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The Role of Cannabinoid Receptor 2 in Zebrafish Toxicities Following Developmental Exposure to THC or CBD

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THE ROLE OF CANNABINOID RECEPTOR 2 IN ZEBRAFISH TOXICITIES FOLLOWING DEVELOPMENTAL EXPOSURE TO THC OR CBD

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ABSTRACT

As the laws concerning the regulation of *Cannabis Sativa* change across the globe, maternal use of cannabinoid-containing products during pregnancy is a greater concern than ever before. Little research has been conducted on the consequences of maternal use and developmental exposure to Δ9-tetrahydrocannabinol (THC) and cannabidiol (CBD. In this study, larval zebrafish (*Danio rerio*) were used to measure CBD and THC-mediated adverse outcomes including effects on larval length, eye area, and behavior in the *cnr*$^{+/+}$ and c*nr2*$^{-/-}$ zebrafish strains where we discovered that cannabinoid exposure results in a decrease in each of these endpoints. Adult zebrafish were used to further examine the effects that receptor knockout has on behavior which also resulted in decreased behavior. This study found that developmental exposure to THC and CBD did result in toxicities, however, toxicities were lessened in zebrafish that lacked cannabinoid receptor 2 suggesting that this receptor has an important role in modulating the effects of cannabinoid exposure and is possibly involved in protective pathways.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

CBD	Cannabidiol
CB2-KO	Cnr2 knockout
Cnr1	Cannabinoid Receptor 1
Cnr2	Cannabinoid Receptor 2
Hpf	Hours Post Fertilization
THC	Δ^9-tetrahydrocannabinol

I. INTRODUCTION

1.1 Cannabinoids as Therapeutics

The most commonly used illicit drug in the United States, marijuana, is derived from the *Cannabis sativa* plant, but it is also known for its many medicinal indications (Ahmed et al., 2018). *Cannabis sativa* is a complex plant with over four hundred chemical components where more than sixty of these are cannabinoid compounds (Atakan 2012). Cannabinoids are lipophilic ligands that are found in all cannabis plant preparations with the best characterized being the psychoactive compound Δ9-tetrahydrocannabinol (THC) and the non-psychotropic cannabidiol (CBD). Marijuana on the market currently typically has a THC composition from 12 – 30% on average while the range for CBD is from 1 – 20% (Royal Queen Seeds 2020).

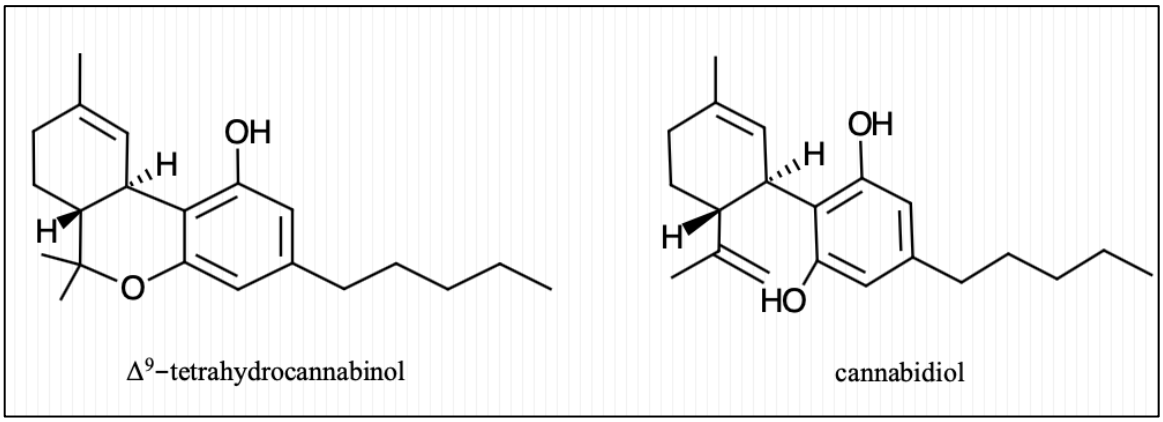

Figure 1: Structures of THC and CBD
Made in ChemDoodle 11.3

The actions of *Cannabis sativa* are considered to be primarily transduced by two transmembrane G-protein-coupled receptors known as cannabinoid receptor 1 (CB1R in humans, Cnr1 in fish) and cannabinoid receptor 2 (CB2R in humans, Cnr2 in fish) along with the subsequent secondary-messenger gene transcription changes. Although Cnr1 and Cnr2 are the most widely-acknowledged and characterized cannabinoid receptors, several other receptors from G-protein-coupled receptors to ion channels and nuclear receptors also interact with cannabinoids (Kano et al., 2009a). One important difference to note for this study revolves around the previously mentioned cannabinoid's affinities for binding to Cnr1 and Cnr2. THC has a high affinity for Cnr1 and Cnr2 with low K_i values in the nanomolar range. CBD's affinity, however, is much lower for both receptors, but it can interact with the cannabinoid receptors at low concentrations. CBD is also a weak Cnr1 antagonist and Cnr2 inverse agonist (Maccarrone et al., 2015). In humans, CB1 and CB2 proteins are encoded by the genes CB1 and CB2, respectively. However, throughout this paper, I will be using the zebrafish nomenclature Cnr1 and Cnr2 to represent the cannabinoid receptor proteins and *cnr1* and *cnr2* for the receptor genes.

Cnr1 is encoded by the gene *cnr1* and is the most prominent cannabinoid receptor subtype in the central nervous system, with the cnr1 receptor appearing first in the preoptic area at 24 hpf (Lam et al., 2006). Cnr1 expression is highest in the olfactory bulb, hippocampus, basal ganglia, and cerebellum regions of the CNS (Mackie, 2005). As a result of this expression, Cnr1's role in the endocannabinoid system revolves largely around regulating brain functions such as mood, anxiety, appetite, memory consolidation, and the control of locomotor activity (Gong et al., 2006). For example, Cnr1 modulates the mobility of the gastrointestinal tract and can control appetite from the hypothalamus

in the CNS and can regulate energy balance and metabolism through food intake from the gastrointestinal tract as well. Although it is predominately found in brain tissue, Cnr1 is also in adipose tissue, skeletal muscle, bone, skin, eye, and reproductive tissue (Maccarrone et al., 2015) .

Cannabinoid receptor 2 was discovered in the macrophages of the human spleen three years after Cnr1 and is encoded by the gene *cnr2* in zebrafish (Munro et al., 1993). *cnr2* is predominately expressed in immune cells and was found have transcript levels that were over 10-fold higher than *cnr1* transcript levels in leukocytes (Krug and Clark, 2015). Along with its role in immunity, Cnr2 has moderate expression in other peripheral tissues including the cardiovascular system, GI tract, liver, adipose tissue, bone, and reproductive tissues (Gong et al., 2006). It was not, however, observed in a high extent in the brain when compared to its expression in immune cells or Cnr1's presence in the brain (Atwood and MacKie, 2010). Although Cnr2's expression in the central and peripheral nervous systems is limited, Cnr2 plays a less understood role in neurological activities such as nociception, drug addiction, and neuroinflammation (Pacher et al., 2006).

The endocannabinoid system is composed of the endogenous cannabinoid receptors such as Cnr1 and Cnr2, their endogenous lipid ligands, and the enzymes responsible for their biosynthesis and degradation. This system is widely distributed throughout the brain and body and is responsible for numerous significant regulatory functions in the neuronal, vascular, metabolic, immune, and reproductive systems (Pertwee et al., 2010). Most studies of the endocannabinoid system focus on the endogenous agonist N-arachidonoylethanolamine (AEA) and 2-arachidonoylglycerol (2-

AG) (Pertwee et al., 2010). AEA is a high-affinity partial agonist of Cnr1 and almost inactive at Cnr2 while 2-AG acts as a full agonist at both Cnr1 and Cnr2 with moderate to low affinity (di Marzo and de Petrocellis, 2012). Both endocannabinoids are produced on demand in response to increased intracellular Ca^{2+} concentration (Kano et al., 2009);; however, the basal level of 2-AG is approximately 1000 times higher than AEA in the brain, thus it is proposed that 2-AG is the primary endogenous ligand for cannabinoid receptors in the central nervous system (di Marzo and de Petrocellis, 2012). After it was discovered that the endocannabinoid system is involved in short and long-term depression at both excitatory and inhibitory synapses, the endocannabinoid system became the most studied retrograde signaling system in the brain (Ahmed et al., 2018).

1.2 Cnr2 and Cnr2-KO

cnr2 is located on chromosome 4 in mice and 1p36 in humans and is minimally divergent between species with sequence identity being 82% between human and mouse (Munro et al., 1993). When comparing the zebrafish genome to the human reference genome, approximately 70% of human genes have at least one obvious zebrafish orthologue (Howe 2013). Similar to Cnr1, Cnr2 is a member of the G-protein coupled receptor family of proteins and mediates its action via inhibition of adenylate cyclases (Ibsen et al., 2017). The receptor does this through initiation of signaling cascades such as the phospholipase C and inositol 1, 4, 5-triphosphate signaling pathways that both serve to increase the levels of intracellular calcium (Zoratti et al., 2003). Upon cannabinoid receptor interaction with its ligand, the G protein exchanges the inactive guanine in the GDP nucleotide for an active GTP form. The heterotrimeric G-protein dissociates into an α and βγ subunit where the α subunit inhibits adenylate cyclase

through binding which decreases second messenger cAMP levels downstream (Howlett and Mukhopadhyay, 2000). This decrease in cAMP production is a mode by which Cnr2 signaling in response to endocannabinoids helps to maintain immunological homeostasis while in response to exogenous cannabinoids results in an immunosuppressive effect (Cabral et al., 2008).

Exogenous cannabinoids decrease host resistance to a variety of infectious agents. This is because, as previously mentioned, Cnr2 is predominately distributed in cells and tissues of the immune system especially concentrated in the thymus, tonsils, B lymphocytes, T lymphocytes, macrophages, monocytes, and natural killer cells (Schatz et al., 1997). Cnr2 is expressed in highest amount in NK cells, macrophages, and T lymphocytes respectively (Núñez et al., 2004). Cnr2 is also expressed in the CNS during states of inflammation (Malfitano et al., 2014). It is for this reason that cannabinoids, as ligands through their receptors, are of interest for therapeutic treatment of hyperinflammatory immune responses in the CNS due to their anti-inflammatory actions (Cabral and Griffin-Thomas, 2009). For example, in mice, Δ9-THC synthetic cannabinoids CP55940 and HU-210 inhibit cell contact-dependent cytolysis of tumor cells that are mediated by macrophages (Burnette-Curley and Cabral, 1995; Coffey et al., 1996) to suppress natural killer cells (Klein et al., 1991). Δ9-THC suppresses the development of B lymphocytes and T lymphocytes in response to mitogens (Burnette-Curley and Cabral, 1995). These two examples show that exogenous cannabinoids inhibit the activity of a variety of immunocytes and that the immune actions of cannabinoids are functionally linked to Cnr2 to exert their effects on immune tissues (Galiegue et al., 1995).

Although Cnr2 is primarily found in immune tissues, evidence suggests that Cnr2 modulates other neuronal functions. For example, activation of Cnr2 reduces pain (Anand et al., 2009), impulsive behavior (Navarrete et al., 2012), and locomotor activity of rodents (Valenzano et al., 2005). Cnr2 polymorphism is also related to schizophrenia (Ishiguro et al., 2010), depression (Onaivi et al., 2008), and bipolar disorder (Minocci et al., 2011). Due to Cnr2's apparent role in many neurological conditions, Cnr2 knockout studies have been particularly useful to further understand the role of Cnr2 in the body outside of immune tissues. For example, chronic activation of Cnr2 increases anxiety in rodents while chronic blockade decreases anxiety (García-Gutiérrez et al., 2013). Cnr2 knockout mice also display a decline in long-term memory and hippocampus-dependent fear memory while hippocampus-independent fear memory was unaffected (Li and Kim, 2016a).

1.3 Cannabinoid Use as a Therapeutic Agent

Plant-based cannabis and cannabinoid agents have been used both medicinally and recreationally since as early as 400 AD (Bridgeman and Abazia, 2017). As previously discussed, the endocannabinoid system is responsible for numerous significant regulatory functions, thus changes in endocannabinoid levels can lead to significant problems. Changes in endocannabinoid levels may be related to neurological diseases such as Parkinson's disease (Krishnan et al., 2009), Huntington's disease (Pazos et al., 2008), and Alzheimer's disease (Shen and Thayer, 1998), as well as irritable bowel syndrome (Wong et al., 2012).

Cannabinoid constituents are widely used to alleviate symptoms or treat disease, but their efficacy is not well established. Cannabinoid involvement in several

physiological processes, however, is well known. This regulatory role includes improving energy balance, appetite stimulation, blood pressure, pain modulation, embryogenesis, nausea and vomiting control, memory, learning and immune response as well as in pathological conditions where it exerts a protective role in the development of certain disorders (reviewed in: Fraguas-Sánchez et al., 2018).

Because CBD is one of the cannabinoids with non-psychotropic action and antiseizure efficacy, it has been popularized for its use as a therapeutic to reduce epileptic seizures (Carty et al., 2019). Epilepsy affects more than fifty million people worldwide and can cause cognitive and sensorimotor deficits which greatly compromise the quality of life and increase the risk for premature death (Gloss and Vickrey, 2014; Sabaz et al., 2003). Unfortunately, more than thirty percent of epileptic patients progress to drug-resistant epilepsy which is defined as failure to achieve seizure freedom after trying two anti-epileptic drugs (AEDs) (Sheng et al., 2017) . In order to combat this issue, the Food and Drug Administration (FDA) and Drug Enforcement Agency (DEA) brought to market the first and only approved prescription cannabidiol called Epidiolex (Sheng et al., 2017).

1.4 Effects of Developmental Cannabinoid Exposure

Our investigation is highly relevant in today's society where over the last decade marijuana use by pregnant women has increased by approximately sixty-five percent in the United States particularly among those of low socioeconomic status. Metadata analysis indicates that marijuana use in pregnancy prior to or during pregnancy could contribute to dangerous pregnancy complications such as low fetal birth weight, preterm delivery, and congenital malformation and neonatal complications (Neradugomma et al.,

2018). In a study done on nonusers and users during pregnancy, the mRNA levels of CNR1 and CNR2 were higher in the endometrium sample tissue taken from women who used marijuana during pregnancy than nonusers, and the CNR2 protein levels were higher in the endometrium as well. A successful reciprocal interaction is required between a competent embryo and receptive endometrium for a pregnancy to be successful, and marijuana use seems to compromise this by altering receptor expression in the endometrium (American College of Obstetricians and Gynecologists Committee on Obstetric Practice, 2015) .

Receptors for cannabinoids can be detected in rodent animal models from the earliest stages of development (Schneider, 2009). In zebrafish, both receptors are expressed from as early as one hpf and into adulthood (Saint-Amant and Drapeau, 1998). These play an essential role in neuronal development; thus, it is increasingly important that the behavioral effects of cannabinoid administration both before and during pregnancy is understood. They do this by influencing the expression of neuron-glia cell adhesion molecules which shows cannabinoid's influence on cell proliferation, neuronal migration, and axon elongation (Fernández-ruiz et al., 2004) .

Understanding the effect of cannabinoid administration before and during pregnancy is crucial as CBD has been proven to be an anti-emetic due to its ability reduce vomiting in the Asian house shrew *S. marinus*; however, this mechanism was not mediated through CB1 or CB2 (Parker et al., 2004). Because of CBD's ability to reduce vomiting, concerns have arisen over cannabinoid use as a treatment for nausea during pregnancy especially considering that cannabinoids are capable of freely crossing the placenta and may pose a significant risk to embryonic development (Neradugomma et al.,

2018). Previous zebrafish studies have shown a correlation between embryonic exposure to THC and irregular CNS development, but less is known about CBD's impact on development (Richardson et al., 2017).

1.5 Zebrafish as a Human Model for Disease

Although mammals have more commonly been used to model human diseases, the zebrafish (*Danio rerio*) is rapidly becoming a new popular model organism in biomedical research. The zebrafish model was chosen because it is a vertebrate species with high physiological and genetic homology to humans. Comparison of the zebrafish genome to the human reference genome shows that approximately 70% of human genes have at least one zebrafish homolog (Richardson et al., 2017). Meanwhile, 84% of known human disease-causing genes having a zebrafish counterpart (Ahmed et al., 2018).

The zebrafish model allows for easy genetic manipulation because they have a similar central nervous system (CNS) morphology. Zebrafish embryos also offer a distinct advantage over mammalian models for toxicity and exposure studies as they develop externally in a chorion casing which is advantageous as researchers can visualize and study the different developmental stages starting from the earliest stages of embryogenesis. Along with this, researchers can dose during embryo development (Teame et al., 2019). Zebrafish are also advantageous due to their high fecundity, numerous strains, short generation time, and rapid embryonic development as they form complete organ systems within forty-eight hours of fertilization (Kalueff et al., 2014) .

Some complex brain disorders that have been modeled by zebrafish are depression, autism, psychoses, drug abuse, and cognitive deficits (Souza Anselmo et al., 2017). Zebrafish are also an appropriate model to use to study metabolic dysfunctions as

they contain all organs involved in energy homeostasis and metabolism including appetite and insulin regulation along with a lipid storage system all of which is conserved with that found in humans (Li and Kim, 2016b).

1.6 Study Goals and Hypotheses

We hypothesized that the cannabinoid receptors would play a significant mechanistic role in THC's, but not CBD's, acute toxic effects. The goals of our study were:

1. Evaluate morphological differences such as mortality, larval eye area, and larval length between *cnr*$^{+/+}$ and *cnr2*$^{-/-}$ zebrafish when exposed to CBD or THC.
2. Evaluate the effect of cannabinoid receptors on larval behavior.
3. Evaluate the differences in behavior between *fli, 5D, cnr1*$^{-/-}$*, and cnr2*$^{-/-}$ seven-month-old adult zebrafish.

II. MATERIALS AND METHODS

2.1 Zebrafish Husbandry and Exposures

Tg(*fli1:egfp*), *5D*, *cnr1*$^{-/-}$ [*Tu*(*cnr1*$^{zf679/zf679}$)], *cnr2*$^{-/-}$ *[Tu*(*cnr2*$^{zf680/zf680}$)] zebrafish strains were used in this study. All fish were raised and all experiments were conducted in accordance with the approved Institutional Animal Care and Use Committee (IACUC) guidelines and recommendations. Healthy adult fish were maintained in Aquatic Habitats Zebrafish Flow-through System (Aquatic Habitats, Apopka, Florida) under ambient conditions (pH 7.5–8.0, dissolved oxygen 7.2–7.8 mg/L, conductivity 730–770 mS, and temperature 27°C–29°C). Sexually mature and healthy fish without any signs of deformities or disease were selected as breeders.

The *Tg*(*fli1:egfp*) strain was obtained through the Zebrafish International Resource Center (ZFIN, Eugene, OR) and represents *cnr*$^{+/+}$ which was used as a wild type control. We have previously used this strain to assess the impact of developmental exposure to both CBD and THC (Carty et al. 2018; Carty et al. 2019; Pandelides, Thornton, Faruque, et al. 2020; Pandelides, Thornton, Lovitt, et al. 2020). Wild-type 5D zebrafish were kindly provided by Dr. Robyn Tanguay at Oregon State University and raised under the approved IACUC protocol (18-022). The cannabinoid receptor 1 and 2 mutant strains (*cnr1*$^{+/+}$ and *cnr2*$^{-/-}$) was provided by Dr. Wolfram Goessling (Genetics Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA).

This strain was genotyped prior to producing embryos used in experiments. For egg collection and larval exposures, embryos were obtained by transferring adult into tanks with a 1:1 ratio of males to females the night prior to collection. The next morning, eggs that fell through the protective grate at the bottom of the breeding tank were collected and debris were removed by pouring the water from the tanks through a sieve. The embryos were then randomly sorted into scintillation vials with ten embryos per vial and stored in the 28°C incubator. These vials contained embryo water with 1 embryo per 0.6 mL of embryo water (sterilized deionized water; pH of 7.4–7.7; 60 ppm Instant Ocean, Cincinnati, Ohio). These embryos were exposed under static conditions from 6 hours post-fertilization (hpf) - 96 hpf. Every 24h, embryos in the exposed vials were screened for any developmental defects, and debris such as mortality or sloughed chorions were removed.

2.2 Determining the developmental toxicity to THC and CBD exposure in cnr mutants:

Zebrafish embryos from the *cnr*$^{+/+}$and *cnr2*$^{-/-}$ strains were exposed to 2, 4, 8, 9.5, and 12 μM (0.65, 1.25, 2.4, 3, 3.75 mg/L) THC or 0.25, 0.5, 1, 2, and 4 μM (0.075, 0.15, 0.3, 0.6, 1.2 mg/L) CBD beginning at 6 hpf. A 0.05% DMSO (control) group was included as a carrier control. Exposures were continued under static conditions until 96 hpf. Each treatment consisted of 5 biological replicates with 10 embryos per replicate.

After behavioral assessments, photographs were taken of all surviving larval fish (50 larval fish per treatment, 10 per replicate, n =5 replicates) per treatment group to assess developmental deformities. Larvae were anesthetized in tricaine methanesulfonate (300 mg/L MS-222) buffered with 600 mg/L sodium bicarbonate. They were immediately placed on a microscope slide with a chamber containing 3% methyl

cellulose and a lateral image was captured with a MicroFire® camera (Optronics, Goleta, CA) attached to a Zeiss Stemi 2000-C Stereo Microscope (Jena, Germany) using Picture Frame™ Application 2.3 software (Optronics, Goleta, CA). The phenotypes were scored blindly using ImageJ software (Schneider et al., 2012). Total body length, diameter and area of the eye, presence or absence of developmental abnormalities (yolk sac edema, and pericardial edema) were recorded by three double-blinded reviewers.

2.3 Larval Behavior Analysis

Larval behavior was measured through the larval photomotor response test using a ViewPoint ZebraBox (ViewPoint, Montreal, Canada). This was done by transferring larvae into 96-well plates at the end of the exposure (96 hpf) where they were acclimated to the light and temperature of the room containing the ZebraBox for five minutes. The locomotor assay conditions were comprised of ten minutes under 100% light followed by ten minutes in the dark (0% light), and finishing with ten minutes back in the 100% light condition (Kirla et al. 2016). The endpoints were measured over 2-minute intervals and the total distance traveled during the light and dark phases per larvae was measured and analyzed. Any larvae that were unable to swim due to malformations were excluded from behavioral analysis.

2.4 Adult Behavior Analysis

To determine the role cannabinoid receptor 1 and 2 play in adult behavior, ten adult zebrafish that had been raised in clean conditions since 96 hpf were removed for each of the four strains being examined in this study (*fli*, *5D*, *cnr1*$^{-/-}$, and *cnr2*$^{-/-}$). These zebrafish were removed the morning of the behavioral analysis and each housing tank was separated into two tanks containing 5 males and 5 females in duplicate (10 males and

10 females per strain total). They were left to acclimate for approximately four hours in order to adjust to being in tanks with the same sex only. These tanks were then moved into a room with ambient lighting and temperature and were left to adjust for five minutes. Adult zebrafish were then removed one at a time and placed in an open field that is a well-established paradigm for assaying anxiety-related behaviors (Godwin et al 2012). Their open field behavior was measured for six minutes and then the fish were removed and recombined into their original housing tanks. The analysis used was EthoVision that tracked the total distance, time spent in center, and time spent in the periphery.

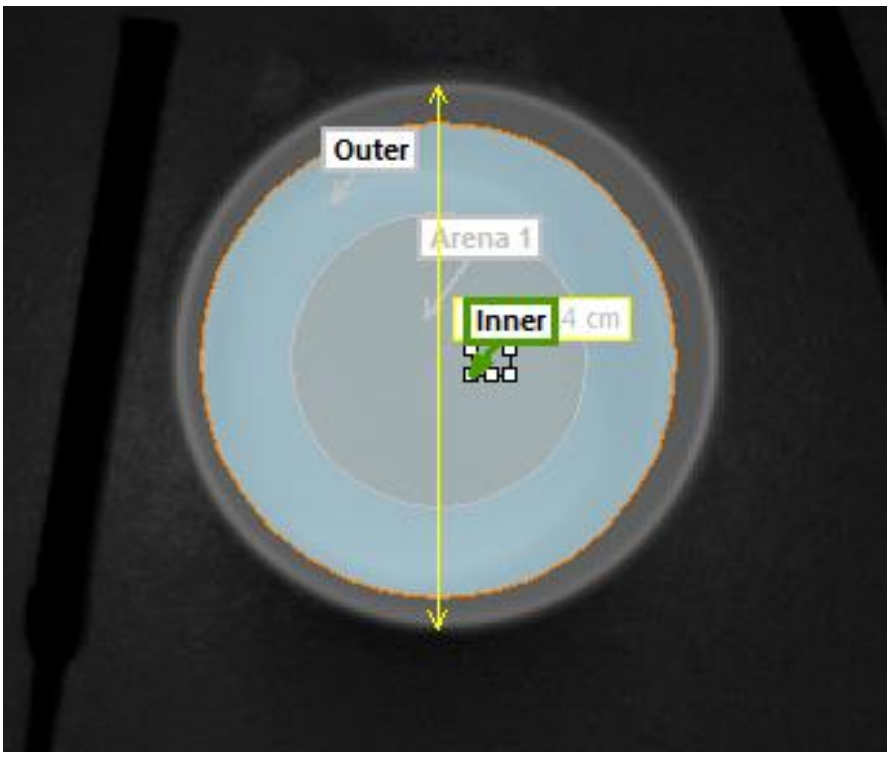

Figure 2: Two Zones of Behavior in the Open Field Test**.** For analysis, the swim arena was divided into two regions- periphery (outer 50% of the arena) and center (inner 50% of the arena). The diameter was 23 cm.

2.5 Statistical Analysis

For mortality percentages, the percentage calculated for mortality was transformed into probit values and the dosage concentrations were plotted in log units. LC50s were calculated from a linear regression performed on the data points.

All data were assessed for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. Larval length and eye area were recorded per fish and then averaged per replicate ($n = 5$). Differences in treatment within each strain were assessed by ANOVA, Dunnett's posthoc, ($p \leq 0.05$), in order to assess the difference between treated and the untreated control fish.

Statistical analysis was conducted on the total distance travelled during the light and dark phases separately. Within each strain, differences in concentration were assed (ANOVA, Tukey's posthoc, $p \leq 0.05$). The differences in strain were assessed by performing pairwise comparisons between the two strains (ANOVA, Tukey's posthoc, $p \leq 0.05$). If the data did not meet the assumptions of parametric tests, an ANOVA on Ranks was performed. All graphing and statistical analysis was conducted using Sigmaplot 14.0 software.

Adult behavior was conducted on the total distance traveled and time spent in periphery. The differences in strain were assessed by performing pairwise comparisons between the two strains (ANOVA, Tukey's posthoc, $p \leq 0.05$). If the data did not meet the assumptions of parametric tests, an ANOVA on Ranks was performed. All graphing and statistical analysis was conducted using Sigmaplot 14.0 software

III. RESULTS

3.1 Role of Cnr2 in THC and CBD induced developmental toxicity

When exposed to THC, there was a dose-dependent effect on mortality in both the *cnr*$^{+/+}$ and *cnr2*$^{-/-}$ strains. In contrast, CBD exposure did have a dose-dependent effect and a significant increase in mortality was only seen at the highest concentration of 4 μM where mortality was 100% and 90 ± 4.47 % in the *cnr*$^{+/+}$ and *cnr2*$^{-/-}$ strains, respectively (Figure 3).

When control (*cnr*$^{+/+}$) larval zebrafish were exposed to THC, LC_{50} mortality was calculated to be 7.2 μM THC (Figure 4A). In contrast, *cnr2*$^{-/-}$ mutants were more sensitive to THC exposure where the LC_{50} was 4.1 μM THC (Figure 4B).

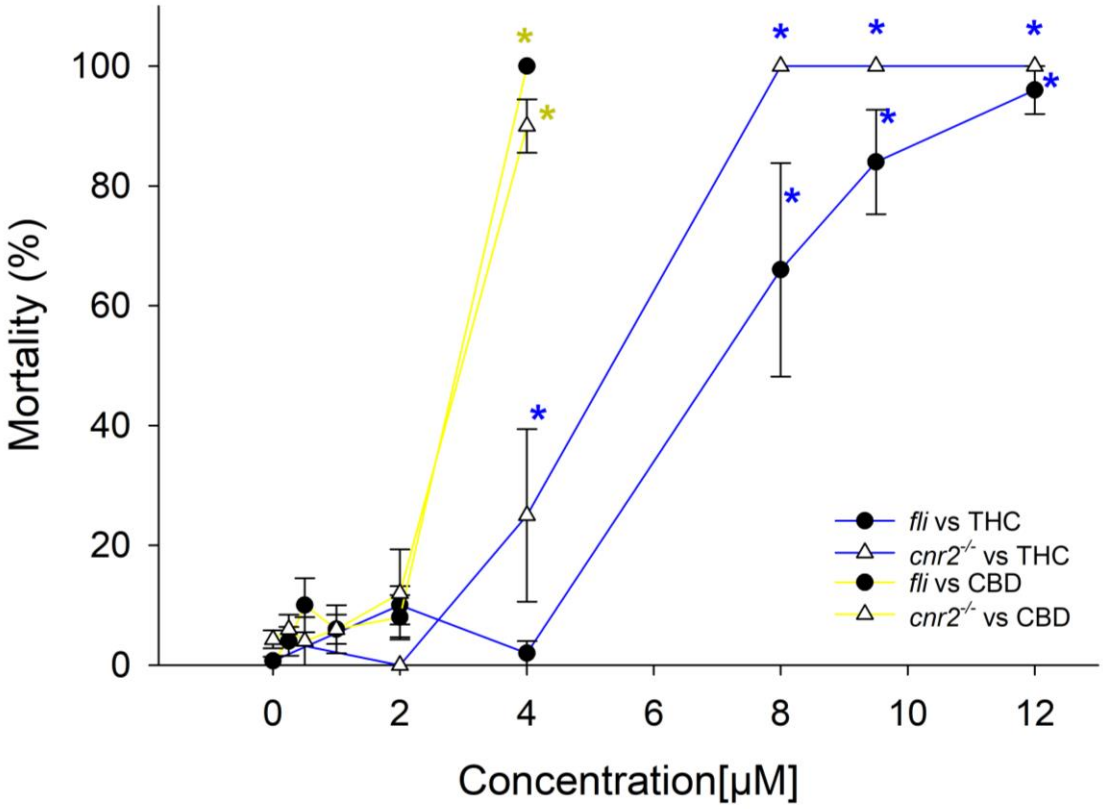

Figure 3: Percent mortality (mean ± SE, n = 5) of 96 hpf larval zebrafish developmentally exposed to THC or CBD. An asterisk indicates a significant difference compared with the within-strain control (ANOVA, Dunnett's posthoc, $p \leq 0.05$).

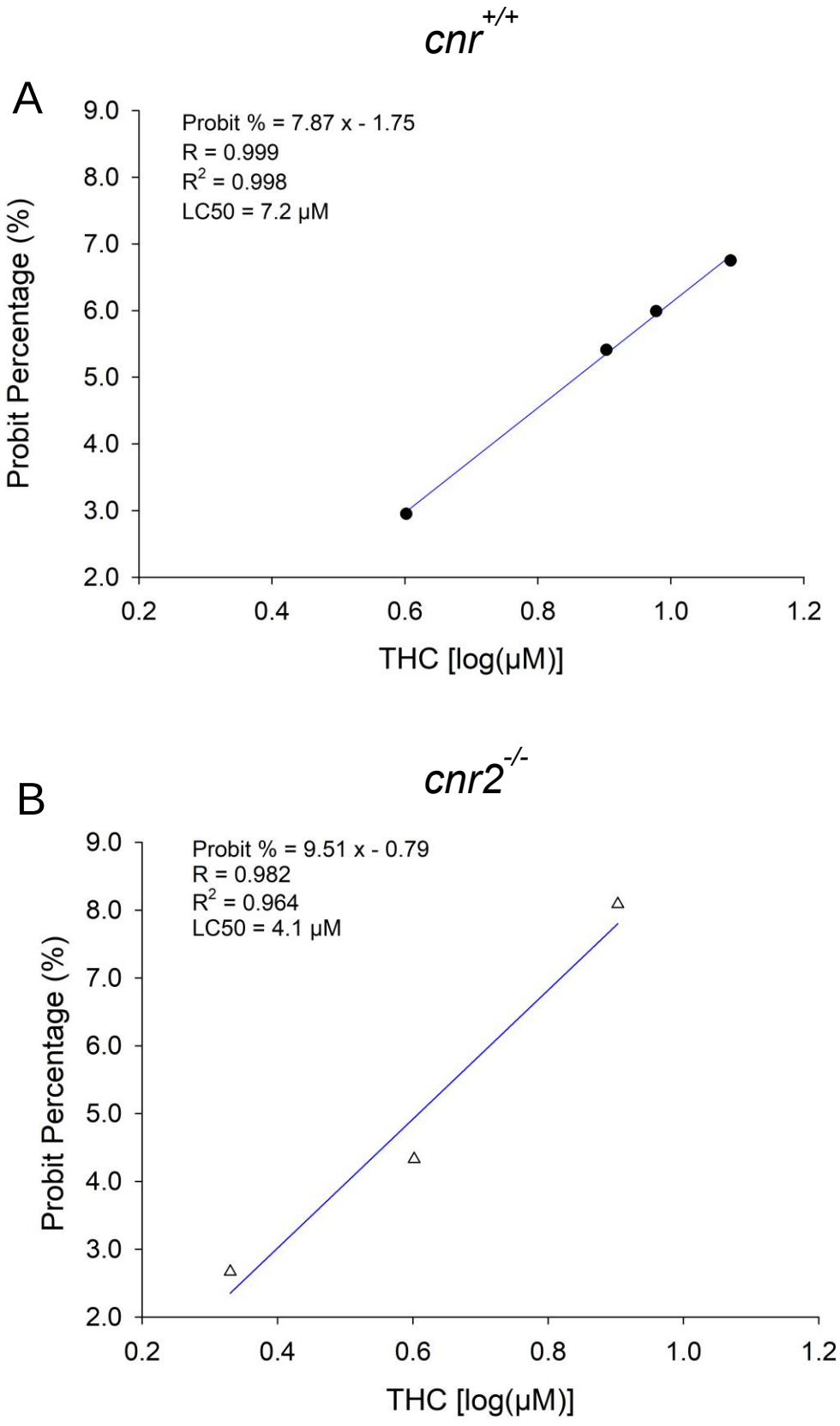

Figure 4: Strain-dependent probit determined LC_{50} comparison of *fli* (A) and *cnr2*$^{-/-}$ (B) both show significantly increased mortality with increased THC concentration.

3.2 Role of *cnr2* in THC and CBD-induced morphological deficits

When $cnr^{+/+}$ larval zebrafish were exposed to THC and CBD, there was a dose-dependent decrease in the length of the fish. Specifically, for THC, there was a significant decline in length as the concentration increased to 9.5 μM (Figure 6A). Similarly, exposure to CBD in the $cnr^{+/+}$ strain resulted in significant decreased larval length starting at 0.5 μM. (Figure 6B).

Exposure to THC in $cnr2^{-/-}$ mutant zebrafish also resulted in a significant decline in length at 4 μM (Figure 6C). When this strain was exposed to CBD, it resulted in a downward trend in length as the dose increased with a significant decrease seen at the highest concentration of CBD (4 μM) (Figure 6D).

Similarly, larval eye area decreased as concentration increased for both THC and CBD in both strains (Figure 7 A-D). One difference between strains was that the $cnr^{+/+}$ wild-type strain had significantly decreased in eye area starting at 0.5 μM (Figure 7B) while $cnr2^{-/-}$ was less affected by CBD exposure as larval eye area did not significantly decrease until 2 μM even though there was a downward trend in length (Figure 7D).

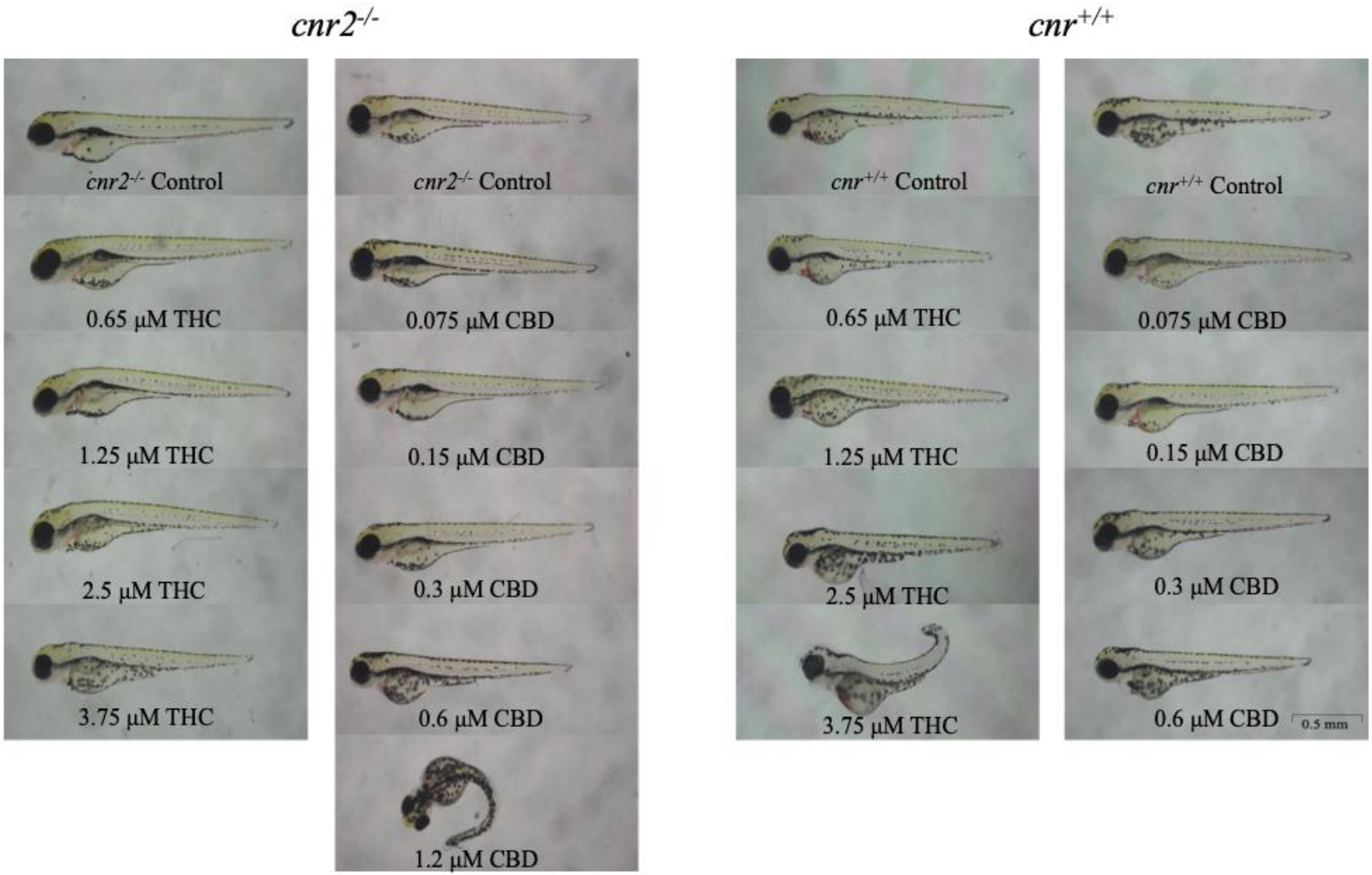

Figure 5: Photos of larval fish taken using a MicroFire® camera using the Picture Frame™ Application 2.3 software. Photos were taken of each of the two strains when they were developmentally exposed to THC and CBD. The phenotypes were scored by three blind reviewers using ImageJ software

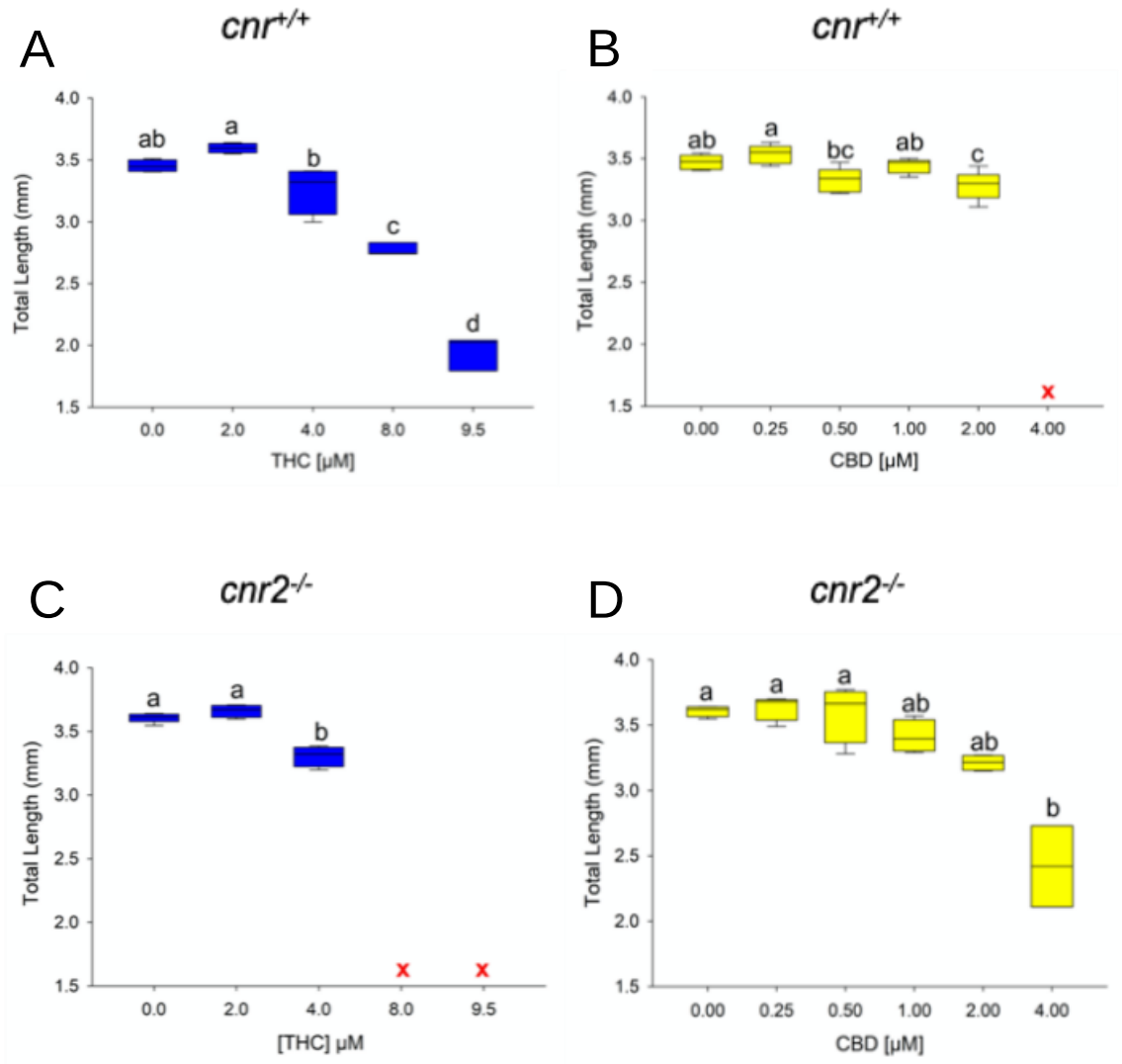

Figure 6: Larval length (mm) of 96 hpf zebrafish that were developmentally exposed to THC and CBD. Different letters indicate a significant distance between groups (ANOVA, Tukey's Posthoc, $p \leq 0.05$). Red X's indicate that there was no survival at that concentration. A box and whisker plot was used where the box represents the interquartile range (IQR), the line within the box represents the median, the area of box above the line represents the upper quartile (75^{th} percentile), the area of the box below the median line represents the lower quartile (25^{th} percentile), and the whiskers represent the remaining quartiles, with any outliers being represented by dots (1.5 x IQR).

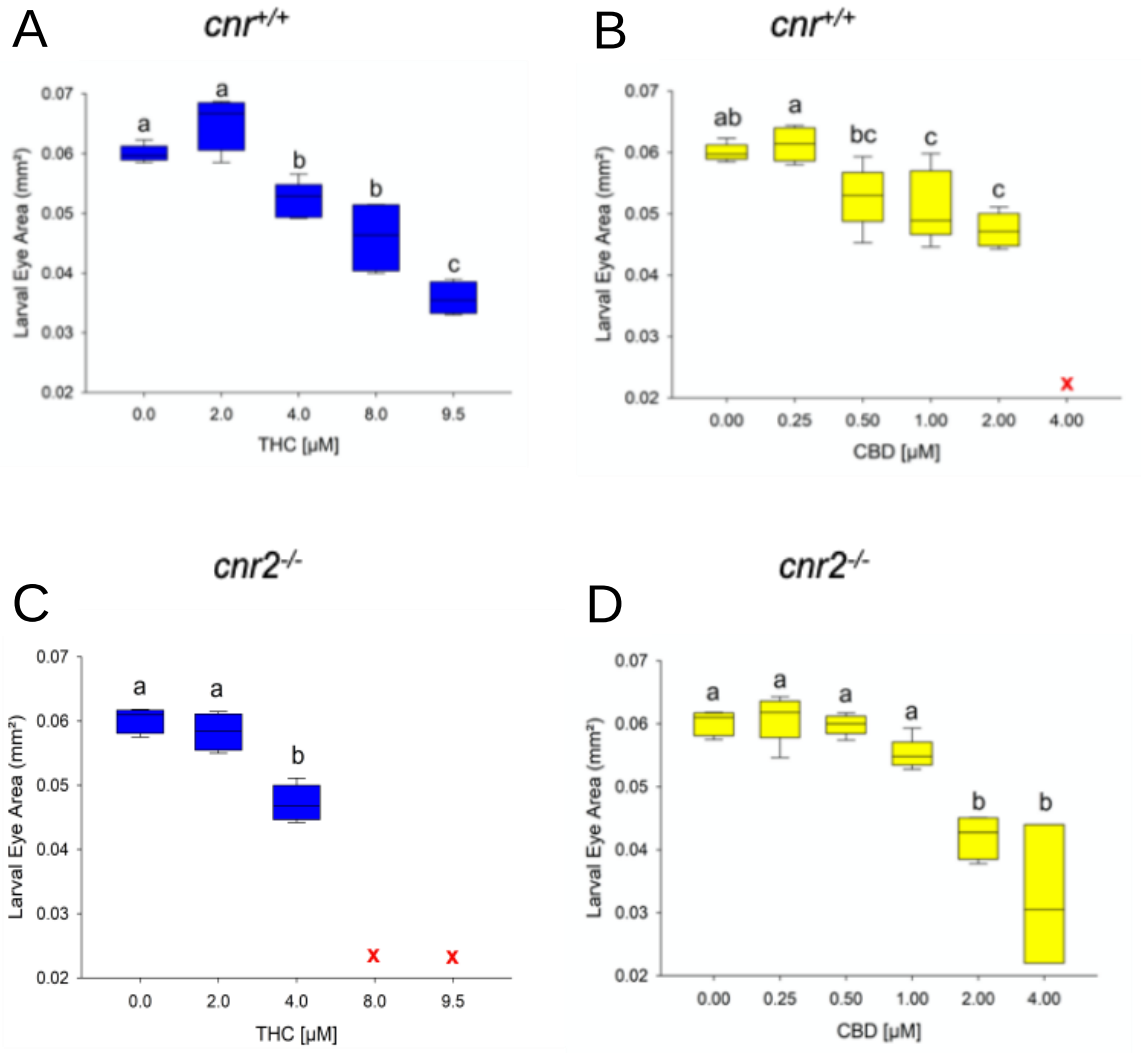

Figure 7: Larval eye area (mm^2) of 96 hpf zebrafish that were developmentally exposed to THC and CBD. Different letters indicate a significant distance between groups (ANOVA, Tukey's Posthoc, $p \leq 0.05$). Red X's indicate that there was no survival at that concentration.

3.3 Role of cnr2 in THC and CBD-induced larval behavioral deficits

When *cnr*$^{+/+}$ and *cnr2*$^{-/-}$ zebrafish were exposed to THC, larval behavior showed a dose-dependent decrease in total movement in both strains during the dark phase. During the light phase, this dose-dependent effect was not observed in either strain and in the *cnr2*$^{-/-}$ strain, locomotor activity increased with concentration during the light phase (Figure 8).

Similarly, when *cnr*$^{+/+}$ and *cnr2*$^{-/-}$ zebrafish were exposed to CBD there was a dose-dependent decrease in activity during the dark phase. During the light phase, this dose-dependent effect was not observed in either strain, and in the *cnr*$^{+/+}$ strain, locomotor activity significantly increased at the highest concentration (Figure 9).

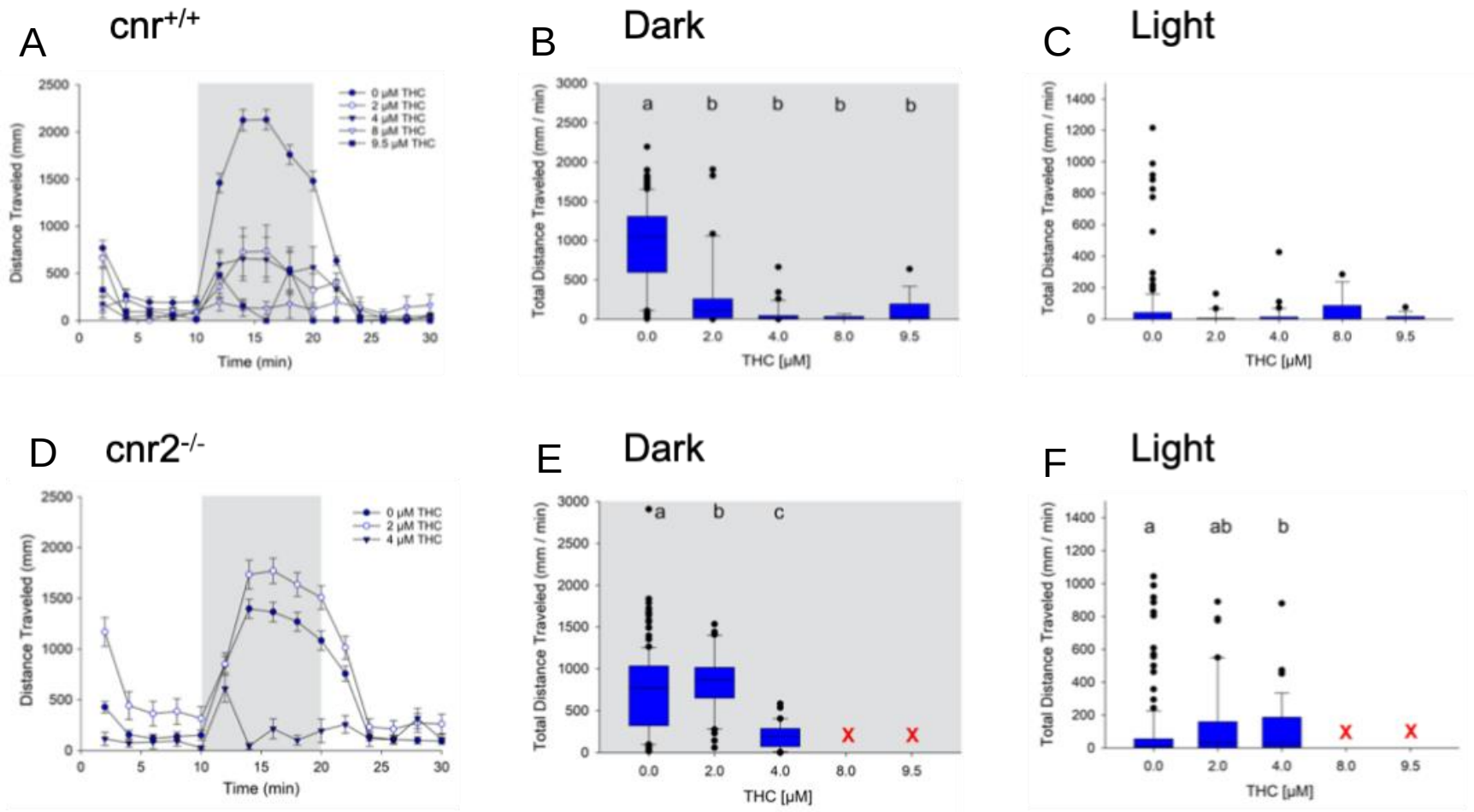

Figure 8: Distance travelled (mm) by larval zebrafish that were developmentally exposed to THC. These graphs show the total distance travelled during the light and dark phase of 96 hpf larval zebrafish. Different letters indicate a significant distance between groups within the light and dark phases (ANOVA, Tukey's Posthoc, $p < 0.05$). Red X's indicate that there was no survival at that concentration.

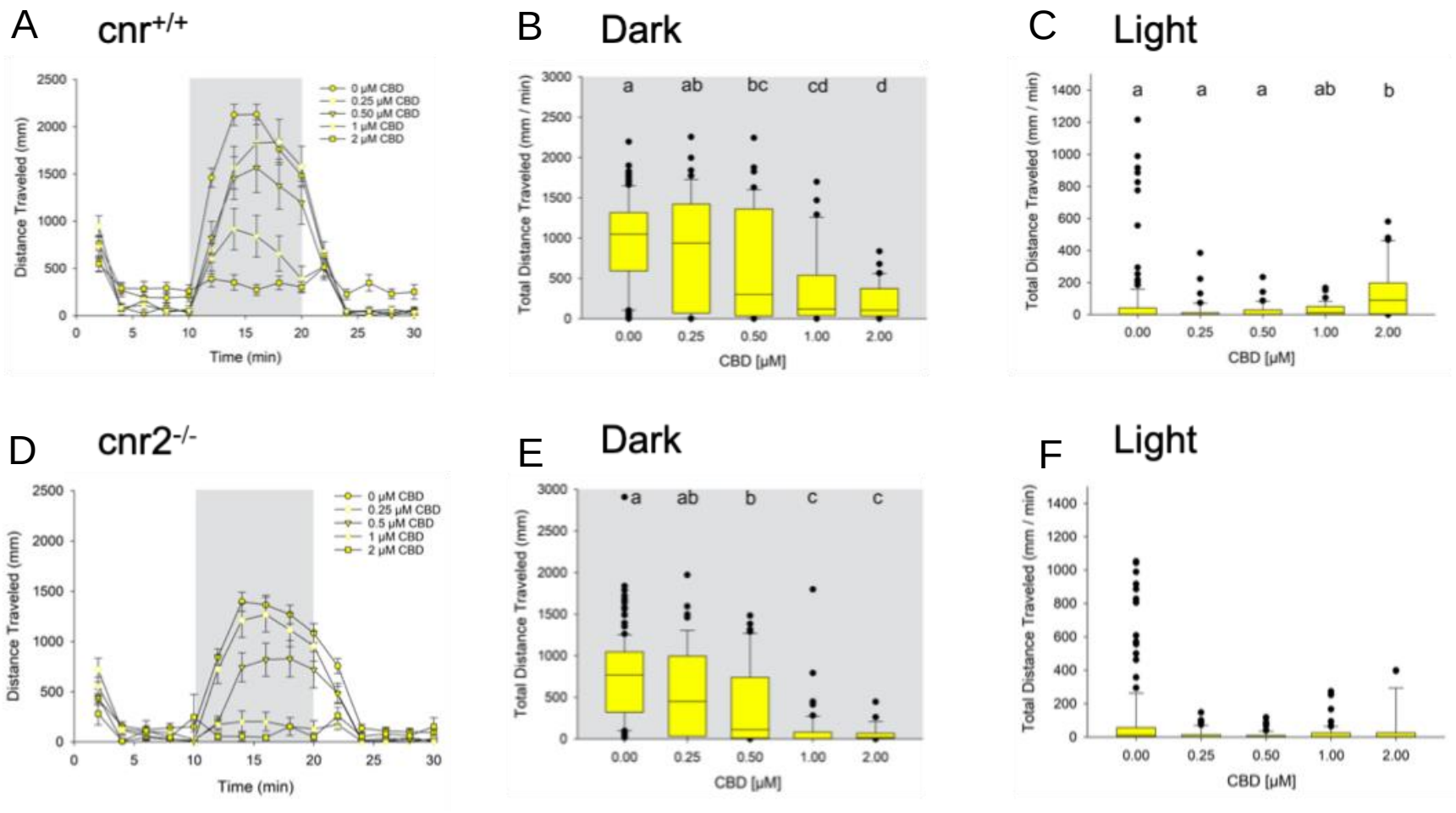

Figure 9: Distance travelled (mm) by larval zebrafish that were developmentally exposed to CBD. These graphs show the total distance travelled during the light and dark phase of 96 hpf larval zebrafish. Different letters indicate a significant distance between groups within the light and dark phases (ANOVA, Tukey's Posthoc, $p < 0.05$).

3.4 Role of *cnr2* in THC and CBD-induced adult behavioral deficits

Adult zebrafish were studied in an open-field test and the total distance traveled (cm) and percent time spent in the periphery was analyzed. When seven-month-old males were examined, there was a significant increase in the total distance traveled by the *cnr2*$^{-/-}$ zebrafish when compared to the *fli*, *5D*, and *cnr1*$^{-/-}$ strains (Figure 10A). The *cnr2*$^{-/-}$ mutant adults also spent significantly more time in the periphery than the *cnr1*$^{-/-}$ mutant strain (Figure 10B).

When seven-month-old adult females were examined, there was a significant increase in the total distance traveled in the *cnr2*$^{-/-}$ strain when comparing this strain to the wildtype *fli* strain (Figure 11A). The *cnr2*$^{-/-}$ strain also spent more time in the periphery when compared to the wild-type *5D* strain (Figure 11B).

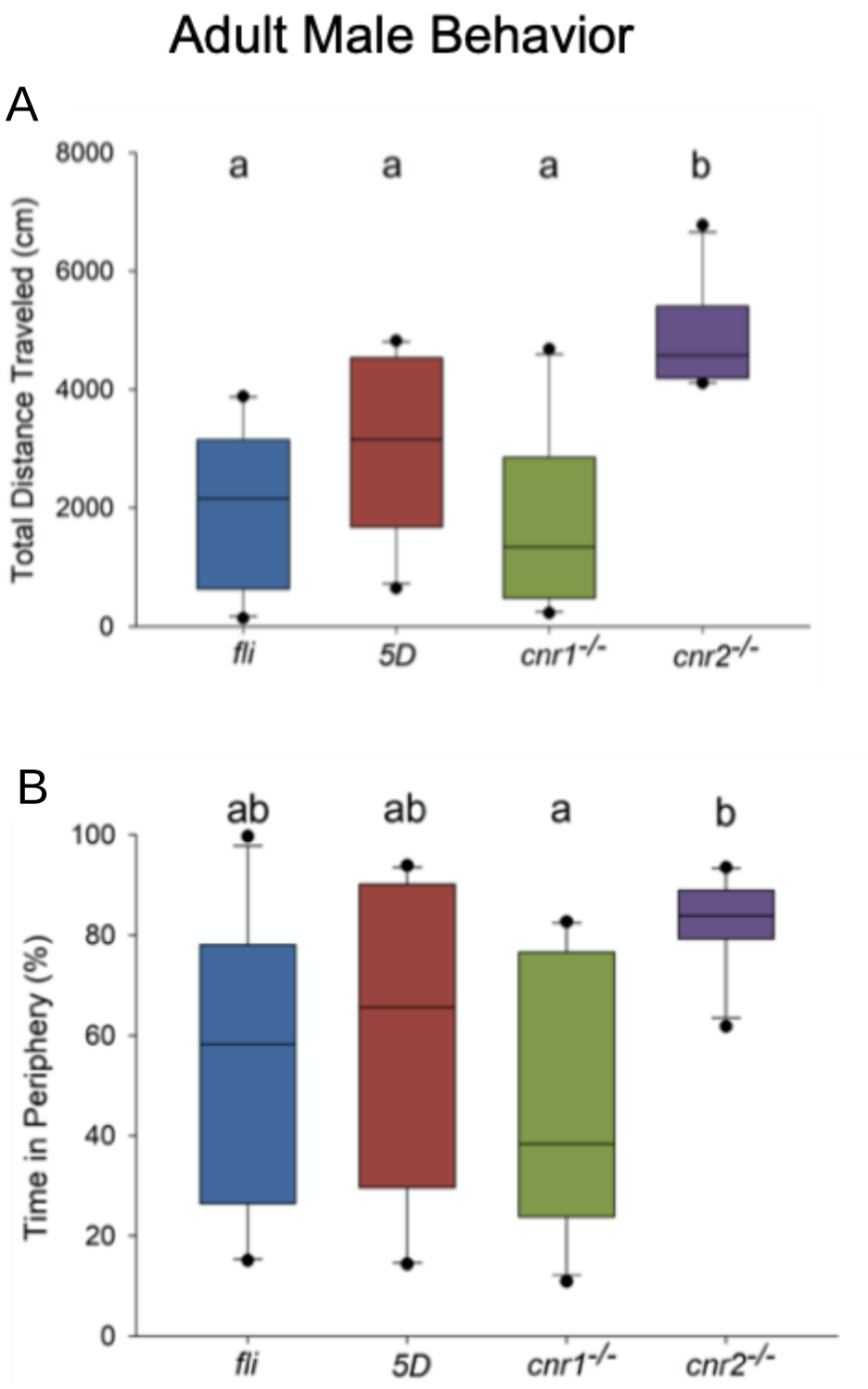

Figure 10: Distance travelled (mm) and time spent in periphery (%) by seven-month old male zebrafish of the *fli, 5D, cnr1*$^{-/-}$*, and cnr2*$^{-/-}$ that were not developmentally exposed to cannabinoids. Different letters indicate a significant distance between groups within the light and dark phases (ANOVA, Tukey's Posthoc, $p < 0.05$).

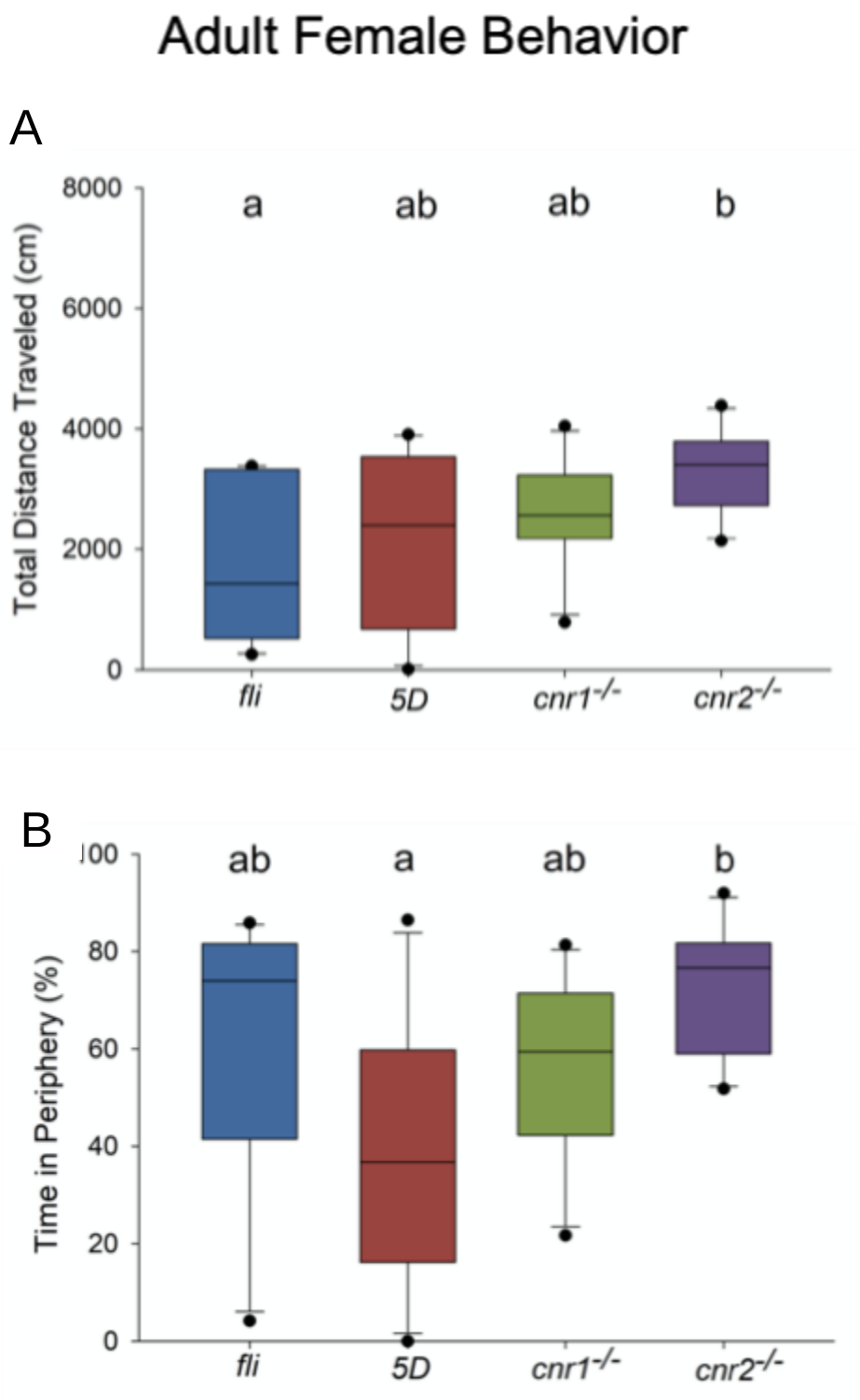

Figure 11: Distance travelled (mm) and time spent in periphery (%) by seven-month old female zebrafish of the *fli, 5D, cnr1*$^{-/-}$*, and cnr2*$^{-/-}$ that were not developmentally exposed to cannabinoids. Different letters indicate a significant distance between groups within the light and dark phases (ANOVA, Tukey's Posthoc, $p < 0.05$).

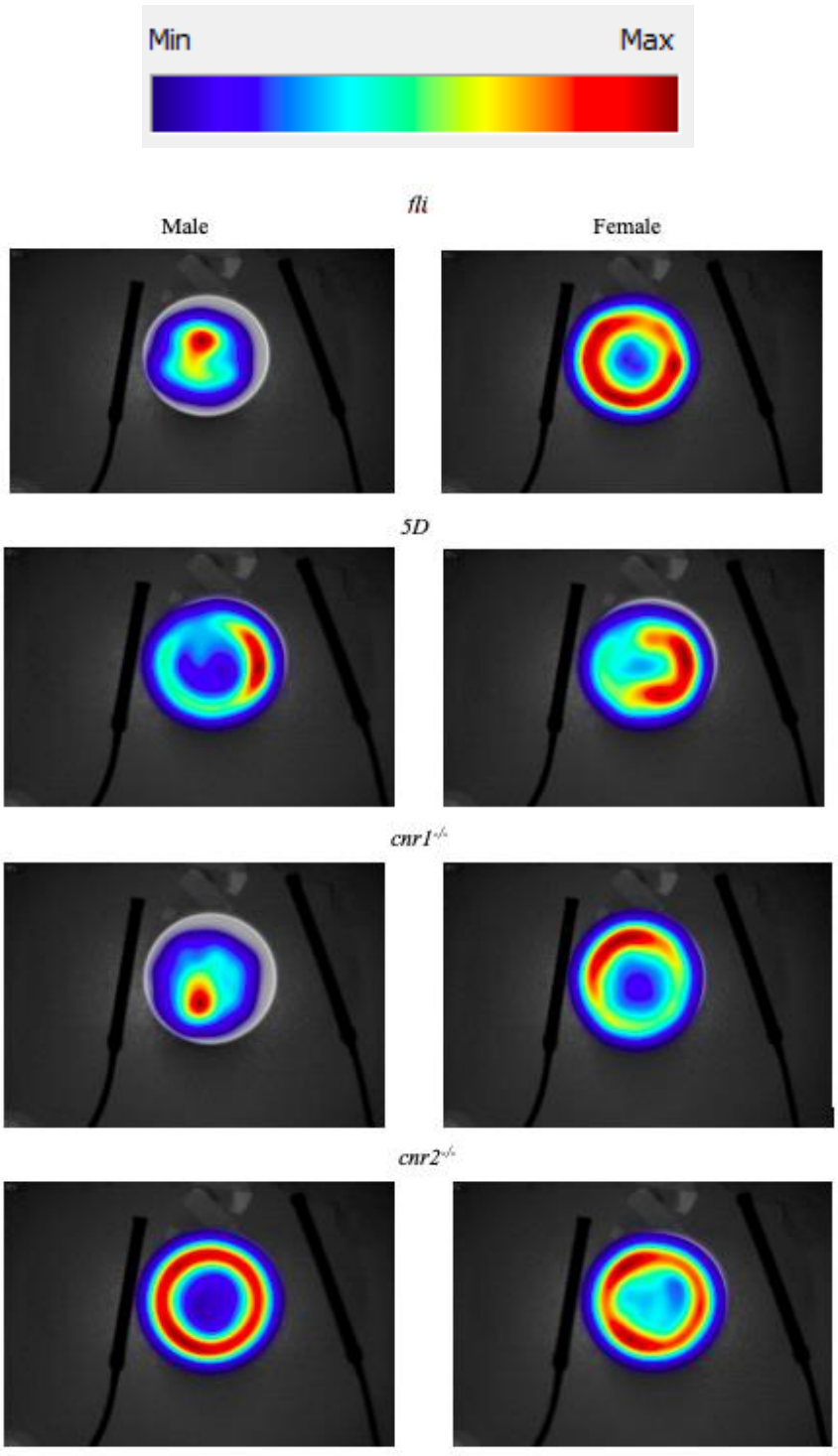

Figure 12: Adult Zebrafish Behavior Heatmap

IV. DISCUSSION

4.1 THC and CBD Toxicity

The effects of THC and CBD are modulated, at least in part, by cannabinoid receptors (Cristino et al., 2020). The results of this study further indicated this modulation because when the *cnr2*$^{-/-}$ fish were exposed to THC, they had significantly increased mortality at lower concentrations than the *cnr*$^{+/+}$ strain. This is consistent with previous studies indicating that cannabinoid receptor knockout disrupts metabolic function which can contribute to the increased toxicity when the *cnr2*$^{-/-}$ stain is developmentally exposed (Liu et al., 2016).

When the *cnr2*$^{-/-}$ strain was exposed to CBD, there was not as significant of an effect compared to the *cnr*$^{+/+}$ strain which implies that there may be a different mechanism of action for each of the cannabinoids. Compared to THC, CBD has a much lower affinity for cannabinoid receptors 1 and 2 and studies indicate that CBD may have modes of action independent of cnr1 and cnr2 (Pertwee, 2008).

4.2 THC and CBD Morphological Effects

A previous study on cnr mutants demonstrated that genetic or chemical inhibition of cannabinoid receptor activity disrupts liver development and metabolic function in zebrafish (Liu et al., 2016). One specific metabolic pathway that is differentially expressed following exposure to CBD or THC is retinol metabolism that has a large role in development of the eye as we have hypothesized in our manuscript (Pandelides et al., 2021). Microphthalmia has been associated with disruption of the genes involved in this

pathway (Tle et al., 2012). The difference in eye sizes seen between THC and CBD developmental exposure supports that these morphological deficits could be due to disruption of metabolism, specifically retinol. The results of our study suggest that this disruption of metabolic function can lead to phenotypic changes such as the decreased length, decreased larval locomotor behavior, and anxious adult behavior. Furthermore, our laboratory has previously shown that this decrease in size due to developmental THC or CBD exposure persists into old age (2.5 years) for female zebrafish (Pandelides et al., 2020).

4.3 Larval Behavioral Assessment

Swimming starts in zebrafish at 48-72 hpf although embryos as early as 27 hpf can swim in response to touch if they are dechorionated. When zebrafish reach 96 hpf, they create a pattern of increased movement in the dark followed by a resting state in the light when exposed to alternating light and dark stimuli (Saint-Amant and Drapeau, 1998). To evaluate behavior of zebrafish, we use a larval photomotor response assay to assess changes in swimming activity following a cyclical light-dark stimulus. The rationale for this assay is that, assuming the larvae are not malformed, an abnormal larval photomotor response in an otherwise normal animal implicates neurotoxicity (Burgess & Granato, 2007). This increased activity under dark conditions is consistent with our results as activity was much higher during the 100% dark conditions than the light conditions in each strain when exposed to either cannabinoid. This is suggested to be due to the evolutionary advantage this affords the zebrafish as the sensitivity to darkness most likely evolved to drive them to seek out light conditions in order to be in conditions that

better allow them to feed and avoid predators (Burgess & Granato, 2007; Emran et al., 2008).

Exposure to THC or CBD generally resulted in a decrease in total distance travelled as cannabinoid concentration increased, with the *cnr2*$^{-/-}$ strain being more sensitive to both THC and CBD exposure than the *cnr*$^{+/+}$ strain. Previous studies have shown that CB1 receptor modulates many of the behavioral effects of THC as *cnr1*$^{-/-}$ strains had a more tolerant response to cannabinoid exposure (Schnörr et al., 2012). The results from the *cnr2*$^{-/-}$ strain suggests that the Cnr2 may have a protective effect against cannabinoid exposure mediated by Cnr2's association with suppressing harmful inflammation activity (Colon-Cruz et al., 2020; Y. J. Liu et al., 2013). There was a larger difference between *cnr*$^{+/+}$ and *cnr2*$^{-/-}$ when the strains were exposed to CBD than when exposed to THC which could be due to the different binding affinity of the cannabinoids for the receptors or the fact that studies indicate that CBD may have modes of action independent of cnr1 and cnr 2 (Pertwee, 2008)..

In addition to the Cnr1 and Cnr2, several other endocannabinoid receptors may influence the effects of THC and CBD on behavior because the endocannabinoid system is not completely understood. One example that fits with our other predictions in this study could be that locomotor behavior in zebrafish exposed to THC and CBD is not due to the receptor's activities on behavior directly, but due to the morphological deficits that the cannabinoids caused during the developmental exposure such as eye area. Eye development could affect behavior because, as mentioned previously, retinol metabolism is essential to eye development and may be disrupted by cannabinoid exposure. Without proper eye development, the zebrafish would not be able to as adequately respond to the

light and dark stimuli. To further add to the understanding of cannabinoid effects on larval locomotor activity future studies should use the larval anxiety-like behavior assay created by Schnörr by examining larval thigmotaxic behavior (Schnörr et al., 2012). This would not only add more to our understanding of the Cnr2 but could also allow for a more reliable comparison of larval behavior to adult zebrafish behavior.

4.4 Adult Behavioral Assessment

To examine the effect of cannabinoid receptor presence and loss on behavior in unexposed seven-month adult zebrafish, we performed an open-field test specifically looking at total movement and thigmotaxis. The open-field test was derived from the traditional rodent behavior-based screening method for validation of pharmacological effects of neuroactive compounds which in our case are cannabinoids (Prut & Belzung, 2003). Thigmotaxis is also known as "wall-hugging" or "wall-following" and is an anxiety-like behavior that presents itself as the propensity of a fish to avoid the center of the arena and stay or move toward the boundary of the environment, or in our case, bucket. Thigmotaxis is believed to be an evolutionarily adapted trait to assist animals in protection and escape which both occur during stressful situations (Champagne et al., 2010; Patton et al., 2010), and we examined the role that cannabinoid receptors have in this stress-response. Our results for adult male zebrafish showed that males of the *cnr2*$^{-/-}$ strain spent significantly more time in the periphery thus exhibiting more anxious behavior when compared to the *cnr1*$^{-/-}$ strain. The females of the *cnr2*$^{-/-}$ strain also spent significantly more time in the periphery, but it was only significant in comparison to the wildtype *5D* strain. These results are consistent with other studies that saw Cnr2 overexpression reducing anxiety-like behaviors (García-Gutiérrez and Manzanares,

2011), and thus knocking out this receptor would reduce the positive benefit generated by overexpression and result in an increased occurrence of these behaviors. In adult males, these results may suggest that Cnr2 plays a larger role in modulating anxiety behaviors than Cnr1.

For total distance traveled, both males and females of the *cnr2*$^{-/-}$ strain showed a significant increase in movement when compared to the *fli* strain. However, differences in significance between sex were observed because not only were males of the *cnr2*$^{-/-}$ strain significantly more active when compared to the *fli* strain, but also when compared to every other strain analyzed in the study. These differences in significance may indicate that sex plays a factor in overall locomotor.

4.5 Conclusion

This study found that developmental exposure to THC or CBD in zebrafish results in significant effects on morphology and larval behavior that was consistent with previous studies. Seven-month adult behavior also differed between strains indicating cannabinoid receptor involvement in behavior. Overall, these data demonstrate that exposure to THC and CBD during development causes significant adverse outcomes. However, the effects of exposure to THC and CBD were lessened in zebrafish that lacked cannabinoid receptors 2 suggesting that this receptor has an important role in modulating the effects of cannabinoid exposure.

Our study on developmental exposure with different strains shines a bit of light on how the use of cannabinoids during development may affect the embryo's development through our evaluated endpoints of morality, larval length, eye area, larval behavior, and adult behavior. There is still need for more work to determine if the cannabinoid

receptors are truly conferring the results presented in this study. As mentioned previously, a logical next step would involve further research into the adverse effects caused by metabolic disruption, specifically metabolism of retinol. This would allow for a more well-rounded understanding of whether the cannabinoid receptors are directly involved in the adverse effects seen in mortality, morphology, and behavior, or if it is a consequence of the receptor's involvement in other processes.

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