Research Project: (-)-α-pinene Anxiolytic Effects and Boldness in Zebrafish (*Danio rerio*)

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Introduction

In 2016 recreational cannabis use became legal in Canada and this has opened the interest people have in cannabinoid products and their effects (Government of Canada, 2021). The legalization of cannabis has supported different strains of cannabis and products being available on the market as well as the promotion of different claims they may have for the consumer. It is important for these claims to be researched and this provides the potential for more studies into different components of cannabis to understand what their impacts are.

Some compounds present in cannabis substances are terpenes; one of the most common terpenes is pinene. Pinene is a monoterpene hydrocarbon ($C_{10}H_{16}$) used in different perfumes, oils, medicines, and food (Sigma n.d). Pinene is found to be produced naturally in conifers resulting in a pine scent used to attract pollinators (Weston-Green et al., 2021). Plants produce this terpene to help protect them in their interactions with mold, fungus and predators and is shown to have a variety of effects on people (Weston-Green et al., 2021).

Zebrafish are an excellent model organism being increasingly used in studies including anxiety-like behaviour modification from exposure to different terpenes (Szaszkiewicz et al., 2021). Borges et al. (2020) found there were anti-inflammatory effects on zebrafish when they were exposed to a product that had **α**-pinene and found that the pinene contributed to it and more medicinal findings have been linked to **α**-pinene (Saheli et al., 2019). There has also been some research into if humans can distinguish between these odorants and these found that people could discriminate between the enantiomers of **α**-pinene (Laska & Teuber, 1999). More recently, research has shown slight differences on the interactions of the enantiomers in medicinal tests and so there would be benefit in isolating the different enantiomers of **α**-pinene to get more information on the impact each have (Rufino et al., 2014, as cited in Salehi et al. 2019).

The software EthoVision XT (v. 11, Noldus, VT, USA) has been used in similar studies for tracking the motion of the zebrafish and using the data to look at their behaviour (Szaszkiewicz et al., 2021). Observing the fish movements in this arena will allow us to deduce how anxious the fish is in the open field test by where in the arena they are spending most of their time. If they spend more time in the outer thigmotaxis zone that is a sign of anxious behaviour but if they are towards the middle and showing a change in locomotion we may observe anxiolytic effects of the terpene (-)-**α**-pinene. In the novel object test if the fish spends more time in the center approaching the novel object test then the fish are displaying more boldness, which we hypothesize the (-)-**α**-pinene will have an anxiolytic effect decreasing anxiety and increasing boldness in the fish.

Methods

Zebrafish Husbandry

Our zebrafish are a wildtype strain and were bred in house at MacEwan University originally from a brood stock from the University of Ottawa in March 2018. The zebrafish are housed in an Aquatic Habitats (AHAB, Aquatic Ecosystems, Inc. Apopka, FL, USA) three-shelf bench top system (Szaszkiewicz et al., 2021). The system contains 24 tanks that hold 15 fish each and 6 large tanks that can fit 50 fish. Laboratory staff ensure daily checks are conducted on water pH ensuring it is between 6.5 and 8.0, dissolved oxygen is 5-10ppm, and temperature is between 26-28 ºC. This system contains environment stickers on the back and bottom of the tanks for enrichment and are typically housed in groups. They are fed food (GEMMA Micro, Westbrook, Maine, United States of America) around the same time each day by staff unless otherwise instructed for research in which case they would be fed after testing. The lights are set on a timer to ensure a consistent schedule for the fish is maintained with the lights going on at

8am and off at 8pm while the lab is technically open from 8am-6pm for those with authorized access. The addition of (-)-**α**-pinene was added to the existing protocol for these research procedures that was already approved by the MacEwan Animal Research Ethics Board (AREB) to ensure that the study would follow Canadian Council for Animal Care (CCAC) standards. The previous approved experiment by Szaszkiewicz et al. (2021) this is modeled after made sure to follow ARRIVE guidelines for animal research.

Procedure

The (-)-**α**-pinene is sourced from Sigma Aldrich (Ontario, Canada). Previous test experimentation with **α**-pinene used concentrations of 0.02%, 0.01% but with no observable effects and to replicate the methods also used these concentrations as well as a higher dose with the enantiomers to see how they might impact the fish. Using the (-)-**α**-pinene concentrations of a 0% control, 0.01%, 0.02% and 0.1% the study will follow a similar acute administration following Szaszkiewicz et al. (2021) procedure. These concentrations were converted into their molar equivalents and put into the program Prism for analysis. There was one control group with 3 different (-)-**α**-pinene concentration groups 0.01%, 0.02% and 0.1% and the zebrafish were randomly distributed to each. Approximately 15 fish were randomly assigned per group. The fish were exposed to either the control or (-)-**α**-pinene by placing them in dosing tanks for 10 minutes, after which the fish were placed in the testing arena for the open field test for 10 min and then ran for another 10 min for the novel object test after inserting the LEGO ® figurine. We used a particular testing arena with a diameter of 26cm that has a ‘center’ zone in the center with a diameter around 8.5cm, a transition zone surrounding the center zone and a thigmotaxis zone with around 8.5cm from the walls of the arena which surrounds the transition zone (Szaszkiewicz et al., 2021). Throughout the open field test and novel object test movement and

location of the fish were observed within the testing arena circles either in the inner, transition or thigmotaxis layer using EthoVision software for tracking.

The (-)-**α**-pinene solutions were made as needed. Solutions were made using a 600ml beaker after filling it with 400ml of habitat water and recording the habitat water pH (should be between 6.8-7.5). Then the correct amount of (-)-**α**-pinene (40 µl for 0.01%, 80 µl for 0.02% and 400 µl for 0.1%) was vigorously mixed with the habitat water and the pH was measured again (should be between 6.8-7.5), except for the control in which no (-)-**α**-pinene was added. Since one person was responsible for preparing the (-)-**α**-pinene terpene solutions and running the tests, the tests were not blind but there was consistency in the process. To keep the temperature the same for the fish (between 25-29°C) all dosing beakers, arena and tanks in the testing arena no longer hooked up to habitat shelf were put on heat mats (Hydrofarm Horticultural Products, Petaluma CA).

The appropriate number of fish tested on a particular day were netted from the large naive pool of fish and put in one of the separate smaller housing tanks. Once ready to conduct the experiment the housing tank with naive fish were brought carefully to the testing room. In the testing room white rectangular dividers were placed around the tank to prevent disturbance for the fish and so they cannot see the ones being dosed. To help reduce any visual stimulation for the fish the dosing beaker was covered with a white piece of plastic around it. The fish being dosed were also not be able to see their conspecifics because Dean et al. found that seeing their conspecifics impacted their anxiolytic response to ethanol and so want to control their surroundings to reduce other potential variables therefore increase replicability of the study (2021). A zebrafish would be taken from the housing tank using a net and put into the 600ml dosing beaker with the appropriate terpene concentration with the white dividers surrounding it

and remain in it for 10 minutes. The arena for the open field test filled with 5cm of habitat water is a hard white plastic container placed on the heat mat. After the 10-minute dosing period, the dosing beaker was poured into an empty beaker with a net over top to separate the fish from the water easily. Once the fish is netted it is put in the arena halfway between the center and thigmotaxis zones for the open field test where the EthoVision is set up with the heat maps to record the movement of the fish in the various areas of the arena for 10minutes. After the 10 minutes a LEGO ® figurine (2cm x 4.25 cm) is attached to the middle of the arena with a LEGO® piece facing the same direction in each trial for the novel object test and the fish was observed and recorded for another 10 minutes. The LEGO ® figurine is multicolored to rule out any potential colour preferences for the fish and is the same figurine used in similar experiments with **α**-pinene. After the 10 minutes the fish was removed with a net and placed in a beaker with habitat water to be sexed and then put into a habitat tank for the fish that have been experimented on and will need to be euthanized by qualified staff at a later date. Every 4 trials the habitat water in the be replaced.

Statistics

Analysis of the data will use GraphPad Prism (Version 9.1.0; GraphPad, San Diego, CA, USA). Statistics was completed with the assistance of Prism using the the D'Agostino-Pearson omnibus normality test, if there is normal distribution the parametric data was subject to the one-way ANOVA test and data that is not normally distributed and therefore is non-parametric was analyzed by the Kuskal Wallis test. The parametric or nonparametric data will then be subject to either the post-hoc Dunnett's or post-hoc Dunn's multiple comparison test respectively. EthoVision software will measure the heatmaps of the fish and detect their distance moved, velocity, meandering (measuring the degree of change in their movement) and mobility. Fish that

pixels on the tracking software demonstrate 5% change or less considered immobile, 60% or higher considered highly mobile any fish immobile for 100 seconds in either test was removed from the study when examining time in zones for consistency with other experiments and to rule them out as outliers. Fish with higher mobility than 100secs have been left in when observing locomotion for an increased sample size and examination of the impacts of (-)-α-pinene.

Results

Open Field Test

Locomotion. Using the data without exclusions due to immobility (Fig. 3) we observed a significant difference in both distance moved and velocity between the controls and concentrations using the One-Way ANOVA test ($F(3, 62)=14.71$, $P <0.001$). Using the Kruskal-Wallis test we observed no significant difference between groups in immobility (Fig. 1E; $P=0.055$) and in meandering (Fig. 1C; $P=0.467$) but we saw a significant difference in high mobility (Fig. 1D; $P <0.001$). Using the Dunn's multiple comparisons test of each group to the control there appeared to be a significant difference between the control and 0.1% group in the distance moved (Fig. 1A; $P <0.001$), velocity (Fig. 1B; $P <0.001$) and high mobility (Fig. 1D; $P <0.001$).

Time in zones. Using the data that excluded results with immobility over 100 seconds (Fig. 3) and the Kruskal-Wallis test there was significant difference noted for the center zone (Fig. 1F; $P <0.001$), the transition zone (Fig. 1G; $P <0.001$) and thigmotaxis (Fig. 1H; $P <0.001$). Using the Dunn's multiple comparison test the 0.1% treated group compared to the control showed that there were significant differences found in the center zone (Fig. 1F; $P=0.004$), transition zone (Fig. 1G; $P=0.003$) and thigmotaxis (Fig. 1H; $P=0.004$).

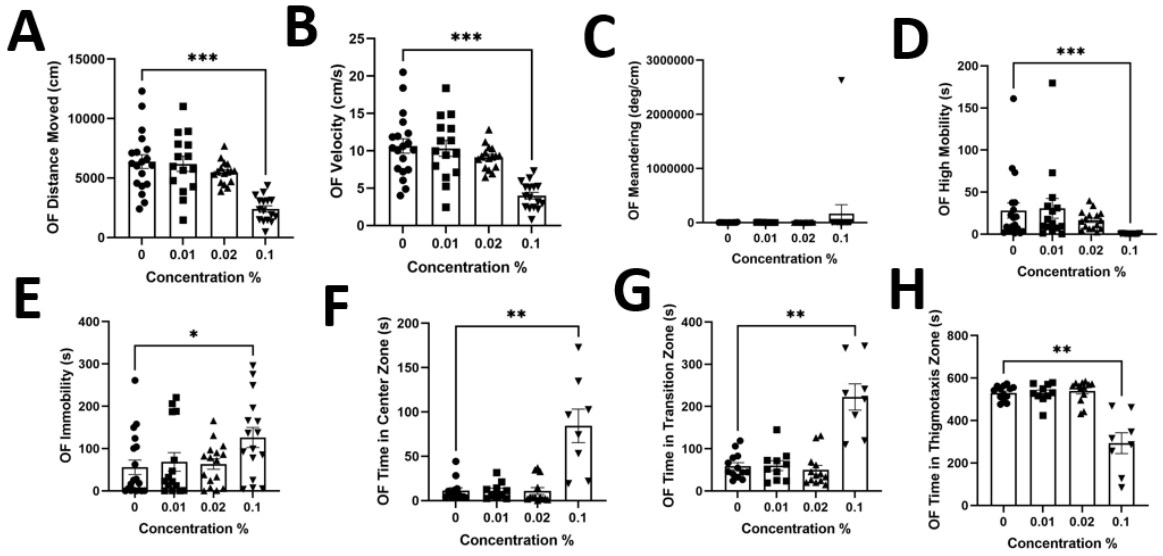

Figure 1- *Open Field Test Graphs.* The effects of acute (-)-**α**-pinene administration on zebrafish behaviour assessed by the open field test. Fish locomotion was determined in the open field test by measuring distance moved (A), velocity (B), meandering (C), time spent highly mobile (D), and time spent immobile (E). The average time spent in the center zone (F), transition zone (G), and thigmotaxis zone (H) zone was also observed during the open field test. All data are presented as mean ± S.E.M. Significant differences between controls and (-)-**α**-pinene treated groups are indicated by *(P < 0.05), **(P < 0.01) and *** (P < 0.001).

Novel Object Approach Test

Locomotion. Using the data without exclusions due to high immobility (Fig. 3) comparing the control to the concentration from the One-Way ANOVA test we observed a significant difference in both distance moved (Fig. 2A; $F(3, 62)=9.711$, $P <0.001$) and velocity (Fig. 2B; $F(3, 62)=9.709$, $P <0.001$). Using the Kruskal-Wallis test we observed no significant difference between groups in meandering (Fig. 2C; $P=0.834$), and immobility (Fig. 2E; $P=0.138$)

but saw a significant difference in high mobility (Fig. 2D; P <0.001). Using a Dunn's multiple comparisons test of each group to the control there appeared to be a significant difference between the control and 0.1% group in the distance moved (Fig. 2A), velocity (Fig. 2B), and high mobility (Fig. 2D), all at P <0.001.

Time in zones. Using the data that excluded results with immobility over 100 seconds (Fig. 3) and the Kruskal-Wallis test there was no significant difference noted for the center zone (P=0.297) but there was significant difference observed in the transition zone (P=0.048) and thigmotaxis (P=0.049). However, using the Dunn's multiple comparison test no significant differences were found in any of the groups compared to the control.

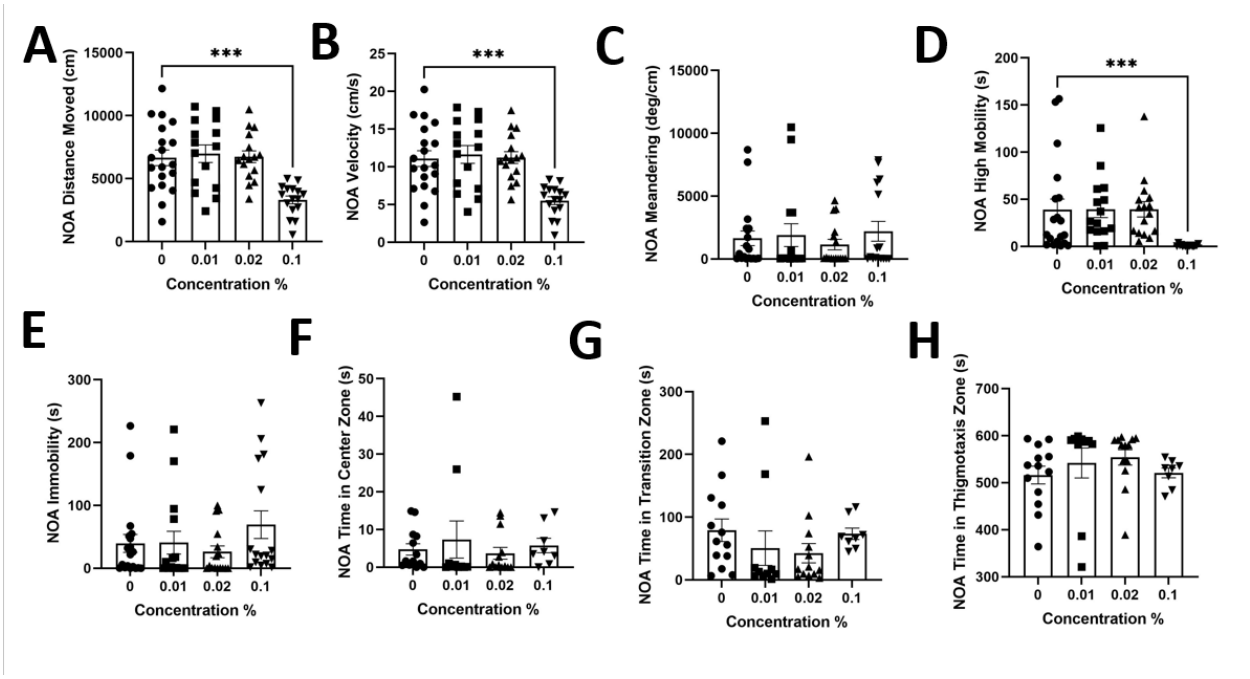

Figure 2 *Novel Object Approach Test Graphs.*

The effects of acute (-)-**α**-pinene administration on zebrafish behaviour assessed by the novel object approach test. Fish locomotion was determined in the novel object approach test by measuring distance moved (A), velocity (B), meandering (C), time spent highly mobile (D), and

time spent immobile (E). The average time spent in the center zone (F), transition zone (G), and thigmotaxis zone (H) zone was also observed during the test. All data are presented as mean ± S.E.M. Significant differences between controls and (-)-**α**-pinene treated groups are indicated by *** (P < 0.001).

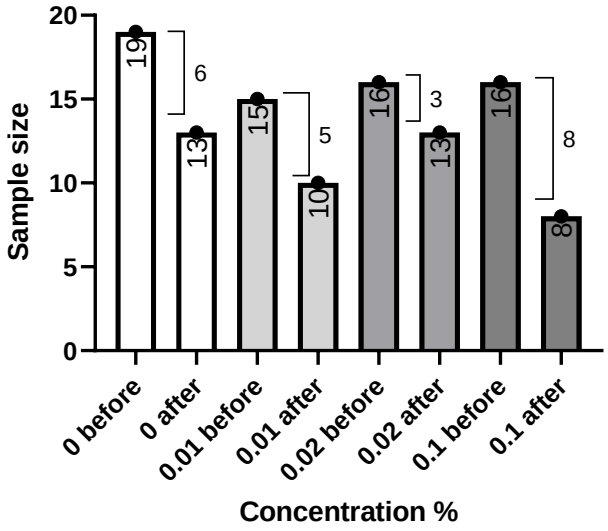

Figure 3- *Sample size before and after immobility >100sec omitted.* The graph shows the number of zebrafish included in the statistics in the locomotion results (before) which includes the data from 19 control (0%), 15 0.01% group, 16 0.02% group and 16 in 0.1% group. It also shows the number of fish included in the statistics when examining the time spent in each zone (after) after the ones with immobility over 100secs were omitted which included 13 fish in the control group (0%), 10 in the 0.01% group, 13 in 0.02% group and 8 left in the 0.1% group.

Discussion

This study investigated the effect of exposing different concentrations (control/0%, 0.01%, 0.02% and 0.1%) of the terpene (-)-**α**-pinene to zebrafish and observed their behaviour in an open field test and novel object approach test. A significant difference was observed in the open field test between the control and 0.1% concentration group in the center, transition and thigmotaxis zones (P < 0.01), The location of the fish in the open field test demonstrates that the

substance is having an impact on anxiety like behaviour at the 0.1% concentration. There was a smaller but still significant difference found in the open field test with immobility ($P < 0.05$) (Fig. 1E). There was a preference towards a higher amount of time immobile in the open field test between the control and 0.1% group this could indicate a relaxed state if in line with the preference towards the center, however results on the connection to immobility in this way were not found in the open field test results of Szaszkiewicz et al. with different terpenes (2021). As a result, this difference in immobility could be unique to (-)-**α**-pinene but could also be from the inclusion of the high immobility fish used in the immobility results compared to the location in zones and shows the high immobility that the greater concentration caused in some of the trials (Fig. 3). In the time spent in zones for the novel object approach test (Fig. 2F-H) no significant differences were found which indicate the exploratory behaviour was not impacted and there was not an impact on boldness observed in this study. The results found no significant differences in meandering for either test (Fig. 1C, and 2C) which is consistent with the lack of this measure of erratic behaviour also found when zebrafish were exposed to the terpenes limonene, linalool and myrcene (Szaszkiewicz et al., 2021). Significant differences were seen ($P < 0.001$) amongst velocity (Fig. 1B, and 2B), high mobility (Fig. 1D and 2D) and distance moved (Fig. 1A and 2A) among both tests between the control and fish exposed to 0.1% (-)-**α**-pinene showing that the substance was having a negative or slowing effect to their motion at a higher dose. It also shows that there was still a residual effect of some kind by the novel object approach test. There is opportunity for more analysis of the data to include the immobile fish and with further testing to look at how it could impact the results. In conclusion, the results suggest that due to the decrease of time spent in the thigmotaxis during the open field test that it could be showing that the higher dose of 0.1% (-)-**α**-pinene produce some anxiety like reducing effects. It also suggests that a

higher concentration of (-)-**α**-pinene influences locomotion decreasing the amount the fish is active and warrants future investigation into the effects of other (-)-**α**-pinene concentrations and terpenes.

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