td>
<td>14.1 ± 2.5</td>
<td>98.1 ± 1.2</td>
<td>84.0 ± 5.7</td>
<td></td>
</tr>
<tr>
<td>F0 CBD 0.15 (3)</td>
<td>66 ± 14*</td>
<td>92.8 ± 6.0</td>
<td>55.3 ± 7.6</td>
<td>23.6 ± 10.5</td>
<td>97.5 ± 2.5</td>
<td>49.5 ± 9.3</td>
<td></td>
</tr>
<tr>
<td>F1 Control 0.05% DMSO</td>
<td>71 ± 18</td>
<td>97.9 ± 2.1</td>
<td>79.0 ± 9.2</td>
<td>63.2 ± 18.0</td>
<td>98.6 ± 1.4</td>
<td>74.3 ± 10.6</td>
<td></td>
</tr>
<tr>
<td>F1 THC 0.6 (5)</td>
<td>55 ± 23</td>
<td>99.5 ± 0.5</td>
<td>79.1 ± 6.3</td>
<td>50.3 ± 14.4</td>
<td>95.7 ± 2.6</td>
<td>76.1 ± 8.4</td>
<td></td>
</tr>
<tr>
<td>F1 CBD 0.006 (7)</td>
<td>82 ± 19</td>
<td>96.5 ± 2.9</td>
<td>76.6 ± 7.3</td>
<td>21.0 ± 7.5</td>
<td>87.7 ± 4.9</td>
<td>66.2 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>F1 CBD 0.03 (6)</td>
<td>46 ± 13</td>
<td>98.1 ± 1.9</td>
<td>94.7 ± 2.7</td>
<td>30.9 ± 10.4</td>
<td>96.7 ± 2.1</td>
<td>89.1 ± 5.5</td>
<td></td>
</tr>
</tbody>
</table>

± SEM

*One-way ANOVA; Dunnett’s (p<0.05)
3.2 Open Field Test

An open field chamber was used to analyze the locomotion and anxiety behavior of F0 and F1 fish. There was a non-significant dose-dependent increase in freezing duration for all F0 fish treated with THC or CBD ($p \geq 0.05$, Figure 7). F1 fish parentally exposed to 0.6 mg/L THC spent significantly less time in the periphery of the open field than the control fish ($p \leq 0.05$). However, this did not hold true for any other experimental concentration groups (Carty et al., 2019).
**Figure 7:** Summary of behavioral data (mean ± SEM) collected from a 6 min Open Field Test (n=12–18) from adult, 18-month old F0 (developmentally exposed) or 12-month old F1 (unexposed) zebrafish treated with increasing concentrations of CBD and THC. For time in periphery data, statistical analysis was performed using a one-way ANOVA for every group other than the F1 THC where a t-test was utilized (p ≤ 0.05) For freezing duration, statistical analysis was performed using Kruskal-Wallis test (p ≤ 0.05) though no significance was found (Carty et al., 2019).
3.3 Learning

A T-maze was utilized to assess the learning and memory of 30-month old F0 zebrafish by analyzing two variables: total path length and latency (time to get to the food ring and staying in the positive reservoir for 20 seconds). The control fish did not exhibit a pattern of learning for latency, as there was no significant trend in decreased latency as the trial went on. There were no other significant differences exhibited by the experimental groups for latency in the dosed fish. Additionally, there was no significant trend of learning in relation to path length in the control fish. However, by day 5, the control group’s path length was lower than all of the experimentally dosed groups, however, this was not a significant decrease These results were found using a one-way ANOVA (p > 0.05, Figure 8). The group of fish exposed to the highest concentration of THC also had a decrease in path length and latency, though it was not significant (p > 0.05). In addition, the percentage improvement was calculated for both path length and latency. Using an ANOVA to analyze the data, an overall decrease in success was observed in each of the treatments (p > 0.05, Figure 9).
Figure 8: Summary of learning and data (mean ± SEM) collected from a 10 min trial (n=3-7) from adult, 30-month old F0 (developmentally exposed) zebrafish treated with increasing concentrations of CBD and THC. A one-way ANOVA was used to analyze the data and no significance was found (p > 0.05).
Figure 9: Learning improvement data (mean ± SEM) collected from a 10 min trial (n=3-7) from adult, 30-month old F0 (developmentally exposed) zebrafish treated with increasing concentrations of CBD and THC. A one-way ANOVA was performed, although no significance was found (p > 0.05). The results from day five were normalized to day one of the trials for each treatment, respectively.
4. DISCUSSION

Prior to this study, concentrations of THC higher than the 0.6 mg/L that was used in this study were found to cause differential expression of genes in addition to developmental malformations and behavioral effects in zebrafish larvae. CBD also caused related effects at concentrations seven times lower than that of the THC that was tested (Carty et al., 2018). It is important to keep in mind that, in conjunction with the results found in this study, differential neurodevelopmental gene expression and larval behavior differences were both found (Carty et al., 2019). Based on these implications, the aims of this study were two-fold: to explore the effects of lower concentrations of THC and CBD in a developmental exposure and to assess the multigenerational reproductive, behavioral, and learning and memory consequences of the exposure. To do so, the concentrations of THC and CBD that were utilized did not cause dysmorphologies (Carty et al., 2019). In addition to the reproductive assay, the Open Field Test, and the T-maze, levels of gene expression for c-fos, bdnf, and dazl were assessed. In short, there were differential levels of gene expression found coinciding with the concentrations and reproductive and behavioral outcomes (Carty et al., 2019). We hypothesized that THC would have greater reproductive and developmental toxicity than CBD.

In the reproductive assessment, the F0 fish exposed to THC or CBD demonstrated decreased fertilized egg production. However, the F1 fish did not have any reproductive abnormalities. This could warrant further research into how THC and CBD alter the process of gamete formation. Since the F0 reproductive assessment was conducted at 6 mpf and the F1 reproductive assessment was conducted at 11 mpf, it would be beneficial
going forward to conduct an assessment when both generations are at the same age. Babies born to mothers who are cannabis users have reportedly lower birth weights than those who are not exposed to THC or CBD (Gunn et al., 2016). To add to this, low birth weight is associated with: increased morbidity and mortality, increased psychopathology, and decreased intellect (Gunn et al., 2016). Babies of mothers who use marijuana have also been found to have more visits to the NICU and ICU as well as increased preterm births (Gunn et al., 2016). Marijuana is known to exacerbate negative effects on male reproductive health. In particular, levels of anandamide (AEA) and 2-arachidonoylglycerol (2-AG) were lower in infertile sperm. Additionally, vanilloid (TRPV1) receptor binding was lower in sperm that was infertile. AEA and 2-AG exert effects through the ECS, particularly through CB1-R, CB2-R, and TRPV1 (Lewis et al., 2012). These results are relevant to consider for couples that use marijuana and are also trying to conceive a child. Further study is needed to investigate the dangers of this and the mechanisms underlying the decreased fertility.

An Open Field Test was employed to explore the locomotion and anxiety behavior of adult zebrafish. CBD has dose-dependent anxiety-reducing effects and has been used clinically in order to treat anxiety (Crippa et al., 2018). Thigmotaxis, the anxious tendency to swim near the outer periphery of an environment, was reduced in zebrafish exposed to anxiolytic drugs (Baiamonte et al., 2016). Under anxious circumstances, both thigmotaxis and freezing behavior increased in zebrafish (Stewart et al, 2014). In the F0 fish exposed to THC or CBD in this study, there was a dose-dependent increase in freezing duration. Also, thigmotaxis was decreased in F1 individuals that were bred from fish exposed to 0.6 mg/L of THC. This result shows that
the exposure had anxiolytic effects on this group of fish since they spent less time in the periphery. Because freezing is indicative of anxiety, these F0 fish must have experienced increased anxiety. These contradictory results suggest a complex relationship between cannabinoid exposure and anxiolytic effects and time of exposure. The clear trend in the data of the F1 fish illustrates results that are the opposite compared to the exposed F0 fish. One possible explanation for these results is that the Open Field Test was conducted at 18 mpf for F0 fish and 12 mpf for F1 fish. However, this could also highlight the multigenerational effects of the developmental exposure to THC and CBD. The multigenerational side to this could be due to epigenetic effects, however more research needs to be conducted. Despite these complex behavioral outcomes, what is clear is that cannabinoid exposure led to behavioral changes in exposed fish and their offspring. This provides support for the gene expression portion of the experiment that was conducted prior to these tests. The genes \textit{c-fos} and \textit{bdnf} are differentially expressed in the F0 fish and have cognitive ramifications on the subsequent generation. We propose that it would be prudent to measure stress hormones in conjunction with performing these tests to deduce whether there is a cognitive or anxious origin of the modified behavioral outcomes (Carty \textit{et al.}, 2019).

The understanding that cannabinoids can have multigenerational effects is important going forward in regulating the CBD and THC use by pregnant women. Because cannabinoid exposure led to behavioral changes in dosed fish and their offspring, this could suggest possible epigenetic effects. The F1 fish in the study that had parents exposed to THC was the only group to exhibit differential thigmotaxis. This suggests an epigenetic effect in response to THC. To add to this, there were 1,027
differentially methylated regions in an F1 rodent model when their parents, the F0 generation, were exposed to THC during adolescence (Watson et al., 2015). However, there is not enough research on the epigenetic ramifications of marijuana exposure (Szutorisz & Hurd, 2016). In the future, it will be of use to investigate the possibility of epigenetic effects of CBD and to delve deeper into THC-mediated epigenetic effects.

The T-maze was used in this study to explore the effects of developmental exposure to THC and CBD on learning and memory. Rodent models have been historically known for exhibiting learning in mazes (Thinus-Blanc, 1996). Learning has previously been evoked in zebrafish using a plus-maze with a food reward (Sison & Gerlai, 2010). In this study, the fish showed learning through exploration patterns and frequency of target arm visits. Sison & Gerlai (2010) suggest that vertebrates could possibly share complex maze-learning abilities. Another study showed that zebrafish did not exhibit learning in a T-maze but did increase their success when a food reward was paired with a color preference test (Kim, et al., 2017). Bault et al. (2015) showed that directional and color preferences can confound T-maze results. A T-maze was shown to evoke learning in an experiment using zebrafish in a study done by Echevarria, et al. (2016). Another T-maze experiment revealed learning deficits in zebrafish developmentally exposed to BPA (Saili et al., 2012).

Developing a working T-maze protocol was a lengthy process because there is no standardized protocol for testing learning and memory in zebrafish. Our work identified key variables that must be considered in setting up the assays. These include maze size, habituation, choice of positive reinforcement, sample size, and sex. Using the second, smaller maze and n = 3-7 fish, we did not observe learning across in the subjects. A
repeated measures of the controls could not be performed due to the low number of fish tested. However, it is predicted that this would show no learning occurred. This could be due to their advanced age of 30 mpf or the setup of the T-maze.

The main outcome from using the third version of the maze was the differential path length on the fifth day of testing. There was an insignificant decrease in path length in the unexposed control fish compared to the exposed fish. If the controls had demonstrated that they had learned, this could have indicated some level of increased learning or cognitive activity in the zebrafish that were not developmentally exposed to cannabinoids. However, there were no overall learning outcomes for this group of unexposed fish because the path length did not significantly lower over the course of the five days. Additionally, there was an overall decrease in Percentage Improvement in learning across the treatments in path length and latency. This means that the fish scored worse as the trials progressed. This could be due to design flaws or the advanced age of the fish. These tests are ongoing and a larger sample size will be obtained in order to provide more reliable evidence of any alterations to learning and memory. In addition, concordant testing in younger fish is needed in order to properly deduce the effects of age on learning and memory. These results could be related to the finding that an up-regulated expression of bdnf in mice is positively correlated with learning and memory impairment (Cunha et al., 2009). There was an up-regulation in bdnf gene expression during different stages of early development of the exposed F0 fish used in this experiment (Carty et al., 2019). Thus, more experiments could be conducted in order to explore this relationship and its implications. Additional modifications could be made to this version of the T-maze, such as taking the fish out of the maze right when it crosses
into the positive reservoir rather than waiting 20 seconds. Using younger fish could also be applicable because learning is impaired in older fish. In a perfect experiment, these alterations would produce results that show significant increases in Percentage Improvement in learning.

This study is important because it is the first multigenerational, developmental exposure study in zebrafish using THC and CBD. The results are relevant on the grounds of the increased clinical use of cannabinoids, particularly to treat drug-resistant childhood epilepsy. The most significant results of this study were: a decrease in fertilized egg production in F0 fish, a dose-dependent increase in freezing duration in F0 fish, and alterations in F1 behavior in response to THC. Rodent models have indicated that adolescent exposure to marijuana led to epigenetic changes in their genome (Watson et al., 2016). A developmental exposure in rats could add value to the results of this study. It is not trivial to do behavior in just any animal model, so exploring the effects in a variety of organisms will be beneficial going forward. In addition, it has been revealed that simultaneous exposure to THC and CBD has different results than an exclusive exposure to either compound (Todd, et al., 2017). Thus, a developmental exposure to both THC and CBD at once is needed. Our hypothesis that THC would have greater developmental toxicity than CBD was disproved in that CBD had more definite adult behavioral differences in the F0 generation. Accordingly, there were also greater differences in gene expression (Carty et al., 2019). It is important to understand that THC also had concrete toxicological effects as well. One study found that about 20% of pregnant women under the age of 24 tested positive for marijuana use (Young-Wolff et al., 2017). Altogether, the outcomes of this study should be considered for those who are
pregnant and using THC and CBD without quality control. Further investigation into the mechanisms by which cannabinoids function and the hazards of developmental exposure is necessary.
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leads to early life-stage hyperactivity and learning deficits in adult zebrafish.”


