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ORIGINAL ARTICLE

Developmental exposure to cannabidiol (CBD) alters longevity and health span of zebrafish (*Danio rerio*)

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Abstract Consumption of cannabinoid-containing products is on the rise, even during pregnancy. Unfortunately, the long-term, age-related consequences of developmental cannabidiol (CBD) exposure remain largely unknown. This is a critical gap given the established Developmental Origins of Health and Disease (DOHaD) paradigm which emphasizes that stressors, like drug exposure, early in life can instigate molecular and cellular changes that ultimately lead to adverse outcomes later in life. Thus, we exposed zebrafish (*Danio rerio*) to varying concentrations of CBD (0.02, 0.1, 0.5 μM) during larval development and assessed aging in both the F0 (exposed generation) and their F1 offspring 30 months later. F0 exposure to CBD significantly increased survival (~ 20%) and reduced size (wet weight and length) of female fish. While survival was increased, the age-related loss of locomotor function was unaffected and the effects on fecundity varied by sex and dose. Treatment with 0.5 μM CBD significantly reduced sperm concentration in males, but 0.1 μM increased egg production in females. Similar to other model systems, control aged zebrafish exhibited increased kyphosis as well as increased expression markers of senescence, and inflammation ($p16^{ink4ab}$, *tnfα*, *il1b*, *il6*, and *pparγ)* in the liver. Exposure to CBD significantly reduced the expression of several of these genes in a dose-dependent manner relative to the age-matched controls. The effects of CBD on size, gene expression, and reproduction were not reproduced in the F1 generation, suggesting the influence on aging was not cross-generational. Together, our results demonstrate that developmental exposure to CBD causes significant effects on the health and longevity of zebrafish.

Keywords Cannabinoids · Aging · *Danio rerio* · Inflammaging · Senescence

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Introduction

Cannabidiol (CBD) is a non-psychoactive constituent of cannabis that has long-been consumed as a potential remedy for a variety of ailments. There is currently (2020) only one FDA-approved drug containing CBD, Epidiolex, which is indicated for specific forms of childhood epilepsy. Given the rapidly changing regulations of cannabis and cannabinoids, CBD has been utilized by practitioners in some regions of the USA to treat psychosis, anxiety, and seizures with effective doses ranging between 1 and 50 mg/kg/day (reviewed in: Millar et al. 2019). Legalization of the growth of hemp, which is a cultivar of *Cannabis sativa*, has allowed for CBD-related products available on the market without FDA approval. Hemp-derived CBD is currently marketed as an ingredient in food, beverages, dietary supplements, cosmetics, and personal care

products (Dabrowska and Johnson 2019). The increased consumption of CBD products and cannabis has been noted in pregnant women, particularly during the first trimester (Volkow et al. 2019). Parental use is of concern as the potential adverse outcomes of in utero exposure to pharmaceutically relevant concentrations of cannabinoids like CBD is not well-characterized.

The Developmental Origins of Health and Disease (DOHaD) paradigm links stressors during embryonic development or early in life stages to permanent changes in the body's structure, function, and metabolism, leading to adverse outcomes such as disease later in life (Barker 2007). For example, perinatal exposure to chemicals like dieldrin increased the risk of Parkinson's disease (Richardson et al. 2006), while lead is linked to amyloid precursor protein dysfunction and Alzheimer's disease (Reviewed in: Zawia and Basha 2005). In contrast, preconditioning biological systems during critical developmental periods may bolster resilience to age-related dysfunction (Calabrese and Mattson 2017; Gidday 2015; Hodges and Ashpole 2019). Dietary restriction due to litter crowding in early life leads to increased life span (Sun et al. 2009). CBD has shown potential benefits long after its consumption as peri-adolescent exposure prevented the onset of behavioral abnormalities in a rat model of schizophrenia many months later (Peres et al. 2018). The effects of CBD on life span and health span, however, are currently unknown.

Recently, zebrafish (*Danio rerio*) have emerged as a model organism to ascertain age-related changes in biology and behavior (reviewed in: Beis and Agalou 2020). Zebrafish exhibit cellular and molecular changes during aging similar to mammals, and live ~3 years on average (Adams and Kafaligonul 2018; Arslan-Ergul and Adams 2014; Kishi et al. 2009). Zebrafish demonstrate age-related decline in cognition and perception (reviewed in: Adams and Kafaligonul 2018). Age-related mitochondrial dysfunction and oculopathy (Wang et al. 2019), nervous system aging and disease (Van houcke et al. 2015), age-related changes in DNA methylation (Shimoda et al. 2014), oncology (Barriuso et al. 2015), Peutz-Jeghers syndrome (van der Velden and Haramis 2011), telomere attrition (Wagner et al. 2017), osteoarthritis (Hayes et al. 2013), osteoporosis (Zhang et al. 2018), immune system, and endocrine decline have all been described in zebrafish (Arslan-Ergul and Adams 2014; Beis and Agalou 2020; Carneiro et al. 2016). In addition, the endocannabinoid system is well-conserved in zebrafish (reviewed in: Oltrabella et al. 2017), thus making them an excellent model to study the effects of both cannabinoids and aging.

Previous research in our laboratory established that embryonic exposure to high doses of CBD (≥2 μM) caused larval developmental teratogenicity, and altered the expression of several genes (Carty et al. 2018). Importantly, lower doses that did not induce teratogenicity and fall within the lower end of the human therapeutic range resulted in long-term changes in fecundity (Carty et al. 2019). Negative effects of high CBD exposure are also recapitulated in mammalian models. For example, in utero exposure to CBD (17 mg/kg) caused eye and brain dysmorphologies in mice as well as zebrafish (Fish et al. 2019). Together, these studies were limited to early-life changes in physiology following developmental CBD exposure and thus do not report changes in the biological processes of aging.

The aim of this study is to assess the DOHaD with CBD by exposing fish to CBD during development and assessing aging in both the exposed F0 generation and their subsequent F1 offspring. Fish were exposed to 0.02–0.5 μM CBD beginning at 6 h post-fertilization (hpf) (gastrula), equivalent to week 4 of human pregnancy (Fish et al. 2019), until larval development at 96 hpf, when the majority of organogenesis has occurred. Previous studies in zebrafish indicate that these are therapeutically relevant doses as exposure to 0.25–0.5 μM CBD leads to a tissue concentration of 1.2–8.61 mg/kg in the fish (Carty et al. 2019, 2018) and Epidiolex dosing in humans ranges from 2.5 to 50 mg/kg/day (Arzimanoglou et al. 2020). At 12 months of age, they were enrolled into the aging study, and the effects of aging were assessed when they were 30 months old. Aging was expected to decrease survival, locomotor behavior, and fecundity, as well as cause increased kyphosis, senescence, and inflammation (Beis and Agalou 2020; López-Otín et al. 2013). While it is known that embryonic exposure to high doses of CBD can acutely impact zebrafish development, the effect of CBD on aging phenotypes is not known. We hypothesized that exposure to CBD during embryogenesis would result in significantly altered behavior, reproduction, growth, and inflammation.

Methods

Zebrafish care and exposure

Tg(fli1:egfp) zebrafish were obtained through the Zebrafish International Resource Center (ZFIN, Eugene, Oregon). Beginning at ~6 hpf, zebrafish embryos were

exposed to sublethal concentrations (Carty et al. 2018), namely, 0.02, 0.1, and 0.5 μM (0.006, 0.03, 0.15 mg/L) CBD, or 0.05% DMSO control water (0.6 mL water:fish) in static conditions without water changes until 96 hpf. Measured concentrations for 0.15 mg/L CBD were 0.07 ± 0.05 mg/L CBD as reported in (Carty et al. 2019). The methodology of exposure and rearing of the F0 generation are described in (Carty et al. 2019). Adult developmentally exposed F0 fish were spawned at 6 months of age to produce an F1 generation. One year following developmental exposure, F0 (exposed) or F1 (parents exposed) fish were enrolled into the aging study and cultured until aged (2.5 years). Fish were kept in Aquatic Habitats ZF0601 Zebrafish Stand-Alone System (Aquatic Habitats, Apopka, FL) with zebrafish water (pH 7.0–7.5, 60 parts per million (ppm) Instant Ocean, Cincinnati, OH) at 25–28 °C, 14:10 light-dark cycle. Fish were fed twice daily with Gemma (Gemma Micro 300, Skretting). At 2.5 years old, aged fish were assessed for age-related effects. Two separate young cohorts were cultured in the same room, system, and conditions as the aged fish to act as a young control (7-month-old cohort when F0s were assessed). Culture and exposure protocols were IACUC-approved.

Fecundity and larval endpoints

At 30–33 months of age, two males and two females for the F0 (exposed) or F1 (parents exposed) were placed into static breeding tanks ($n = 3$ spawning tanks per treatment group) with 750 mL system water from our primary zebrafish culture unit and housed in a 28 °C temperature-controlled room. Water was changed during spawning events. Mating pairs were allowed a 1-week acclimation period for which one spawning event took place. During weeks two and three, eggs were collected for three consecutive days to obtain eggs from all tanks and to ensure both females per tank spawned (Reed and Jennings 2011). Following each egg collection, debris and water were removed and replaced with clean embryo medium (60 ppm Instant Ocean; pH 7.5–7.8). Egg production was calculated as the average number of eggs per breeding tank. Collected eggs were subsequently counted and screened for fertilization, mortality, malformations, and hatching every 24 h until 96 hpf. At 96 hpf, photos were taken of a subset of 24 larvae per group to assess developmental deformities. Larvae were anesthetized in 300 mg/L tricaine methanesulfonate (MS-222) and 600 mg/L sodium bicarbonate. They were immediately placed on a microscope slide with a chamber containing 3% methyl cellulose and a lateral photo 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). Blind to treatment, measurements and scoring of the anatomical structures were recorded using ImageJ software (Schneider et al. 2012). First, the scale was set to the number of pixels per millimeter using a 1-mm micrometer. Then, the total body length along the spine and the diameter of the eye were measured, following with the presence absence of developmental abnormalities being scored by three double-blinded reviewers. Following reproductive assessment, fish were returned to their respective tanks until use for the open-field test.

Open-field behavior

Tanks of young (7 months old) and aged F0 and F1 (30–33 months old) fish were acclimated to a darkened behavioral testing room (27–28 °C) prior to open-field behavioral assessment. Individual fish were then transferred to a water-filled bucket (diameter of 23.5 cm and a depth of 24.8 cm) and allowed to freely swim and explore for 5 min while their response was video-captured overhead by Noldus Ethovision 14 software. The testing area was lit to 9 Lux. At the completion of the trial, the fish were removed from the open-field arena and placed in a holding container until euthanasia. For analysis, the swim arena was divided into two regions—periphery (outer 50% of the arena) and center (inner 50% of the arena). Distance, swim speed, mobility, and time spent in each region of the arena was then calculated by Ethovision and decoded by a blinded observer.

Phenotypic assessment of adults

Immediately following open-field testing, fish were euthanized in 300 mg/L MS-222 and 600 mg/L sodium bicarbonate. After euthanasia, wet weight and length of the fish were measured, and the fish were laid prone on a flat measuring surface. Pictures of the lateral side of the fish were taken for subsequent analysis of the curvature of the back, more specifically, for the presence of kyphosis. Images were analyzed by three double-blinded observers. Each rater used a scale of 1–5, with 1 signifying no curvature, 3 signifying mild curvature, and 5 signifying severe curvature (Fig. 3e). Raters were given

reference photos for the varying levels of curvature. Inter-observer scoring showed 76% identity and over 90% similarity. The median score for each fish was determined and used for comparison between groups. After which, the brain, gonads, liver, and muscle tissue of the fish were dissected, flash-frozen in liquid nitrogen, and stored at − 80 °C until further analysis. During dissection, any observed incidence of tumors were grossly assessed and recorded.

RT-qPCR

Adult F0 and F1 zebrafish whole liver ($n = 6$ per sex per concentration) tissue was homogenized in TRIzol (Invitrogen no. A33251) followed by RNA extraction using an RNeasy mini-kit (Qiagen no. 74104) in conjunction with gDNA removal via RNase-Free DNase set (Qiagen no. 79254) following manufacturer's recommended protocol. RNA was quantified and evaluated for purity (260/280 ratio = 1.9–2.1) on a NanoDrop 2000 (Thermo Fisher, Waltham, MA). RNA (250 ng) was then reverse transcribed to 10 ng/μL cDNA following manufacture's protocol (Invitrogen kit no. 4304134). RT-qPCR was performed using an Applied Biosystems 7200 real-time cycler with SYBR Green detection chemistry (Applied Biosystems no. 4309155) following manufacture's protocol (25 μL reaction volume). Parameters for RT-qPCR were as follows: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by 95 °C for 15 s–60 °C for 1 min–95 °C for 15-s dissociation curve. Primers were optimized as in (Corrales et al. 2014) and detailed in Supplemental Table 1. Samples were screened in duplicate followed by $2^{-\Delta\Delta CT}$ method evaluation (Livak and Schmittgen 2001).

Statistical analysis

RT-qPCR was assessed using one-way analysis of variance (ANOVA) on the ΔCT followed by Dunnett's post hoc test compared with the old controls ($p \leq 0.05$). The aged and young controls were compared using t tests ($p \leq 0.05$). The gene expression data were summarized in tables displaying the average log(2)ΔΔCt ± standard error of the mean. Reproductive success, open field, adult size, and weight were also analyzed using the one-way ANOVA followed by Dunnett's post hoc test compared with the old controls ($p \leq 0.05$), and the old and young controls were also compared by t test ($p \leq 0.05$). These data were displayed using box and whisker plots. Categorical data such as kyphosis, yolk sac edema, pericardial edema, and adult survival were analyzed using chi-squared ($p \leq 0.05$), or Fisher's exact test ($p \leq 0.05$). All graphing and statistical analyses were conducted using Sigmaplot 14.0 software.

Results

Locomotor behavior following CBD exposure

Male and female zebrafish were exposed to increasing concentrations of CBD (0.02, 0.1, and 0.5 μM) during embryo-to-larval development (6–96 hpf). Fish were then enrolled into a survival analysis study at 12 months of age. At 30 months of age, 60% of control male and female fish had died (Fig. 1a, b). Male fish treated with increasing doses of CBD had significantly increased survival than controls (Fig. 1a). Similarly, females treated with 0.1 and 0.5 μM CBD during larval development had a non-significant trend of increased survival at the 30-month time point. Given the survival at 30 months, behavior and aging phenotypes were then assessed in a cross-sectional study. Locomotor function was significantly reduced in advanced age, as male and female aged control fish swam shorter distances (Fig. 2a, b) at reduced speeds (Fig. 2c, d) than young, 7-month-old control fish. Overall mobility in the open-field arena was also significantly decreased in the aged control fish (Fig. 2e, f). Early-life exposure to CBD did not improve locomotor abilities in aged male of female fish (Fig. 2a–f), even at the concentrations that showed increased survival (Fig. 1). There was no significant effect of CBD developmental exposure on the time spent in the periphery vs center of the open-field arena (data not shown).

Fecundity following CBD exposure

Reproductive potential in the aged cohorts was measured by quantifying egg and sperm production and the survival of fertilized eggs. Aged control males produced significantly fewer sperm than young controls (Table 1). The lower two concentrations of CBD did not reduce sperm production; however, exposure to 0.5 μM CBD during larval development resulted in significantly less sperm production in aged males, compared with age-matched controls (Table 1). Aging was also associated with decreased fecundity in females, because aged fish produced 85% fewer eggs than young control fish

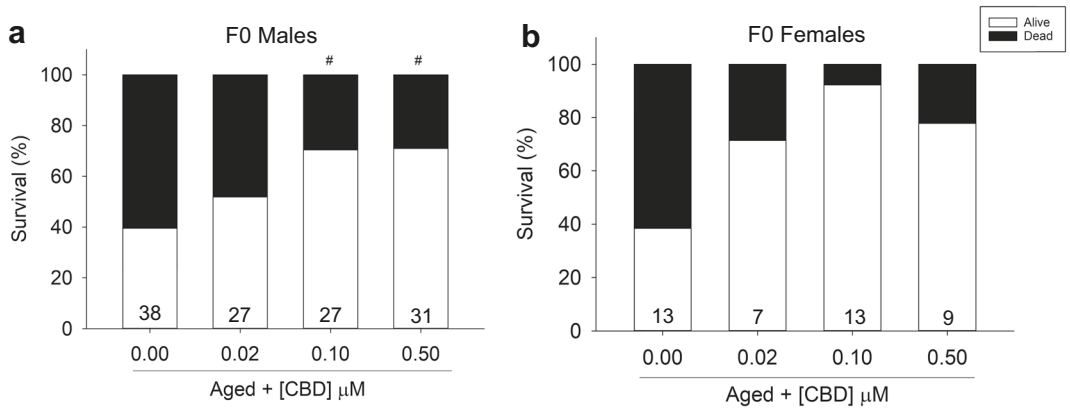

Fig. 1 Survival (%) measured at 30 months of age of adult F0 zebrafish developmentally exposed to CBD and enrolled into the study at 12 months old. **a** Male % survival from 12 to 30 months, $n = 27–38$. **b** Female % survival from 12 to 30 months, $n = 7–13$. The number displayed at the base of the bar is the total number of fish per treatment enrolled into the study at 12 months old. Number sign indicates a significant difference compared with aged controls (Fisher's exact test $p \leq 0.05$)

(Table 1). Early-life exposure to 0.1 μM CBD partially rescued egg production in aged females, producing 464% more eggs than aged controls (Table 1). Despite this increase in egg number, only 48.2% of fertilized eggs from 0.1 μM CBD-exposed fish survived 96 h post-fertilization which was similar to the survival of the offspring from the other aged fish (Table 1). Early-life exposure to the other concentrations of CBD had no effect on the age-related loss in fecundity, and all treatment groups saw over 97% successful hatching within 72 h of fertilization; however, the high concentration of CBD (0.5 μM) during development was associated with a non-significant reduction in the survival of offspring 24 h and 96 h post-fertilization in the aged cohort (Table 1). Aside from reduced eye diameter in certain treatment groups, surviving offspring did not exhibit robust abnormalities, their overall length was similar, and no significant differences in yolk sac or cardiac structure were observed (Supplemental Figure 1A-G).

Phenotypic changes in aged fish exposed to CBD

Following behavior and fecundity assessments, zebrafish were euthanized at 30 months and overall body size was assessed. No difference in body length or mass was observed in male fish exposed to vehicle or CBD during development (Fig. 3a–c). Aged female fish were significantly longer and heavier than young controls (Fig. 3b–d). High concentrations of CBD (0.1 and 0.5 μM) throughout larval development resulted in significant reductions in both length and mass in aged females (Fig. 3b–d). Despite the difference in size, the condition (*k*) factor (a measure of general fish health) of the adult fish was not significantly different (Supplemental Figure 2A-B).

Similar to other vertebrates, aged zebrafish exhibit increased incidents of kyphosis in advanced age as we observed in both aged male and female cohorts (Fig. 3e–g). Early-life exposure to CBD did not significantly affect the incidence of kyphosis in advanced age within males, despite a visual trend of reduced severity (Fig. 3e–f). Similarly, CBD exposure in the females did not significantly rescue the incidence of kyphosis (Fig. 3e–g).

Very few tumors were observed in any of the treatment groups at 30 months of age. Within the male cohort, 2 of 14 (18%) aged controls had obvious tumors, primarily found in the gonads, and no differences were observed in any of the CBD-treated aged males (Supplemental Figure 3A). Gross tumors were neither observed in young control fish nor in aged female controls (Supplemental Figure 3B).

Senescence and inflammation in aged fish exposed to CBD

Gene expression of several inflammatory and senescence markers were assessed in the livers of the aged fish. Compared with young fish, aged male controls exhibited significantly increased expression of *p16* and pro-inflammatory *tnfα* and *il-1β*. In contrast, the expression of *il-6* and *pparγ* was significantly reduced in the aged control males, compared with young controls. Early-life exposure to CBD did not alter *p16*, *tnfα*, or *il-6* expression in advanced age, but high concentrations of CBD resulted in reduced expression of *il-1β*

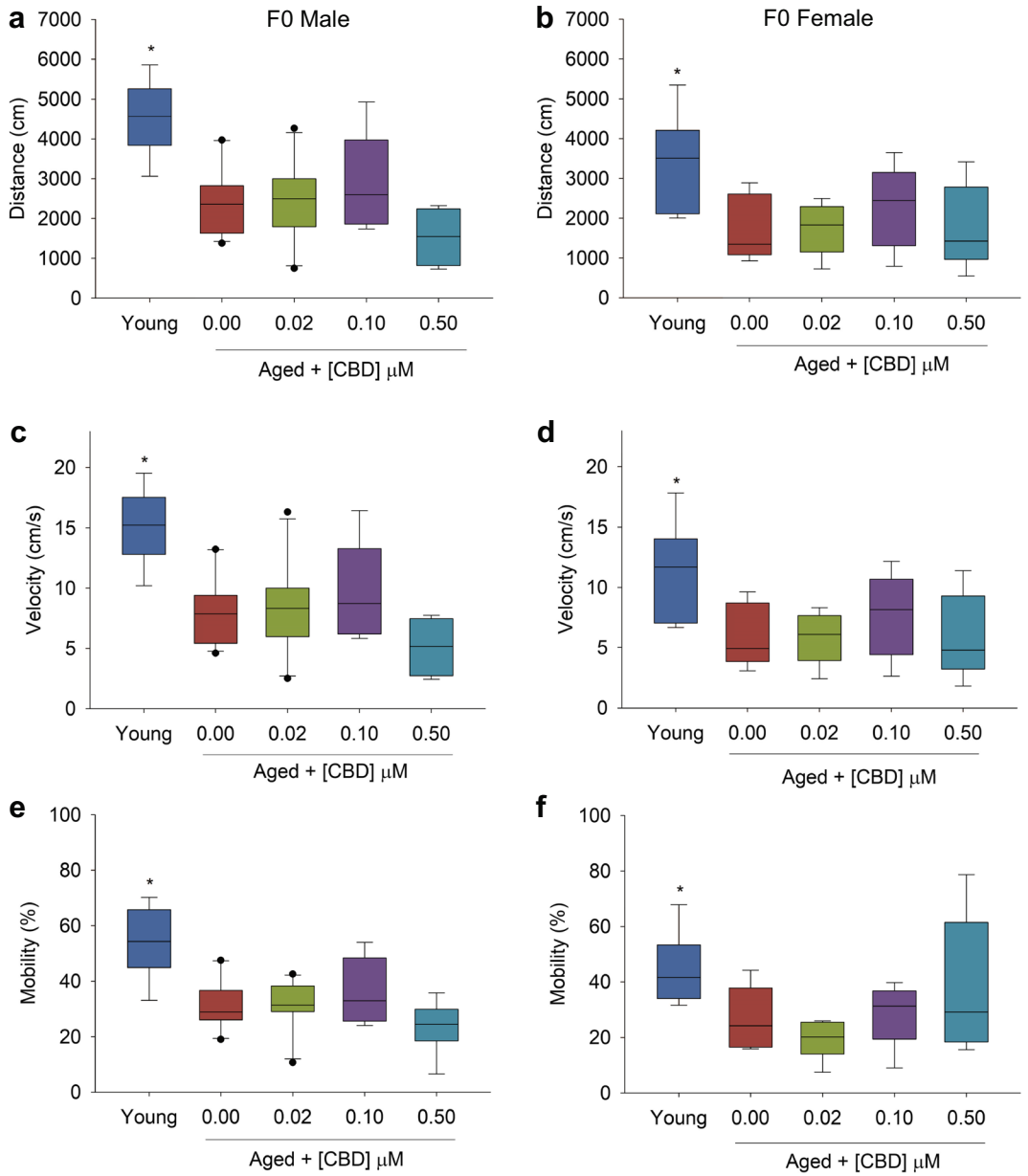

Fig. 2 Open-field behavior of adult F0 (young = 7 months old, aged = 30 months old) zebrafish developmentally exposed to CBD, $n = 8$. **a**, **b** Male and female zebrafish distance traveled (cm). **c**, **d** Male and female velocity (cm/s). **e**, **f** Male and female mobility (%). Asterisk indicates a significant difference between aged and young controls (*t* test, $p \leq 0.05$). No significant difference between CBD treatment and aged controls (ANOVA, Dunnett's post hoc, $p > 0.05$)

(Table 2). Within females, aging also resulted in increased expression of *p16*, *tnfα*, *il-1β*, and *il-6* (Table 2). Unlike the male cohorts, no differences in *pparγ* expression were observed in aged female control fish (Table 2). High concentrations of CBD during development did not alter *p16* expression in advanced age, but did result in a significant decrease in *tnfα* and *il-1β* expression (Table 2). *pparγ* was also significantly reduced with 0.5 μM CBD exposure and *il-6* levels were significantly decreased in fish treated with either 0.1 or 0.5 μM CBD (Table 2).

Table 1 F0 fecundity and survival of CBD-exposed (μM) zebrafish, data presented as mean ± SE

Nominal developmental treatment (μM) at 6 hpf	*n*	Avg no. of eggs per tank per week ± SE	% survival at 24 hpf ± SE	% hatch at 48 hpf ± SE	% hatch at 72 hpf ± SE	% survival at 96 hpf ± SE	Sperm/g testes* 10^6 ± SE
Young control	6	491 ± 71.7*	66.2 ± 5	48.3 ± 5.3*	98.5 ± 1.4	62.8 ± 4.3	31.2 ± 3.1*
Aged control	6	73 ± 43	59.9 ± 6.6	18.3 ± 8.6	100 ± 0	56.4 ± 7.2	13.4 ± 1.5
0.02 CBD	6	158 ± 62.7	49.5 ± 10.2	19.8 ± 6.8	97.7 ± 1.4	40.6 ± 8.8	9.1 ± 2.5
0.1 CBD	6	339 ± 79.6#	50.3 ± 12.9	35.1 ± 15.6	99 ± 0.7	48.2 ± 12.1	11.4 ± 3.6
0.5 CBD	6	126.9 ± 43.3	23.3 ± 8.1	38.9 ± 16.4	99.5 ± 0.5	19.7 ± 7.5	4.8 ± 0.8#

*$p \leq 0.05$ *t* test, aged control vs young control

#$p \leq 0.05$ ANOVA, Dunnett's post hoc vs aged control

F0: aged control = 30 months old, young control = 7 months old

n = number of breeding events (3 consecutive days of egg collection/week = 1 breeding event)

Correlation analysis with CBD exposure

To determine the relationship between the observed parameters, a correlation matrix was generated (Fig. 4). *tnfα*, and *pparγ* expression as well as body length and weight were strongly ($r^2 \geq 0.95$) inversely correlated with survival in male zebrafish, while *il-6* expression as well as length and weight were inversely correlated with survival in females. *pparγ* expression correlated with sperm and egg production (0.78–0.83) as well as the expression of pro-inflammatory *tnfα* and *il-1β* in males and females. The size (weight and length) of the female fish correlated moderately to strongly (0.52–1.00) with the expression of *il-6*, *il-1β*, *tnfα*, *p16*, and *pparγ*. Yet in male fish, only the length of the fish correlated with *il-1β*, *tnfα*, and *pparγ*.

Multi-generational effects of CBD exposure on aging

To assess whether early-life CBD exposure influenced aging in the subsequent generation of offspring, a F1 (first generation of offspring) cohort was spawned from the CBD-treated fish when they were 6 months of age. The F1 generation was then enrolled into the aging study at 12 months of age, similar to the F0 parents. No differences in survival (Fig. 5a, b), locomotor behavior (Fig. 5c, d, Supplemental Figure 4A-D), body length (Fig. 5e, f), body weight (Fig. 5g, h), or incidence of kyphosis were observed in the F1 generation (Fig. 5i, j). Also, there were no significant changes in fecundity or fertility in aged F1 fish observed, but there was a trend of decreased egg production with increasing concentration of CBD parental exposure (Supplemental Table 2). Interestingly, *p16* and *il-1β* expressions were significantly increased in the aged male offspring of fish treated with 0.02 μM CBD (Table 3). This was sex-specific as no changes in gene expression were observed in any of the female offspring of the CBD-exposed fish (Table 3).

Discussion

The potential adverse outcomes of in utero exposure to pharmaceutically relevant concentrations of cannabinoids like CBD are not well-characterized even in the face of increased societal prevalence. It is well-accepted that early developmental exposure to certain chemicals can increase the risk for disease later in life (Richardson et al. 2006; Zawia and Basha 2005). Alternatively, some kinds of stress during development can precondition biological systems, in turn reducing the risk of latent diseases (Peres et al. 2018). By exposing zebrafish embryos during development, we investigated the role CBD plays in the DOHaD, focusing on the CBD effects on growth, metabolism, reproduction, cell senescence, and inflammation.

CBD isolated from cannabis is currently FDA-approved for the treatment of two specific childhood forms of epilepsy. This pharmacotherapy is marketed as Epidiolex and the dosing ranges from 2.5 to 10 mg/kg twice daily, with the goal of maintenance dosing of 10–20 mg/kg/day (Arzimanoglou et al. 2020). In zebrafish, waterborne exposure to 0.25–0.5 μM CBD results in measured tissue concentrations of 1.2 to 8.61 mg/kg (Carty et al. 2019, 2018), which suggests our exposure of 0.02–0.5 μM should result in tissue concentrations consistent within or below the human pharmacotherapeutic dose range of Epidiolex. It also is important to note that, like other model

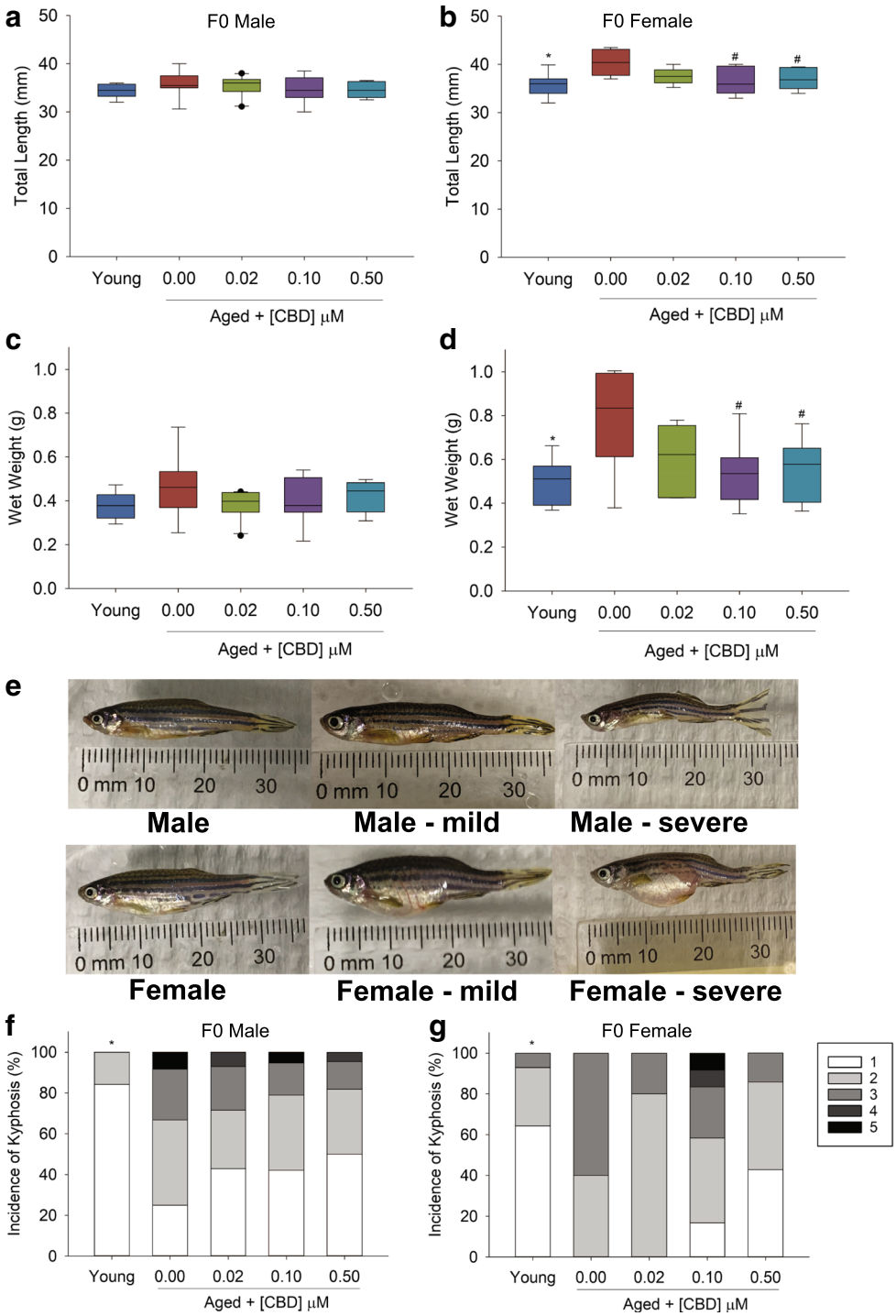

Fig. 3 Weight, length, and spinal curvature (kyphosis) of adult F0 (young = 7 months old, aged = 30 months old) male ($n = 8–22$) and female ($n = 5–12$) zebrafish developmentally exposed to CBD. **a**, **b** Male and female zebrafish total length (mm). **c**, **d** Male and female wet weight (g). **e** Male and female spinal curvature grading from healthy (1) to medium (3) to severe (5). **f**, **g** Male and female kyphosis (%). Asterisk indicates a significant difference between aged and young controls (*t* test, $p \leq 0.05$). Number sign indicates a significant difference compared with aged controls (ANOVA, Dunnett's post hoc, $p \leq 0.05$ or Fisher's exact test $p \leq 0.05$)

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

Males	*n*	*p16* ± SE	*tnfα* ± SE	*il-1b* ± SE	*il-6* ± SE	*pparα* ± SE	*pparγ* ± SE
Young control	6	− 0.76 ± 0.61	− 2.63 ± 0.72*	− 3.24 ± 0.30*	1.03 ± 0.22*	0.56 ± 0.42	1.46 ± 0.09*
Aged control	6	0 ± 0.4	0 ± 0.78	0 ± 0.6	0 ± 0.16	0 ± 0.19	0 ± 0.23
0.02 CBD	6	− 0.63 ± 0.43	− 0.90 ± 0.82	0.01 ± 0.33	− 0.30 ± 0.02	− 0.19 ± 0.25	− 0.65 ± 0.28
0.1 CBD	6	− 0.23 ± 0.41	− 2.75 ± 0.26#	− 1.04 ± 0.64	0.14 ± 0.28	− 0.26 ± 0.76	− 1.15 ± 0.42
0.5 CBD	6	− 1.20 ± 0.61	− 2.31 ± 0.63	− 2.06 ± 0.35#	− 0.35 ± 0.09	0.27 ± 0.19	− 1.05 ± 0.28
Females	*n*	*p16* ± SE	*tnfα* ± SE	*il-1b* ± SE	*il-6* ± SE	*pparα* ± SE	*pparγ* ± SE
Young control	6	− 1.13 ± 0.29	− 4.27 ± 0.65*	− 2.84 ± 0.14*	− 0.76 ± 0.19*	0.68 ± 0.54	− 0.23 ± 0.38
Aged control	6	0 ± 0.43	0 ± 0.8	0 ± 0.56	0 ± 0.25	0 ± 0.31	0 ± 0.37
0.02 CBD	6	− 0.98 ± 0.36	− 1.44 ± 0.58	− 1.21 ± 0.6	− 0.88 ± 0.36	− 1.58 ± 0.8	− 1.45 ± 0.51
0.1 CBD	6	− 0.71 ± 0.6	− 1.95 ± 0.8	− 1.06 ± 0.52	− 1.28 ± 0.33#	− 0.87 ± 0.47	− 1.25 ± 0.31
0.5 CBD	6	− 0.87 ± 0.44	− 3.39 ± 0.50#	− 2.63 ± 0.35#	− 1.11 ± 0.26#	− 1.15 ± 0.44	− 2.57 ± 0.48#

*$p \leq 0.05$ *t* test, aged control vs young control

#$p \leq 0.05$ ANOVA, Dunnett's post hoc vs aged control

species, fish metabolism is different than humans. While our dose range is conservative compared with the human doses, other animal models such as mice (using a pharmacokinetic scaling factor of 12.6) are often treated with doses that far exceed the human therapeutic range (50–246 mg/kg CBD) (Dalterio et al. 1984; Ewing et al. 2019). There are no other FDA-approved indications for CBD although it is now widely consumed as an over-the-counter supplement. There are no FDA recommendations on dosing for this type of consumption, and poor quality control guidelines make it hard to determine whether people are consuming what they expect and in the concentrations they expect (as reviewed by VanDolah et al. 2019).

We observed significant effects on both the health span and longevity of developmentally exposed zebrafish. CBD exposure significantly increased male survival in a concentration-dependent manner, while in females there was only a trending increase. This trend is most likely due to the low sample size of female fish enrolled in the study. CBD significantly affected male and female fecundity in a sex-dependent manner. While a significant reduction in fecundity of both male and female fish was observed due to aging, CBD treatment caused an even further decrease in sperm production relative to the aged-matched controls. These results were similar to our previous study (Carty et al. 2019), where there was a reduction in embryo survival at 24 h post-fertilization in the same population of treated fish when they were 6 months old. While sperm quantity was not measured in the previous study, 24 h post-fertilization (hpf) survival can be influenced by sperm quality. A reduction of sperm quantity and quality following exposure to CBD has been observed in other animal models including mice and sea urchins (Carvalho et al. 2018; Schuel et al. 1987). Notably, these exposures were conducted in adolescent or adult organisms, not during embryonic development. The regulation of the endocannabinoid system (ECS) is necessary for normal sperm function and male fertility (Amoako et al. 2013; Battista et al. 2008). For example, seminal plasma N-arachidonoylethanolamide (AEA) levels are lower in men with asthenozoospermia or oligoasthenoteratozoospermia (Amoako et al. 2013). Given that CBD is known to inhibit both fatty acid amide hydrolyase (FAAH) and fatty acid binding proteins that regulate the degradation of endocannabinoids like AEA, it is possible that embryonic exposure to CBD caused long-term effects within the testes via altered endocannabinoid signaling.

In contrast to the male effects observed in this current study, female fish were partially rescued dose-dependently by treatment of CBD, producing significantly more eggs at the 0.1 μM CBD dose. Female mice oocytes express several known targets of CBD including G protein coupled receptor 55 (GPR55) and transient vanilloid type 1 channel (TRPV1); antagonism of these receptors can disrupt oogenesis (Cecconi et al. 2019). The response of increased fecundity at an intermediate concentration in females and reduced sperm at a higher concentration in males suggests a biphasic or hormetic response to CBD for zebrafish reproduction.

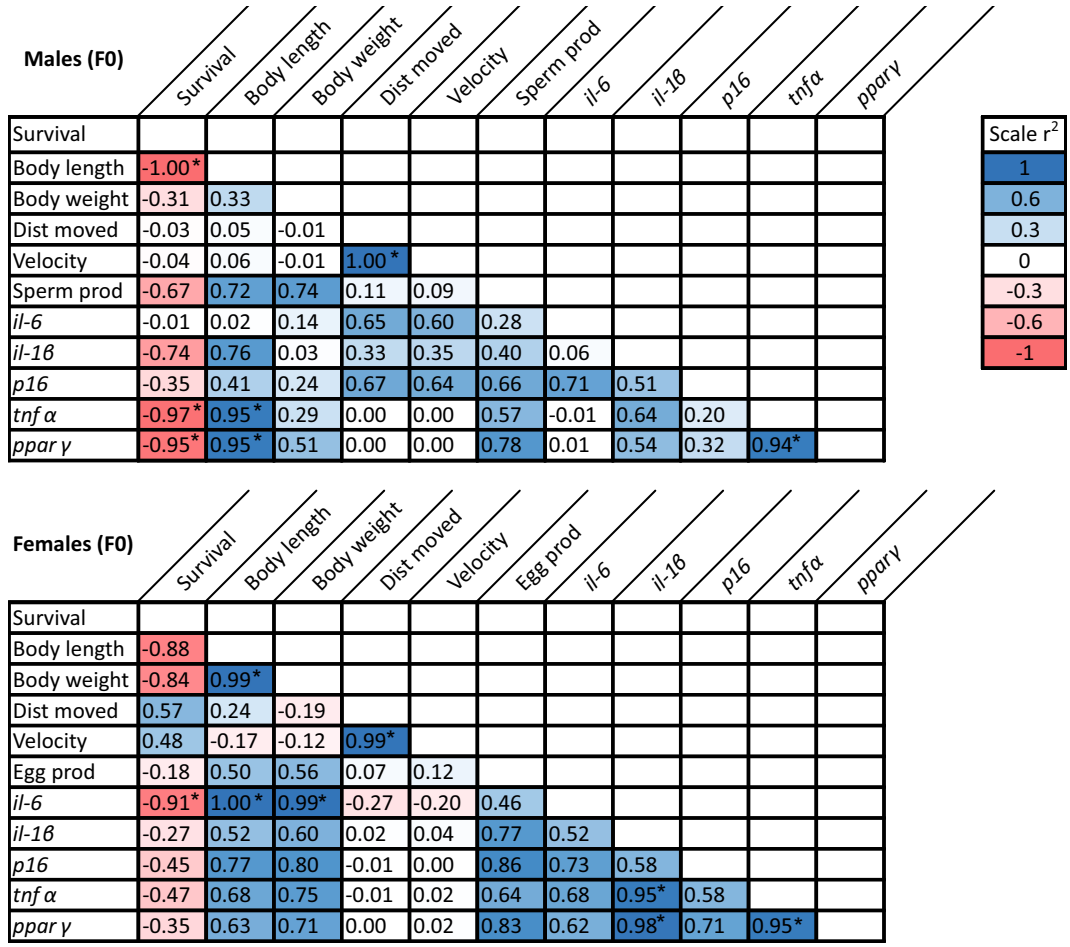

Fig. 4 Correlation matrix of endpoints used in this CBD developmental exposure study. The values for each parameter were averaged by treatment and then plotted and analyzed by linear regression; the values presented in the table are the resulting r^2

A biphasic response to CBD was also demonstrated in the expression of certain genes of mice livers following exposure (Ewing et al. 2019). While the majority of genes were dysregulated in a dose-dependent manner, *Fmo1*, *Lgr5*, and *Lss* were upregulated at lower doses and downregulated at higher, demonstrating CBD can cause both dose-dependent and biphasic effects (Ewing et al. 2019). The contrasting male and female reproductive results suggest that male zebrafish may be more sensitive to developmental exposure to CBD. Increased egg production in this current study is in contrast to our previous study, where we reported fewer eggs produced by the same population of 0.5 μM CBD-exposed fish when bred at 6 months of age (Carty et al. 2019). Decreased egg production measured early, and increased egg production later, could be a result of delayed female fish growth and development.

Delayed development due to CBD exposure is supported by the outcome that female CBD-treated fish were significantly smaller, considering both weight and length, than the age-matched controls. In humans, maternal exposure to cannabis results in infants with lower birth weight (Gunn et al. 2016). In addition, zebrafish exposed to high concentrations (3.2–12.7 μM) of CBD during embryo development resulted in significantly shorter larvae measured at 48 hpf, but the long-term effects on zebrafish growth were not assessed (Ahmed et al. 2018). In zebrafish, maturation is size-dependent and correlates very strongly with developmental progress (Parichy et al. 2009). Furthermore, caloric intake in female zebrafish affected the rate of oocyte growth rather than total oocyte numbers (Leibold and Hammerschmidt 2015). Thus, exposure of female fish to CBD could have delayed developmental

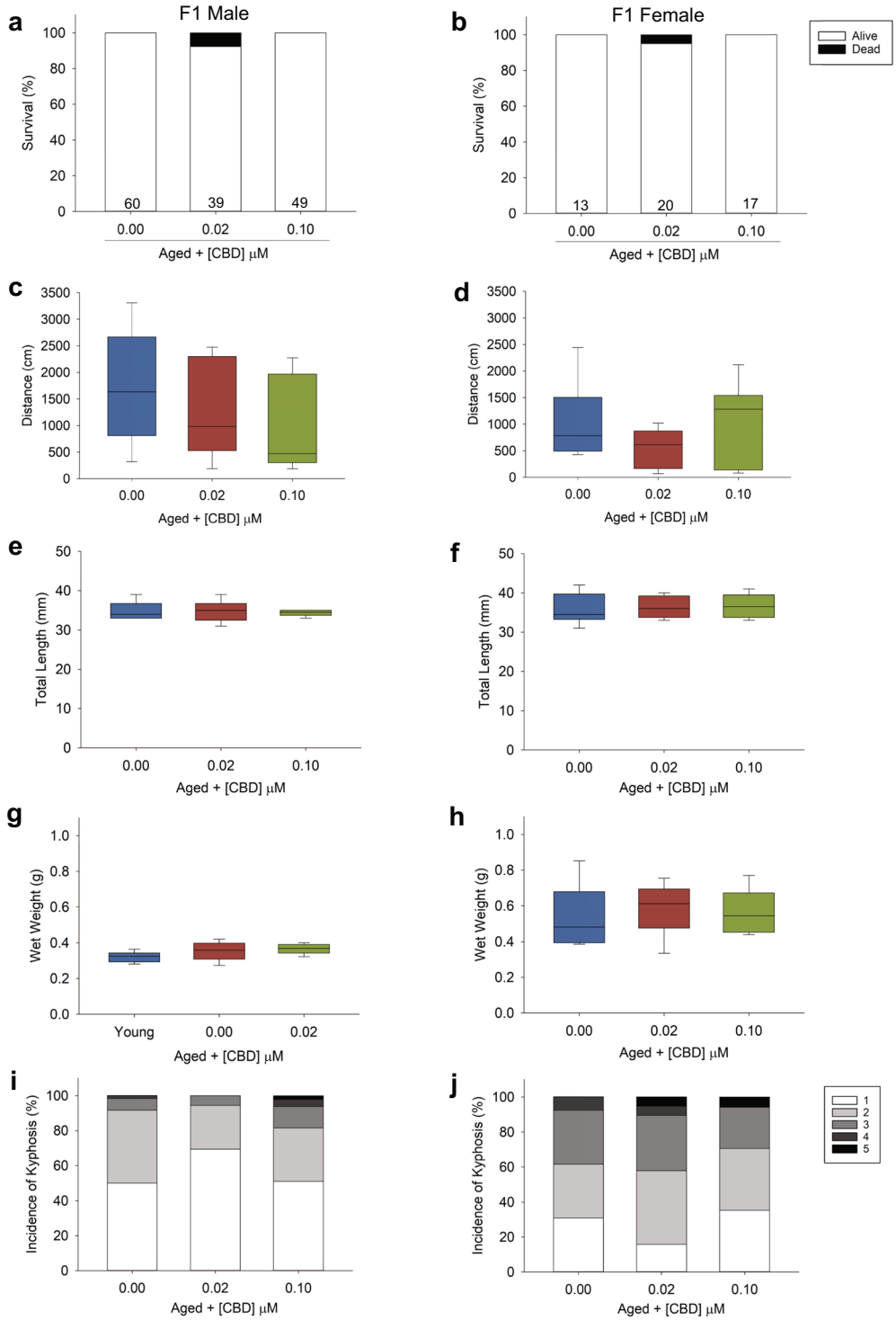

Fig. 5 Survival, distance traveled, length, weight, and incidence of kyphosis of male and female F1 zebrafish (parents exposed to CBD). **a**, **b** Male, $n = 39–60$, and female, $n = 13–20$, survival (%) from 12 to 30 months old. The number displayed at the base of each bar is the total number of fish per treatment enrolled into the study at 12 months old. **c**, **d** Male and female zebrafish distance traveled (cm), $n = 6$. **e**, **f** Weight; **g**, **h** length; and **i**, **j** spinal curvature (kyphosis) of adult F1 male ($n = 11–60$) and female ($n = 6–19$) zebrafish. No significant difference between F1 parentally exposed fish and aged controls (ANOVA, Dunnett's post hoc, $p > 0.05$ or Fisher's exact test $p > 0.05$)

Table 3 Male and female F1 (mean ± SE) gene expression (log(2)ΔΔCt) relative to the aged controls, $n = 6$. These fish were the offspring from parent F0 developmentally exposed fish to 0.02, 0.1 μM or solvent control. Exposure groups were normalized to 18 s, and the ΔCT values were assessed for significance by one-way ANOVA with Dunnett's post hoc test ($p \leq 0.05$). Number sign indicates a significant difference compared with aged controls (ANOVA, Dunnett's post hoc, $p \leq 0.05$)

Males (μM)	*n*	*p16* ± SE	*tnfα* ± SE	*il-1b* ± SE	*il-6* ± SE	*pparα* ± SE	*pparγ* ± SE
Aged control	6	0 ± 0.59	0 ± 0.9	0 ± 0.43	0 ± 0.16	0 ± 0.09	0 ± 0.19
0.02 CBD	6	2.87 ± 0.42#	3.49 ± 1.3	3.38 ± 0.51#	0.81 ± 0.26	− 0.21 ± 0.11	− 0.18 ± 0.24
0.1 CBD	6	1.45 ± 0.13	1.13 ± 0.85	0.04 ± 0.46	0.80 ± 0.4	0.46 ± 0.3	0.15 ± 0.33
Females (μM)	*n*	*p16* ± SE	*tnfα* ± SE	*il-1b* ± SE	*il-6* ± SE	*pparα* ± SE	*pparγ* ± SE
Aged control	6	0 ± 50	0 ± 0.57	0 ± 0.52	0 ± 0.26	0 ± 0.48	0 ± 0.18
0.02 CBD	6	− 0.08 ± 0.33	0.30 ± 0.58	− 0.03 ± 0.41	0.10 ± 0.14	0.28 ± 0.38	− 0.64 ± 0.24
0.1 CBD	6	0.06 ± 0.31	0.29 ± 0.82	− 0.13 ± 0.36	0.11 ± 0.33	0.11 ± 0.33	0.06 ± 0.27

$p \leq 0.05$ ANOVA, Dunnett's post hoc vs aged control

event timing or heterokairy, relative to controls, which could have caused the effects observed on fecundity.

Additionally, reduced growth could have been caused by a significant change in development or behavior that affected food intake. Exposure to CBD significantly reduced body weight gain in rats, which was completely blocked when co-treated with a cannabinoid receptor type 2 CB2 receptor antagonist (Ignatowska-Jankowska et al. 2011). This result in rats is surprising because CBD displays very weak affinity for both cannabinoid receptor type 1 (CB1) and CB2 receptors (Zou and Kumar 2018), although it is possible that CBD influences CB2 signaling by increasing endocannabinoid tone and its associated agonism of CB2 (Bisogno et al. 2001). CB2 receptors were linked to food intake (Ishiguro et al. 2010); thus, perturbation of this signaling pathway could have led to changes in the regulation of food intake, ultimately resulting in the differences in fish size observed.

Dietary restriction increases the life and health spans in many eukaryotic species (Adams and Kafaligonul 2018; Beis and Agalou 2020). A restriction in caloric intake, or reduced metabolism, could have contributed to the increased the life span of zebrafish observed in this current study. Survival correlated strongly with the length of the male and female fish which could imply that treatment groups that ate and grew more had a higher mortality than the treatment groups who did not. However, one confounding factor is that as fish died, there was a change in fish tank density. Controls changed from 10 fish/tank at 12 months to 5 fish/tank (3 L) by 30 months, and CBD-treated fish ranged from 11 to 13 fish/tank at 12 months and dropping to 6–10 fish/tank by 30 months. In the lab, adult zebrafish are housed in tanks ranging from 4 to 15 fish/tank. Because fish were fed ad libitum twice daily, and were within typical densities, there should not have been major changes in food availability despite the changes in numbers per tank. Furthermore, effects on size were sex-based, with males still reaching their maximum size, and females not. In addition, the fish displayed similar healthy ($k = 1$) condition factors which further supports that while their size differed, their health did not. While sex-specific difference in life span and health span are commonly observed in aging studies (Austad 2019), caloric restriction or density affects alone should have impacted both male and female fish. While not likely, it should be acknowledged that food availability due to changes in tank density could have confounded the F0 results.

Similar to other studies (Arslan-Ergul et al. 2016; Wang et al. 2014), we observed significant increases in senescence and inflammation in our aged zebrafish. Treatment with CBD reduced the expression of inflammatory genes in a dose-dependent manner. The acute anti-inflammatory effects of cannabinoids including CBD are well-documented (Malfait et al. 2000; Nagarkatti et al. 2009; Stančić et al. 2015). The reduced expression of inflammatory genes observed in this current study suggests that exposure during embryo development may cause enduring alterations to the immune system. In addition to reduced hepatic expression of inflammation markers, we also saw a reduction in the expression of *pparγ*.

PPARα and γ receptor modulation is thought to mediate some of the neuroprotective, anti-inflammatory, and metabolic effects of cannabinoids (reviewed in: O'Sullivan 2016). Indeed, CBD was demonstrated to bind and activate PPARγ in vitro (Hegde et al. 2015; O'Sullivan et al. 2009). PPARγ agonists mitigate

inflammatory processes associated with chronic and acute neurological insults (Kapadia et al. 2008). CBD also protects against β-amyloid neurotoxicity and inflammation in rats, which is reduced by PPARγ antagonism (Esposito et al. 2011). Similarly, the PPARγ antagonist GW9662 reverses the anti-aging effects of ellagic acid (Rahimi et al. 2019). In addition to directly agonizing *pparγ*, CBD regulates the mTOR pathway, reduces the expression of pro-inflammatory cytokines, and induces *pparγ* expression (Giacoppo et al. 2017). The altered expression of *pparγ* observed suggests lipid dysregulation. A change in metabolism is consistent with the changes in growth, inflammation, and reproduction observed. As CBD targets the mitochondria in order to regulate intracellular Ca^{2+} levels (Ryan et al. 2009), future studies should establish if developmental exposure to CBD modulates age-induced mitochondrial dysfunction. Furthermore, altered calcium signaling during early development may be a driving force for a wide range adult diseases such as reduced fertility, neurodegenerative disorders, Alzheimer's, and immunodeficiency (reviewed in: Paudel et al. 2018). Considering the anti-inflammatory and metabolic effects of CBD and our results showing that the changes in inflammation are still present 30 months following exposure, future studies should characterize whether early-life exposure to CBD alters necroptosis and its associated molecular and behavioral phenotypes in advanced age (reviewed in: Royce et al. 2019).

The majority of the effects observed in the parental F0 population following CBD exposure were not observed in the F1 generation. The only significant difference observed was an increase in the expression of inflammatory genes in the 0.02 μM CBD (F0 parents exposed) males. However, the results are difficult to interpret as the highest dose of CBD (0.5 μM) in which the most significant age-related effects were observed, was not kept for the transgenerational study.

The current study demonstrates that a single developmental exposure to CBD can cause significant effects on the longevity and health span of zebrafish. Exposure to CBD during development affected the metabolism, growth, expression of genes, survival, and reproduction of the zebrafish even into old age. The current study adds insight into the potential role of in utero exposure to CBD in DOHaD. While some effects observed were beneficial, such as increased survival and reduced inflammation, other effects were toxicological, such as decreased sperm production. An increase in survival without a concomitant increase in locomotor function or decrease in kyphosis could indicate a worsened age-related behavioral burden. While it is difficult to establish the exact mechanism through which CBD caused the wide range of observed effects, exposure to CBD significantly altered zebrafish development. Future studies should elucidate how developmental exposure to CBD can cause these long-term effects. Together, our results highlight that there are lifelong, sex-dependent outcomes following exposure to CBD during crucial developmental periods.

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Author contributions KW, NA, and ZP conceived and designed the experiments; NA and ZP analyzed the data; AF, AW, CT, NA, and ZP performed the experiments; and KW, NA, and ZP prepared the manuscript.

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Compliance with ethical standards

Conflict of interest The authors have declared that they have no conflicts of interest.

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