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Brief exposure to (-) THC affects zebrafish embryonic locomotion with effects that persist into the next generation

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Cannabis is one of the most widely used drugs, and yet an understanding of its impact on the human brain and body is inconclusive. Medicinal and recreational use of cannabis has increased in the last decade with a concomitant increase in use by pregnant women. The major psychoactive compound in cannabis, Δ^9-tetrahydrocannabinol (THC), exists in different isomers, with the (-) trans isomer most common. Prenatal exposure to THC can alter neural and behavioral development, but it is unknown how exposure to (-) trans-THC ((-)THC) during very early stages of development impacts fetal growth and movement, and whether effects persist to adulthood, or into the next generation. Here we exposed zebrafish (*Danio rerio*) to a single exposure of (-)THC (0.001 mg/L (3.2 nM) to 20 mg/L (63.6 µM), for 5 h) during gastrulation (5.25 hpf to 10.75 hpf) when key neurons involved in locomotion such as the primary motor neurons and Mauthner cell first appear. We then examined the impact on embryo morphology and locomotion, adult behavior, and locomotion in the next (F1) generation. Embryos treated with (-)THC experienced changes in morphology, were shorter in length and experienced altered hatching and survival. Spontaneous coiling of 1 dpf embryos was reduced, swimming after touch-evoked responses was reduced and basal swimming in 5 dpf larvae was also reduced. Adult zebrafish tested in the open field test and novel object approach test demonstrated no differences in locomotion, anxiety-like behavior, nor boldness, compared to controls. The (-)THC F1 generation embryos at 1 dpf showed reduced coiling activity, while swimming after touch-evoked responses was reduced in 2 dpf animals but basal swimming at 5 dpf remained similar to controls. Taken together, exposure to (-)THC only once for 5 h during gastrulation has a significant impact on locomotion in embryos and larvae, a minimal impact on adult behavior, and effects that persist into the next generation.

Keywords Cannabinoid, Motor, Sensory, Locomotor, Behavior

The major psychoactive ingredient in cannabis is Δ^9-THC which has four stereoisomers that vary in their double bond position and cis or trans ring junction: (+) trans Δ^9 THC, (-) trans Δ^9 THC, (+) cis Δ^9 THC and (-) cis Δ^9 THC[1]. The production of (-) trans Δ^9 THC (hereafter referred to as (-)THC) is energetically favored and is found in greater quantities in natural cannabis compared with the other stereoisomers[1,2]. (-)THC is used for medicinal purposes, for appetite stimulation and as an antiemetic following chemotherapy. It has also been used as a treatment for chronic pain, multiple sclerosis and epilepsy[2–4]. Moreover, with legalization, more women of reproductive age are using cannabis[5]. Indeed, recent studies indicate that not only are more pregnant women using cannabis during the first trimester, but cannabinoids may be prescribed during pregnancy to combat morning sickness[6]. THC rapidly enters the blood stream following inhalation and blood plasma levels rise within 1–2 min of the first exposure[7]. The bioavailability varies substantially with each individual and factors such as weight, gender, age, genetic predisposition, health and physiological background impact the extent to which THC and other cannabinoids affect an individual[8]. Once it is inhaled or ingested, THC readily crosses the

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blood-brain barrier and can be found in high quantities in the brain[9]. Moreover, it accumulates in fatty tissue and organs such as the heart, liver and spleen[10].

Cannabinoids appear to be less toxic or harmful to the adult central nervous system compared with embryonic organisms[11,12]. Acute or short-term exposure to cannabinoids during prenatal, neonatal and adolescent periods can be harmful[13] due to the critical involvement of the endocannabinoid system in brain development[14]. Neonatal rodents exposed to cannabinoids experience cortical cell death, whereas adults do not appear to show similar effects, and paternal THC exposure in rodents causes neurobehavioral effects in the offspring with long-term consequences[15]. Furthermore, prenatal exposure has been shown to alter the development of major neurotransmitter systems in the brain, which may have long-lasting impacts on the organism[16]. Importantly, zebrafish embryos exposed to cannabinoids for even brief periods of time (~ 5 h during gastrulation) experience deficiencies in multiple neurological systems (motor, sensory and cognitive) early in development[17–19].

Phylogenetic analyses show that the endocannabinoid system (ECS) is highly conserved between zebrafish and humans, establishing zebrafish as an effective model for studying cannabinoid signaling in vivo[4]. Zebrafish express all of the ECS genes necessary for cannabinoid signaling, including genes encoding cannabinoid receptor 1 (*cnr1*), cannabinoid receptor 2 (*cnr2*), fatty acid amide hydrolase (*faah*) and monoacylglycerol hydrolase (*mgll*). Furthermore, zebrafish ECS genes have been shown to be linked to development[20,21], locomotion[22], immune system function[23], cognitive process[24], anxiety[24], energy balancing[25], and addiction[26]. In zebrafish CB_1R mRNA expression has been detected as early as the 3 somite stage (10 hpf) through RT-qPCR[27,28] and in situ hybridization experiments have documented the spatial expression of CB_1R as early as 1 dpf in the preoptic area and the lenses of the eye[28]. Thus zebrafish has shown to be a promising model for the study of the role of the endocannabinoid system during short and long-term development, particularly as it pertains to motor system development and function.

In this study we sought to examine the short-term, long-term, and intergenerational effects of brief (-)THC exposure during early development. Similar to previous studies, we exposed zebrafish embryos during the gastrulation stage (5.25 h post fertilization (hpf) to 10.75 hpf) to (-)THC and focused on locomotion in the first 5 days of development. Next, we reared animals to adulthood (8–11 months) and examined their behavior in open field and novel object approach tests. We then allowed the adults to produce offspring and we examined the offspring (F1 generation) for motor activities. Our data suggest that embryos show deficits in locomotion but when grown to adults, they generally appear normal. However, the next generation of animals (F1) exhibit deficits early in development which larger disappear as development proceeds.

Materials and methods

Animal care and drug exposure

The fish used in this study were wild type zebrafish (*Danio rerio*) of the Tubingen Longfin (TL) strain that were maintained at the University of Alberta Aquatic Facility. All animal housing and experimental procedures in this study were approved by the Animal Care and Use Committee at the University of Alberta (AUP #00000816) and adhered to the Canadian Council on Animal Care guidelines for humane animal use and all researchers complied with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. For breeding, 5 adults, consisting of 3 females and 2 males, were placed in breeding tanks the evening before eggs were required. The following morning, fertilized eggs were collected from the breeding tanks, usually within 30 min of fertilization. Twenty eggs were placed in a plastic petri dish (35 × 10 mm) containing egg water (EW; 60 mg/ml Instant Ocean) exposed to either (-)THC solution (0.001 to 20 mg/L which is equivalent to 3.2 nM − 63.6 µM diluted from a stock solution obtained from Sigma-Aldrich (St. Louis, MO, USA); THC solution 1.0 mg/mL in methanol), or equivalent amounts of methanol during the period of gastrulation (5.25 hpf to 10.75 hpf). The exposure medium was then replaced at 10.75 hpf with 25 mL of fresh EW. Embryos were washed several times in EW and then incubated in fresh EW until further experiments at 48 hpf. Embryos and larvae were housed in incubators on a 12 h light/dark cycle, and set at 28.5 °C.

Zebrafish were reared to adulthood (~ 8 months old) in a recirculating habitat system maintained at 28 °C with a 14/10 light-dark cycle. All other water quality parameters were as previously described[19]. Following adult behavioral testing ("Open Field (OF) Test (Adults)", "Novel object approach (NOA) test (Adults)" Sect.), male and female adults from the vehicle control and the (-)THC groups were bred using the procedure stated above. The fish acquired from these matings (F1 embryos and larvae) were then tested for coiling, touch-evoked responses and swimming, as described later.

Embryo imaging and morphological observations

Embryos were imaged at 2 dpf using a Lumenera Infinity2-1R color microscope camera mounted on a Leica stereomicroscope. Embryos were placed in a 16-well plate with one embryo per well and were anesthetized in 0.02% MS222 (Tricaine methanesulfonate, Sigma-Aldrich). Measurements of embryo length were done using a microscope eyepiece equipped with a micrometer[17]. The number of fish still alive and the number of fish that had hatched out of the chorion were recorded on each day until 5 dpf.

Behavioral observations

Spontaneous coiling activity (1 dpf)

Quantification of spontaneous coiling activity was performed using DanioScope (Noldus; Wageningen, Netherlands) software to analyze video recordings of 1 dpf embryos that were still encased within their chorion. Video recordings of 1 dpf embryos were taken under a dissecting microscope. The spontaneous coiling activity of 1 dpf embryos ($n = 8–54$, per group) shown as a percentage (%) represents the proportion of time that embryos

were actively moving, while the burst count/minute represented the mean number of contractile movements performed by embryos averaged per minute.

Escape response to touch (2 dpf)

Individual 2 dpf larvae ($n = 28–36$, per group) were gently placed into the center of a petri dish (14 cm in diameter) containing 200 mL of egg water, pH 7.0. A thin fishing line of 3 cm was used to stimulate the head of the larvae to trigger an escape response. A fishing line was used because it is translucent and robust enough to accurately target the location of stimulation, without being tracked by the EthoVision software. The petri dish was placed on top of an infrared backlight source and a Basler GenlCaM (Basler acA 1300-60) scanning camera with a 75 mm f2.8 C-mount lens (Noldus) was used for individual larval movement tracking. EthoVision ® XT software (v. 11.5, Noldus) was used to quantify the swimming distance (cm) and swimming velocity (cm/s).

Free swimming (5 dpf)

Locomotion parameters (velocity, swim bouts and activity) were recorded in larvae at 5 dpf ($n = 37–71$, per group) in a 96-well plate. Larvae were gently placed into the center 48 wells of a 96-well plate containing 150 µl egg water, pH 7.0 (Sigma-Aldrich, St. Louis, MO, USA) and were allowed to acclimate for 60 min. Plates were placed on top of an infrared backlight source and a Basler GenlCaM (Basler acA 1300-60) scanning camera with a 75 mm f2.8 C-mount lens, provided by Noldus (Wageningen, Netherlands).

EthoVision ® XT software (v.11.5, Noldus) was used to quantify activity (%), velocity (mm/s), frequency of swim bouts and cumulative duration of swim bouts for one hour. To exclude background noise, a displacement ≥ 0.2 mm was defined as active movement. Activity was defined as % pixel change within a corresponding well between samples (motion was captured by taking 25 samples/frames per second) as reported previously[29].

Adult behavioral testing protocol

All testing was completed in an isolated environmental chamber between the times of 9:00 and 17:00. Arenas for behavioral testing were placed in environmental chambers and were refilled with fresh habitat water prior to each trial. Water temperature for each behavioral test was maintained between 23 and 25 °C for all experimental trials. All subjects were first transported to the environmental chamber in their respective housing tank. Fish remained in their housing tank in the environmental chamber for a minimum of 10 min to recover from potential transportation stress prior to the behavioral tests described below. Fish were tested prior to feeding. In all behavioral tests, video recordings were captured using an overhead camera. Video analysis was performed with EthoVision XT motion-tracking software (v. 11.0, Noldus, Leesburg, VA, USA). Each fish was tested once in each of the behavioral tests by a researcher who was blind to the experimental group.

Open field (OF) test (adults)

The open field test is a reliable assay used in behavioral neuroscience and measures zebrafish locomotion and anxiety-like behavior[30,31]. In this test, adult fish were netted from their respective tanks and released into a circular arena filled with water to a height of 6.5 cm and a diameter of 25 cm. Distance moved and time spent in three virtual zones were quantified using motion-tracking software EthoVision. The three virtual zones included a center zone in the middle of the arena (8.3 cm in diameter), a thigmotaxis zone (25.0 cm in diameter) which was located near the perimeter of the arena and a transition zone located between the center and thigmotaxis zones. Behavior was quantified as time spent within these zones, with a greater amount of time in the thigmotaxis zone being indicative of increased anxiety-like behavior. For each trial, zebrafish were individually netted from their respective treatment tanks and placed in the arena halfway between the center and thigmotaxis zone for a ten-minute trial.

Novel object approach (NOA) test (adults)

The novel object approach test is a well validated measure of boldness behavior. Following the open field test, with the fish in the arena, a multicolored LEGO ® (2 cm x 4.25 cm) novel object was placed in the center of the arena and zebrafish locomotion and location within the various zones of the arena were measured via EthoVision motion-tracking software. The zones used in this test were identical to those used in the open field test. In this test, a greater duration of time spent in the center (or object zone) is indicative of increased boldness whereas a greater duration of time spent in the thigmotaxis zone is indicative of decreased boldness. Trials began approximately 5 s after the addition of the novel object and were 10 min in duration.

Statistics

Values in Fig. 1C, D are reported as means ± SEM (standard error of the mean) whereas in all other figures are reported as violin plots. In all instances, tests for normality/homoscedasticity were first done using the D'Agostino-Pearson normality test. Multiple sample comparisons were achieved using ANOVA followed by Kruskal-Wallis multiple comparison followed by Dunns multiple comparison test (using the statistical software built in to GraphPad Prism, San Diego, CA, USA). Outliers were identified and removed from data sets with the ROUT methods (Q = 1%) using Graphpad Prism.

Results

In this study we examined whether a single exposure to (-)THC in zebrafish embryos, had short-term, long-term and next generation effects. To do this, we exposed zebrafish embryos during gastrulation (5.25–10.75 hpf) to

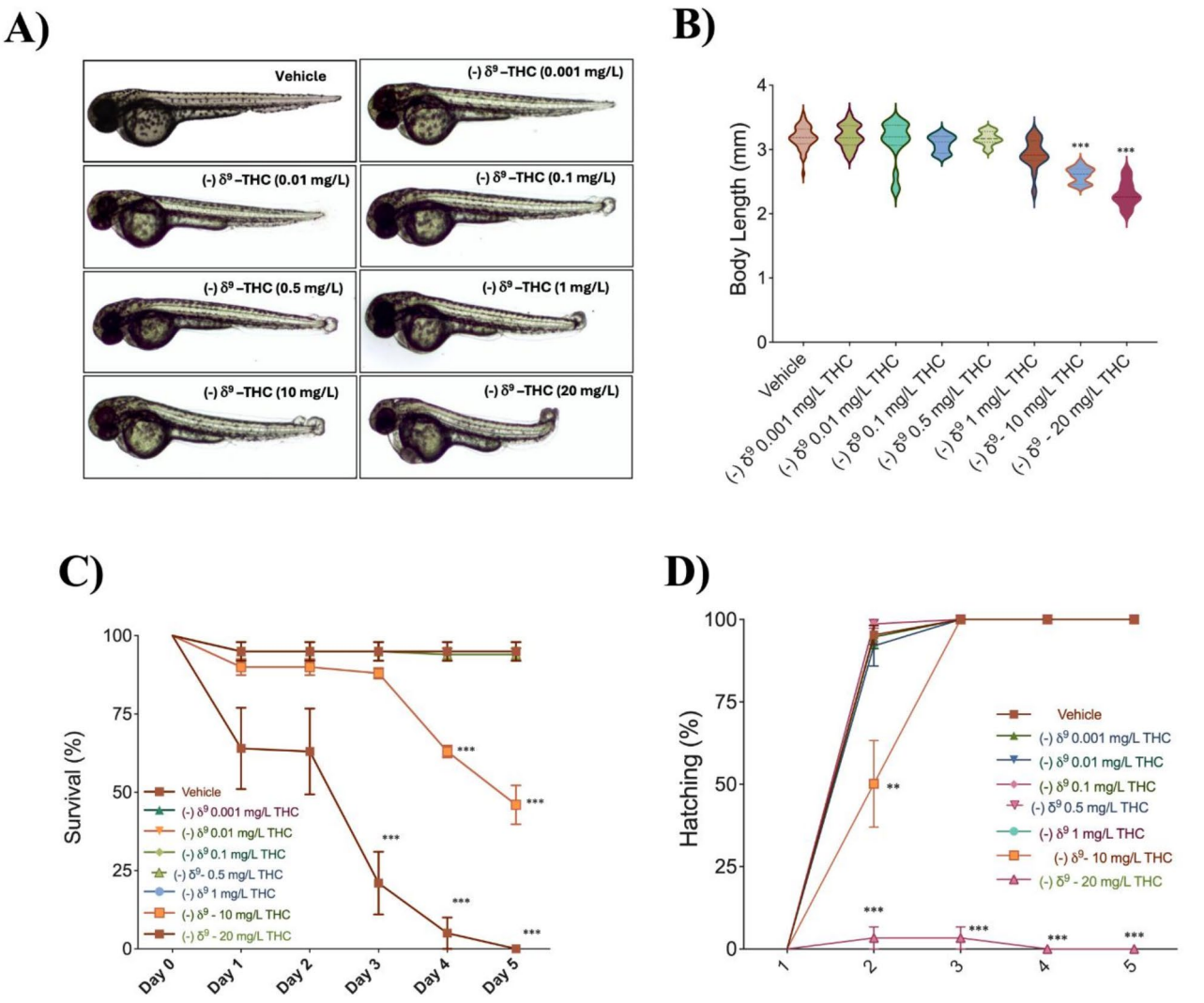


Fig. 1. Effect of (-) THC on the development of zebrafish embryos. (**A**) Embryos were treated with vehicle, or exposed to 0.001 mg/L, 0.01 mg/L, 0.1 mg/L, 0.5 mg/L, 1 mg/L, 10 mg/L, or 20 mg/L (-) THC (from 5.25 hpf to 10.75 hpf) and then allowed to develop in normal embryo media. Images were taken at 48–52 hpf. Representative images are shown. (**B**) Violin plots showing the median body lengths of fish in vehicle control and different concentrations of (-) THC ($N = 3$ experiments and $n = 56$, 26, 18, 8, 27, 16, 13, and 14 for vehicle, 0.001, 0.01, 0.1, 0.5, 1, 10, and 20 mg/L (-) THC-treated fish, respectively). (**C**) Line graph showing the percentage of embryos that survived within the first 5 days of development following (-) THC exposure during gastrulation ($N = 3$ experiments and $n = 20$ embryos for each treatment). (**D**) Line graph showing the percentage of embryos that hatched within the first 5 days of development following (-) THC exposure during gastrulation ($N = 3$ experiments and $n = 20$ embryos for each treatment). For survival and hatching, significance was determined using two-way ANOVA followed by Tukey's multiple comparison tests. For body length, significance was determined using Kruskal-Wallis tests followed by Dunn's multiple comparison test. Groups which share the same letter(s) of the alphabet are not statistically different from one another. * Significantly different from controls $p < 0.05$. **** Significantly different from controls $p < 0.0001$.

concentrations of (-)THC ranging from 0.001 mg/L (3.2 nM) to 20 mg/L (63.6 mM) and examined locomotion in F0 embryos, larvae and adults, and in F1 embryos and larvae.

(-)THC exposure (during gastrulation) affects the morphology, body length, and survival of zebrafish embryos

Exposure to (-)THC had immediate, observable effects on morphology and body length of 2 dpf embryos (Fig. 1A and B). Embryos exposed to 0.1 mg/L (-)THC and above exhibited blebbing in the tail region, which was not statistically quantified (Fig. 1A), while animals exposed to 1 mg/L (-)THC and above were significantly shorter than vehicle controls (Fig. 1B; $p < 0.05$). Lower doses of (-)THC (0.001–0.1 mg/L) did not alter body length.

Animals exposed to 10 mg/L and 20 mg/L of (-)THC had lower survival rates of 46 ± 6% and 0%, respectively, by day 5, compared to controls of 95 ± 3% (Fig. 1C, $N = 3$ experiments with 20 fish per experiment, $p < 0.05$). Hatching rates were also significantly reduced by exposure to the highest concentration of (-)THC (20 mg/L; Fig. 1D, $N = 3$ experiments with 20 fish per experiment, $p < 0.05$). Because most of the fish in the 20 mg/L group did not survive past 2 dpf, we refrained from using them in further experiments.

(-) THC exposure (during gastrulation) affects the locomotion of zebrafish embryos

Next, we tested whether exposure to (-)THC during gastrulation altered locomotion in the first week of development. Zebrafish embryos develop spontaneous coiling activity (i.e. burst activity) within the chorion around 17–19 hpf. This is followed by the development of a touch-evoked escape response around 27 hpf[32] and animals start to swim freely at 72 hpf. Beat and glide type swimming starts to occur by 4–5 dpf. Therefore, we investigated whether (-)THC exposure affected spontaneous activity at 1 dpf, touch-evoked, escape responses at 2 dpf and free swimming at 5 dpf.

Analysis of spontaneous coiling activity at 1 dpf showed that animals exposed to 10 mg/L (-)THC exhibited a reduction in coiling activity, which represents the percentage of time spent coiling, compared to control embryos (Fig. 2A, $n = 21–69$ embryos per group, $p < 0.05$). Moreover, the number of spontaneous coils per minute was significantly decreased in groups exposed to almost all of the concentrations of THC, compared to control embryos (Fig. 2B, $n = 38–76$, $p < 0.05$).

Zebrafish embryos exhibit a robust escape activity referred to as a c-bend, in response to a light touch to the head. We examined the swimming activity immediately following the c-bend and quantified the swimming distance and swimming velocity. Analysis showed that exposure to the lower concentrations of 0.001- 1 mg/L (-)THC did not affect swimming distance or velocity. However, animals exposed to the highest concentration of 10 mg/L (-)THC only swam a distance of 0.44 ± 0.05 cm ($n = 34$) compared with controls of 6.2 ± 1.1 cm (Fig. 2C, $n = 21$, $p < 0.05$). A similar trend occurred for swimming velocity, where lower concentrations of THC had no effect, whereas animals exposed to 10 mg/L (-)THC experienced a significant reduction in velocity (Fig. 2D, $n = 20–35$, $p < 0.05$).

Finally, we recorded the free-swimming activity of 5 dpf larvae for one hour and found that exposure to (-)THC (0.1 mg/L to 10 mg/L) resulted in reductions in swimming activity and swimming distance travelled compared with controls (Fig. 2E, F; $n = 33–82$ in 2E and $n = 42–74$ in 2 F, $p < 0.05$). Taken together these data show that exposure to (-)THC during gastrulation leads to a reduction in locomotor activity in embryonic and larval zebrafish.

Brief exposure to (-) THC during gastrulation has a negligible effect on the behavior of adult zebrafish

To investigate the long-term effects of (-)THC treatment, we reared exposed animals to adulthood and performed an open field test to examine locomotion and anxiety-like behaviour (thigmotaxis), and a novel object approach test to examine boldness and locomotion. We were only able to rear animals treated with concentrations of (-) THC that were lower than 1 mg/L to adulthood with any appreciable numbers to perform the behavioral tests and therefore the concentration ranges for these groups of animals ranged from 0.001 mg/L to 0.5 mg/L.

Open field test

The distance travelled by (-)THC-treated zebrafish was not significantly different compared to vehicle controls (Fig. 3A, $n = 9–59$, $p > 0.05$). Furthermore, the time spent in each of the three zones was not significantly different between controls and any of the treatments (Fig. 3B-D, $n = 9–59$, $p > 0.05$). However, it is worth noting that the variation for the time spent in each of the three zones for animals treated with 0.5 mg/L THC was small compared with all of the other treatments, even though there was no significant difference between the groups.

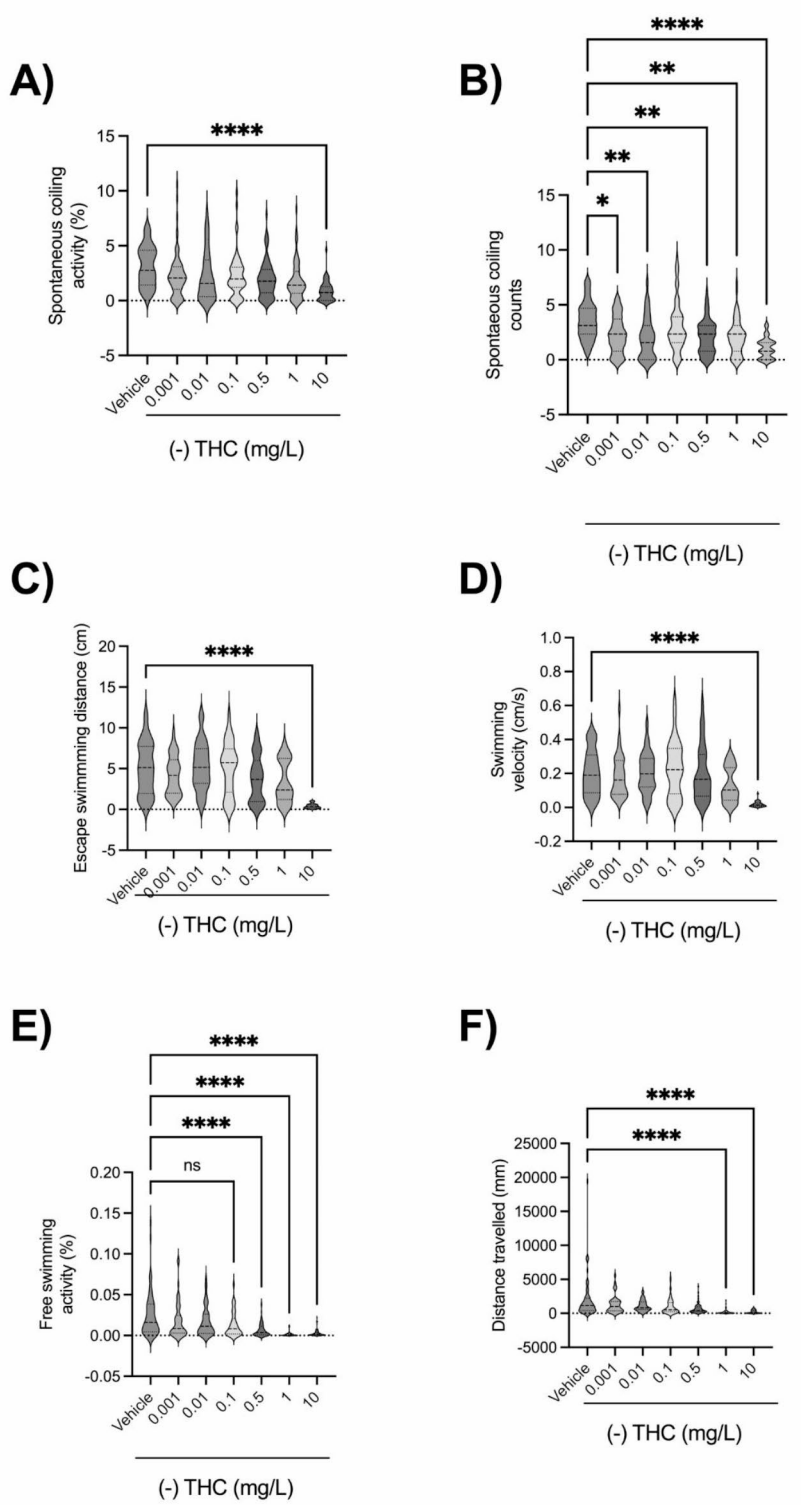
Novel object approach test

There were no significant differences in distance travelled by (-)THC treatment groups compared to vehicle controls (Fig. 4A; $n = 9–59$, $p > 0.05$). Additionally, there were no differences between any (-)THC treatment and control with respect to time spent in the three virtual zones (Fig. 4B-D; ; $n = 9–59$, $p > 0.05$).

The exposure of (-)THC during gastrulation in F0 generation has a transgenerational effect on the locomotion of F1 embryos

To determine if brief exposures to (-)THC during gastrulation had an effect on locomotion in the next generation of animals, we examined coiling, touch-evoked responses and swimming in the F1 generation of embryos whose parents were treated with (-)THC or the vehicle during the gastrulation stage. The F1 embryos exhibited significantly reduced spontaneous coiling activity at 1 dpf for parents treated with 0.001 mg/L to 0.1 mg/L (-) THC (Fig. 5A, $n = 27–45$, $p < 0.05$). Furthermore, the number of spontaneous coils per minute was reduced in all of the treated groups compared with controls (Fig. 5B, $n = 30–67$, $p < 0.05$). Interestingly, the greatest level of significance occurred for the animals treated with the lower concentrations of (-)THC (Fig. 5A-B).

Next, we examined touch-evoked escape responses in 2 dpf animals and found that the distance swam following a touch response was significantly reduced in animals whose parents were treated with 0.1 mg/L and 0.5 mg/L (-)THC (Fig. 5C, $n = 28–48$, $p < 0.05$). Whereas, the swimming velocity was not different amongst any of the groups (Fig. 5D, $n = 28–51$, $p > 0.05$). Finally, we examined free swimming at 5 dpf and found no difference amongst any of the groups when determining swimming activity (Fig. 5E, $n = 21$- b51, $p > 0.05$) or distance (Fig. 5F, $n = 31–46$, $p > 0.05$). Taken together, these data suggest that the offspring of adults that were treated with (-)THC as embryos exhibited differences in locomotion compared to age matched control fish only at the very young ages and that more mature animals appeared normal. These findings show that embryonic exposure to



(-) THC has effects into the next generation of animals but that the deficits may be phased out as development occurs.

Discussion

The goal of the present study was threefold: (1) To investigate the effect of the negative isoform of THC ((-) THC) on locomotion in developing zebrafish, (2) To determine if there are long-term effects on behavior and locomotion in adults, and (3) To determine if locomotion was altered in the next generation of animals. Our key findings can be summarized as follows: First, brief exposure to concentrations above 0.1 mg/L (-) THC can alter morphology while concentrations above 1 mg/L have the potential to affect locomotion in embryos and concentrations above 0.1 mg/L alter movement in larvae. Second, there were no significant effects on locomotion, anxiety-like behavior and boldness in adults. Finally, the offspring of adults that were treated with (-)THC when

◀**Fig. 2**. Effect of transient exposure to (-) THC on locomotion during development of F0 embryos. (**A**, **B**) Spontaneous coiling was recorded from F0 embryos at 1 dpf. (**A**) Violin plot represents mean percentage of time embryos were spontaneous coiling. Fish were treated during gastrulation with vehicle, 0.001, 0.01, 0.1, 0.5, 1, or 10 mg/L (-) THC ($n = 21$, 70, 38, 57, 69, 59, and 50 respectively; $N = 5$ experiments). (**B**) Violin plot represents the median number of spontaneous coils (count/minute). Fish were treated during gastrulation with vehicle, 0.001, 0.01, 0.1, 0.5, 1, or 10 mg/L (-) THC ($n = 66$, 76, 38, 72, 68, 58, and 50 respectively; $N = 5$ experiments). (**C**, **D**) Escape locomotion was recorded from F0 embryos in response to touch at 2 dpf. (**C**) Violin plot represents median distance travelled by embryos in response to touch. Fish were treated during gastrulation with vehicle, 0.001, 0.01, 0.5, 0.1, 1, or 10 mg/L (-) THC ($n = 21$, 35, 30, 29, 30, 29 and 34, respectively; $N = 3$ experiments). (**D**) Violin plot represents median velocity exhibited by embryos during escape response. Fish were treated during gastrulation with vehicle, 0.001, 0.01, 0.5, 0.1, 1, or 10 mg/L (-) THC ($n = 20$, 35, 30, 30, 33, 29 and 35, respectively; $N = 3$ experiments). (**E**, **F**) Locomotor behavior in 5 dpf larvae was determined through 1-hr recordings of the free-swimming activity of individual larvae. (**E**) Violin plot represents median time spent swimming. F0 embryos were treated during gastrulation with vehicle, 0.001, 0.01, 0.5, 0.1, 1, or 10 mg/L (-) THC ($n = 57$, 47, 55, 67, 67, 33, and 82, respectively: $N = 4$ experiments). (**F**) Violin plot represents median distance travelled by larvae in the 1-hour time period. F0 embryos were treated during gastrulation with vehicle, 0.001, 0.01, 0.5, 0.1, 1, or 10 mg/L (-) THC ($n = 57$, 42, 51, 64, 66, 63, and 74, respectively: $N = 4$ experiments). Significance was determined using Kruskal-Wallis tests followed by Dunn's multiple comparison test. * Significantly different from vehicle controls $p < 0.05$. ** Significantly different from vehicle controls $p < 0.01$; *** Significantly different from vehicle controls $p < 0.001$; **** Significantly different from vehicle controls $p < 0.0001$.

they were in the gastrula stage exhibited alterations in locomotion during the embryonic stages but appeared largely normal as development proceeded.

We used a range of concentrations from 0.001 mg/L to as high as 20 mg/L in our preliminary study. We purposely refrained from using the high concentration (20 mg/L) in remaining experiments because the embryos did not survive after 4 days. Human blood plasma concentrations of THC can peak as high as 0.25 mg/L during the smoking of a single cigarette[7]. In this study, we exposed embryos to concentrations ranging from 0.001 to 10 mg/L (0.0033-33.3 µM) (-)THC while the newly fertilized eggs were still in the chorion, or egg casing. Under these conditions, approximately 0.1–10% of toxicants typically cross the chorion[33,34], suggesting that about 0.0001–1 mg/L (-)THC was directly exposed to the embryo.

Here, we examined several types of locomotor activities including spontaneous activity at 1 dpf, escape responses at 2 dpf, and free swimming at 5 dpf. While the escape response is mediated by reticulospinal neuronal activity (M-cell, Mid2cm, Mid3cm neurons)[35–38], swimming is generated by networks of neurons in the spinal cord, including excitatory and inhibitory interneurons, and primary and secondary motor neurons, as well as motor neuron actions on muscle fibers[39,40]. Escape responses and free swimming activity can be categorized as fast (> 30 Hz) and slow frequency (< 30 Hz) swimming[41]. Fast frequency escape responses involve the relay of sensory information to M-cells, which in turn excites a CPG network of neurons in the spinal cord that activates muscle fibers[42]. During fast swimming, more dorsal motor neurons (both primary and secondary) become recruited and activated than ventral motor neurons. White fibers are active during fast swimming but not in slow swimming. In contrast, only the most ventral MNs are active during slow swimming. The red fibers are active during slow swimming and become deactivated during faster swimming. Slow free swimming, which only lasts few seconds, begins to appear at 3 dpf. By 4 dpf, embryos exhibit beat and glide locomotion and by 5 dpf they swim more frequently. Beat-and-glide locomotion consists of swim bouts, i.e., periods of rhythmic tail movement, and alternate periods of rest[43].

Adult zebrafish that were exposed previously to (-)THC (0.001-0.1 mg/L) at gastrulation did not show significant differences in the open field or novel object approach tests relative to controls for any locomotion or zone preference parameter. It is noteworthy that we were only able to rear animals treated with low concentrations of (-)THC (less than 1 mg/L) to the adult stage, and in some of our locomotor tests on F0 animals, these concentrations were generally less effective at altering locomotion. We did not consider the sex of the adult fish and it is feasible that there is a sex difference which may also be determined with additional behavioral tests. Finally, it is noteworthy that the behavior of fish treated with the highest concentration of (-)THC feasible for survival (0.5 mg/L), trended towards very tight ranges even though the median was not significantly different from controls. Our findings are consistent with a previous study which showed that F0 zebrafish embryos treated with low concentrations of THC (less than 2 mg/L) did not show alterations in behavior as adults[21]. Perinatal exposure to CBD and THC in mice over a 5-day period did not affect mobility in adults but did alter repetitive, coping behaviors[44]. This subtle change in antidepressant response suggests neurological changes have occurred due to prenatal cannabinoid exposure, and this possibility still exists in zebrafish.

Our results showed that exposure to (-) THC impacted locomotion of the F1 embryos. Transgenerational effects have been seen in zebrafish where acute exposure to adults resulted in changes to locomotion in F1 embryos[21]. The mechanism by which these effects occur is unknown but may be epigenetic in nature. Multiple human and animal studies suggest that cannabis exposure during development affects epigenetic processes such as DNA methylation and histone modification. For example, prenatal CBD exposure in mice caused behavioral deficits in offspring and perturbation of brain epigenome, i.e., changes in the methylation in the brain through bisulfite sequencing[45]. DNA methylation of human cannabis users differs by at least 10% compared to non-cannabis users and the DNA methylation marks at 6640 CpG sites (cytosine-phosphate-guanine), including at 3979 CpG islands in the gene promoter region were different in the F1 generation. Furthermore, several

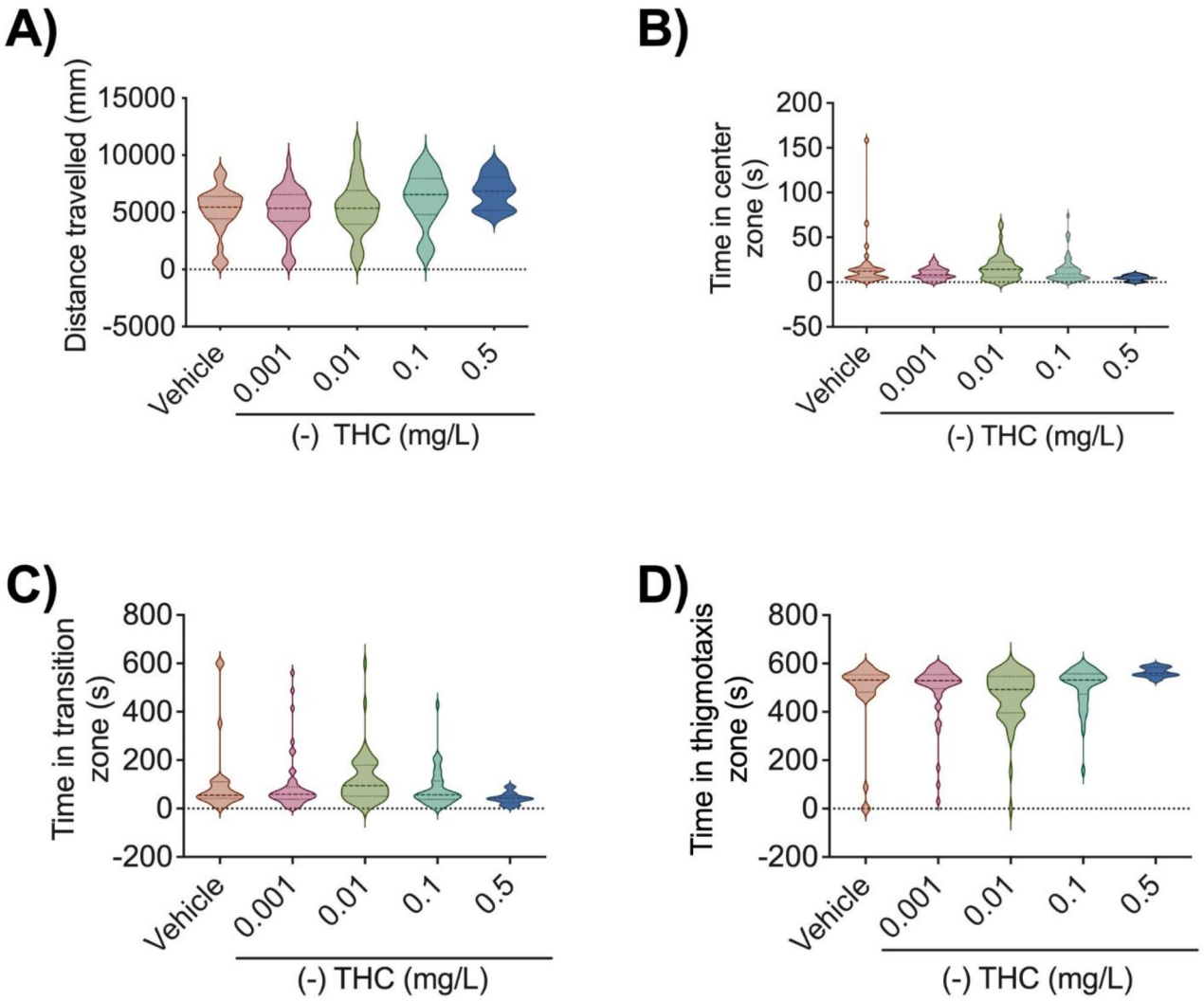


Fig. 3. THC-treated embryos were reared to adulthood and were tested for locomotion in an open field test. Adult zebrafish exhibited normal behavior following a brief exposure to (-) THC as F0 embryos. Open field tests in individual adults (panels **A**–**D**). The open field test was carried out on vehicle controls ($n = 30$) and animals treated with 0.001 ($n = 50$), 0.01($n = 59$), 0.05 ($n = 18$), 0.1 ($n = 44$), and 0.5 ($n = 9$) mg/L (-) THC during gastrulation in the F0 generation. The data is presented in violin plots (**A**-**D**) for (**A**) mean distance travelled, (**B**) time spent in center zone, (**C**) time spent in the transition zone, and (**D**) time spent in the thigmotaxis zone. Significance was determined using KruskaWallis followed by Dunn's multiple comparison tests.

signaling pathways such as oncogenic pathways (i.e., genes encoding for BRAF, PRCACA, AKT1, and FGF), hippo pathways (critical in cancer and embryonic body patterning), MAP kinase pathways, and Wnt signaling pathways were also altered[46].

Taken together, we observed significant impacts of (-) THC on larval zebrafish, and on larval fish in the F1 generation, but not on the adult fish (who bred the F1 generation). The mechanism behind the transfer of the toxic effect of (-) THC may be epigenetic, and via the following: (1) Exposure to cannabinoids might impact any level of the regulatory mechanism mentioned above and disrupt the balance of these processes, thereby influence gene expression[47]. Interestingly, zebrafish embryos exposed to *Cannabis sativa* extracts from 1 dpf to 5 dpf showed no overt negative impacts on movement, but an increase in locomotion at 5 dpf and an over-expression of cnr1 and cnr2 transcripts in 5 day old animals[48] (2) Cannabinoid exposure might affect the demethylation process. DNMTs generate 5-methylcytosine at CpG sites; however, ten-eleven translocation (TET) proteins oxidize 5-methylcytosine into 5-hydroxymethylcytosine that leads to demethylation of the DNA and affect the gene expression. So, cannabinoid exposure might modify the level of DNMTs and TET enzymes and influence gene expression. (3) Cannabinoid exposure might alter posttranslational modification marks of histone tails

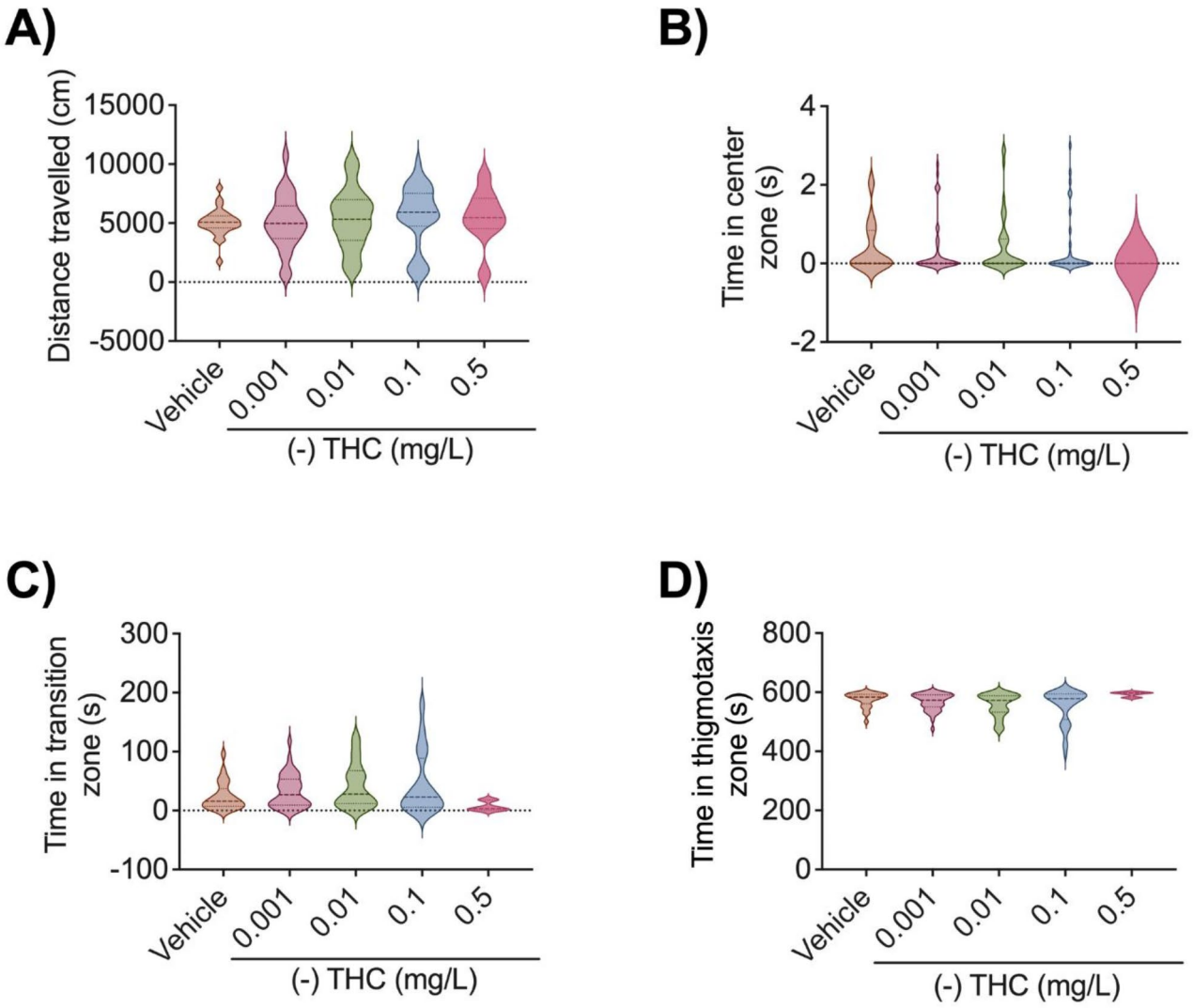
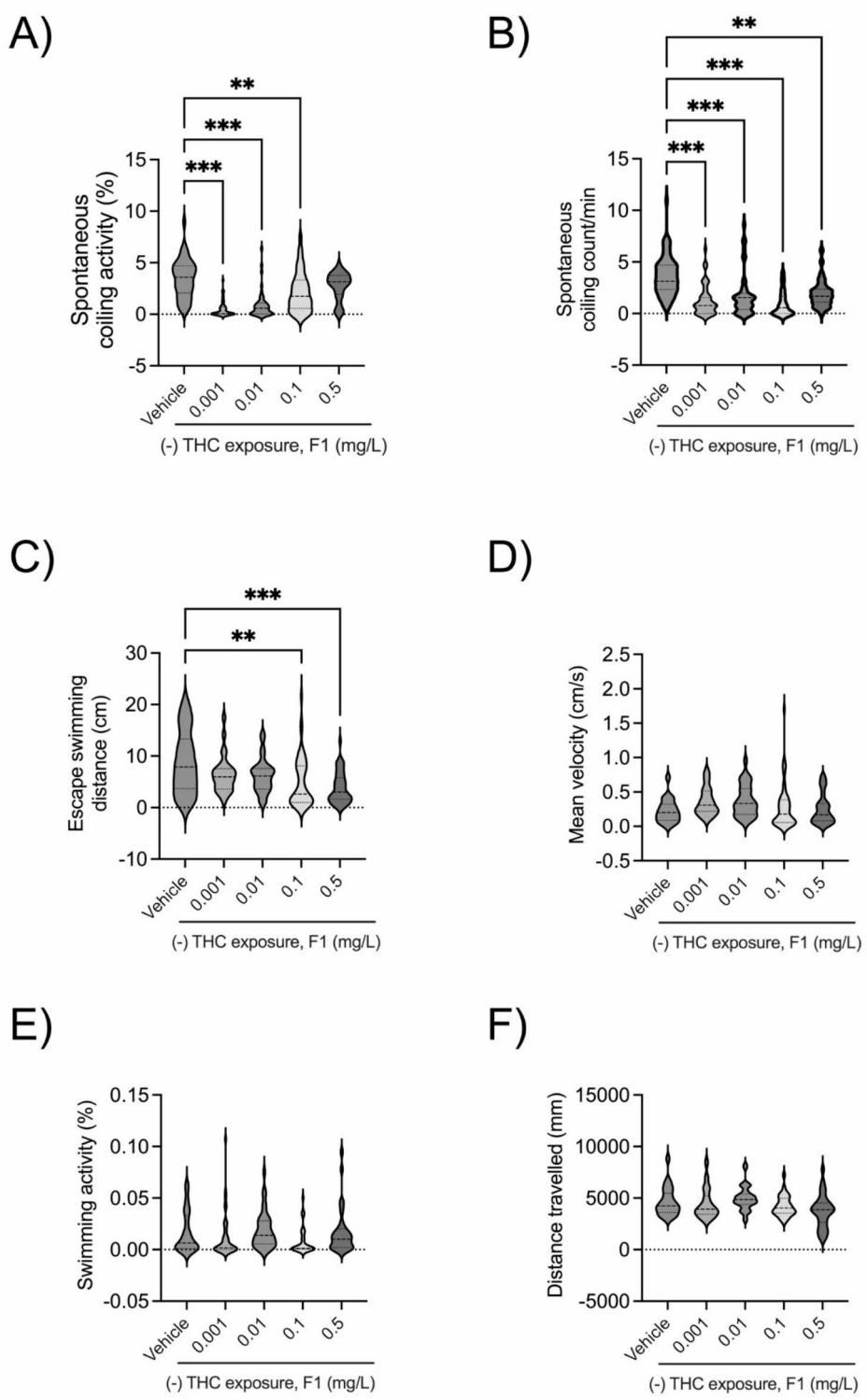


Fig. 4. THC-treated embryos were reared to adulthood and were tested for locomotion in a Novel Object Recognition test. Adult fish had been transiently exposed to either vehicle ($n = 30$), 0.001 ($n = 50$), 0.01 ($n = 59$), 0.1 ($n = 44$), or 0.5 ($n = 9$) mg/L (-) THC during gastrulation as F0 embryos. The data are presented in violin plots (**A**-**H**); (**A**) mean distance travelled, (**B**) time spent in center zone, (**C**) time spent in the transition zone, and (**D**) spent in the thigmotaxis zone. Significance was determined using Kruskal-Wallis followed by Dunn's multiple comparison tests.

such as methylation and acetylation, which are catalyzed by histone methyltransferases (HMT) and histone acetyltransferases (HAT), respectively. Acetylation of DNA is permissive, whereas deacetylation is mediated by histone deacetylases (HDAC) that lead to repression of transcription. Hence, cannabinoids exposure might influence the methylation or acetylation marker of the histone by upregulating or downregulating HMT, HAT, and HDAC enzymes or related proteins. These actions would result in alterations in the expression of critical functional genes. (4) Cannabinoid exposure might also affect the level of non-coding microRNAs (miRNA), which are produced from specific genes and target protein-coding RNA (mRNA) for degradation; these processes would influence protein production[47]. miRNAs can modify gene expression through complementary binding to the target sequence and prevent the binding of the transcriptional machinery, leading to restriction of gene expression of a particular gene. Such miRNAs-mediated regulation of gene expression has been shown for *bdnf*, and other genes involved synaptic plasticity[49].


A)
Spontaneous coiling activity (%)
15
10
5
0
-5
**
***
***
Vehicle
0.001
0.01
0.1
0.5
(-) THC exposure, F1 (mg/L)
B)
Spontaneous coiling count/min
15
10
5
0
-5
**
***
***
***
Vehicle
0.001
0.01
0.1
0.5
(-) THC exposure, F1 (mg/L)
C)
Escape swimming distance (cm)
30
20
10
0
-10
***
**
Vehicle
0.001
0.01
0.1
0.5
(-) THC exposure, F1 (mg/L)
D)
Mean velocity (cm/s)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Vehicle
0.001
0.01
0.1
0.5
(-) THC exposure, F1 (mg/L)
E)
Swimming activity (%)
0.15
0.10
0.05
0.00
-0.05
Vehicle
0.001
0.01
0.1
0.5
(-) THC exposure, F1 (mg/L)
F)
Distance travelled (mm)
15000
10000
5000
0
-5000
Vehicle
0.001
0.01
0.1
0.5
(-) THC exposure, F1 (mg/L)

◀**Fig. 5**. Untreated F1 embryos exhibited altered locomotion following an exposure to (-) THC during gastrulation as F0 embryos. (**A**, **B**) Spontaneous coiling was recorded from F1 embryos at 1 dpf (within the chorion) collected from (-) THC or vehicle-treated parents. (**A**) Violin plot represents the median percentage of time embryos were spontaneous coiling. F1 embryos from vehicle treated parents ($n = 45$), and 0.001 mg/L ($n = 40$), 0.01 mg/L ($n = 45$), 0.1 mg/L ($n = 42$), and 0.5 mg/L (-) THC ($n = 27$). (**B**) Violin plot represents the median number of spontaneous coils (count/minute). F1 embryos from vehicle treated parents ($n = 67$), and 0.001 mg/L ($n = 40$), 0.01 mg/L ($n = 45$), 0.1 mg/L ($n = 67$), and 0.5 mg/L (-) THC ($n = 30$). (C, D) Escape locomotion in response to touch of unexposed F1 embryos at 2 dpf collected from (-) THC or vehicle-treated parents. (**C**) Median distance travelled by embryos in response to touch. F1 embryos from vehicle treated parents ($n = 33$), and 0.001 mg/L ($n = 28$), 0.01 mg/L ($n = 31$), 0.1 mg/L (-) ($n = 48$), and 0.5 mg/L (-) THC ($n = 43$)-treated parents. (**D**) Median velocity during escape response are presented. $N = 5$ experiments. F1 embryos from vehicle treated parents ($n = 21$), and 0.001 mg/L ($n = 28$), 0.01 mg/L ($n = 31$), 0.1 mg/L ($n = 50$), and 0.5 mg/L (-) THC ($n = 51$)-treated parents. (**E** and **F**) Violin plot represents median time spent swimming (**E**) and the median distance travelled (**F**) by larvae in the 1-hour time period for larvae collected from vehicle-treated ($n = 46$), and 0.001 mg/L ($n = 40$), 0.01 mg/L ($n = 37$), 0.1 mg/L (-) ($n = 35$), and 0.5 mg/L (-) THC ($n = 31$) -treated parents. Significance was determined using Kruskal-Wallis followed by Dunn's multiple comparison tests. ** Significantly different from vehicle controls $p < 0.01$; *** Significantly different from vehicle controls $p < 0.001$.

Conclusions

Cannabis use for recreation and as a potential therapeutic agent is increasing throughout the world, yet an understanding of how it may alter embryonic development is unknown. This study is particularly relevant to the potential teratogenic effects of (-)THC if a pregnant mother consumed cannabis in the first 4 weeks of gestation, when most signs of pregnancy are not yet evident. Here we found a significant impact on morphology and locomotion that persisted into the next generation. Future studies are essential to further examine the impact of (-)THC and other cannabinoids on embryonic development and that caution should be taken when considering consumption of cannabis at any stage of pregnancy.

Data availability

Data is provided within the manuscript or supplementary information files.

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Author contributions

M.R.A., T.J.H. and D.W.A. conceived of the study and designed the experiments.M.R.A., L.K. J.S. and A.M.K. performed experiments for the study.M.R.A. prepared Figs. 1 and 2.J.S. prepared Figs. 3 and 4.L.K. prepared Fig. 5.M.R.A., T.J.H. and D.W.A. wrote the manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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