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(RESEARCH ARTICLE)

# The Influence of Tetrahydrocannabinol (THC) on anxiety-like behavior in zebrafish larvae: A light-dark assay study

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## Abstract

Cannabis is a commonly used illicit substance, often consumed for its potential to promote relaxation and alleviate anxiety. This study employed the light-dark transition test to examine the effects of acute delta-9-tetrahydrocannabinol (THC) exposure on anxiety-like behavior in larval zebrafish. At 7 days post-fertilization, zebrafish larvae were exposed to one of several treatments: egg water (control), 1% dimethyl sulfoxide (DMSO, vehicle control), 100 mg/L caffeine in egg water, 10 mg/L alprazolam in DMSO, and 0.01 and 0.1 mg/L THC in DMSO for 2 hours. Their behavior was then recorded during alternating light and dark periods to measure the distance traveled in each condition. Both egg water and DMSO controls demonstrated significantly higher activity levels in the dark compared to the light ($p = 0.003$ and 0.011, respectively). THC exposure exhibited a biphasic effect in the dark period relative to the DMSO control: lower concentrations of THC (0.01 mg/L) increased distance traveled, whereas higher concentrations (0.1 mg/L) decreased it, though these effects were not statistically significant. Additionally, larvae exposed to 0.01 mg/L THC showed higher locomotion in both light and dark periods compared to those exposed to alprazolam, but this difference was not significant. These findings suggest that THC has both anxiolytic and anxiogenic effects in the light-dark transition assay, aligning with some existing research on THC's impact on anxiety. Further studies are needed to draw more definitive conclusions, but this research provides valuable insights into THC's effects on anxiety-like behavior.

**Keywords:** THC; Zebrafish larvae; Acute Exposure; Anxiety-like Behavior; Light-Dark Assay

## 1. Introduction

Approximately 147 million people globally consume cannabis annually, representing about 2.5% of the world population, making it the most widely used illicit drug [1]. Cannabis, commonly known as marijuana, consists of various preparations derived from the Cannabis sativa plant. The primary active compounds in cannabis are cannabinoids, with delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD) being the most prominent. Unlike CBD, THC is psychoactive [2, 3], meaning it influences mental processes such as perception and mood [4] and can induce a euphoric state. While cannabis is often used recreationally for this reason, it also has recognized medicinal properties, and there are reports of individuals using it to alleviate anxiety [5, 6].

THC exerts its medicinal effects primarily through the endocannabinoid system (eCBs), which comprises cannabinoid receptors (CBRs), their endogenous ligands, and associated enzymes and transporters [7]. The two primary cannabinoid receptors, CB1 and CB2, are predominantly located in the central nervous system [2, 7]. THC activates these G-protein coupled receptors, initiating a signaling cascade that can inhibit the release of neurotransmitters such as gamma-aminobutyric acid (GABA) [7, 8]. GABA neurons in the amygdala, a brain region crucial for emotion processing and stress response, play a key role in modulating anxiety and stress [9]. Therefore, as a CBR agonist, THC's interaction with the eCB system and its effect on GABA regulation suggests its potential use in managing anxiety.

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While studies have indicated that some individuals use cannabis to alleviate anxiety [5, 6], there remains debate over whether cannabis might contribute to the development of anxiety disorders [10]. These mental health conditions are among the most common worldwide and arise from dysfunctions in the brain's threat-response circuitry [11, 12]. This dysfunction leads to excessive fear or heightened avoidance in reaction to perceived threats, negatively impacting quality of life. Anxiety can manifest in symptoms such as irritability, difficulty concentrating, sleep disturbances, and a persistent sense of impending danger [12]. According to the WHO [12], only about 25% of those with anxiety disorders globally receive treatment. Current interventions typically involve selective serotonin reuptake inhibitors (SSRIs) or benzodiazepines, but these treatments can have side effects and the potential for addiction [12]. Therefore, there is a pressing need for alternative treatment options.

To explore alternative treatments, researchers have turned to animal models such as zebrafish (*Danio rerio*). Zebrafish possess many neurotransmitters found in humans and their neuroendocrine system can elicit significant physiological responses to stress [13]. Evidence shows that anxiolytic drugs can influence zebrafish behavior, indicating that the pathways involved in anxiety regulation are conserved across zebrafish and mammals [14]. Various assays have been developed to assess zebrafish responses to stressors [13], with novelty and light being identified as key factors that can induce anxiety-like behavior [14]. Zebrafish display a preference for dark areas (scototaxis) and exhibit reduced movement when exposed to light [14]. Additionally, a tendency to stay near the edges of their environment (thigmotaxis) is another sign of anxiety-like behavior [15]. Therefore, in light-dark transition assays, zebrafish typically show increased movement during dark periods compared to light periods [16, 17, 18]. Analyzing these movement patterns in such assays provides valuable insights into their anxiety levels.

This paper aims to investigate the impact of THC on anxiety-like behavior in larval zebrafish using the light-dark assay. Given that the endocannabinoid system (eCBs) is highly conserved in zebrafish, this model can help elucidate whether THC has anxiolytic or anxiogenic effects. The findings could offer valuable insights for clinical research on THC as a potential alternative treatment for anxiety disorders.

## 2. Material and Methods

*Preparation of Solutions*: Egg water was prepared by adding 1.5 mL of a 40 g/L sea salt solution to 1 L of distilled water, following the procedure described by Westerfield [19]. To prevent fungal growth, 2 drops of methylene blue were also added [20]. Caffeine was dissolved in this egg water at a concentration of 100 mg/L. Alprazolam (10 mg/L) and THC (0.01 and 0.1 mg/L) were dissolved in 1% DMSO to enhance solubility.

*Zebrafish Care and Breeding:* Adult male and female zebrafish were housed separately in tanks measuring 18”x12” under controlled conditions: 28°C and a 14-hour light/10-hour dark cycle. The tanks contained fresh fish water with a conductivity of approximately 1,500 µS/cm and a pH of 7. Air stones were used to maintain an oxygen concentration of 8.0 mg/L. The fish were fed powdered fish flakes twice daily. For breeding, healthy males and females were transferred to a separate tank overnight using a fish net, ensuring an equal number of each sex. The following morning, the adult zebrafish were removed from the breeding tank, leaving behind the fertilized eggs, which were then transferred to a container with egg water (EW) maintained at 28°C.

*Treatment Exposure:* At 7 days post-fertilization (dpf), larvae were transferred to white 12-well plates using a pipette, with each well containing 1.5 mL of media. The larvae were exposed to one of six treatment groups: egg water control, DMSO control, caffeine, alprazolam, and two THC concentrations. Each treatment group consisted of 6 larvae. The larvae were exposed to the treatments for 2 hours before being subjected to the light-dark transition assay.

*Behavioral Analysis:* At the conclusion of the 2-hour drug treatment, the 12-well plates were placed in an enclosed box equipped with a fixed glass tray. A white light source mirror reflector was positioned beneath the plates, and a digital infrared camera was mounted above them. Larval activity was recorded for 45 minutes, with the light source switched every 15 minutes to create a light-dark-light cycle, beginning with the light on.

*Data Analysis:* The recorded videos were analyzed using NIH ImageJ software with the manual tracking plugin. The distance traveled per second over the entire 45-minute period was measured for each larva. Additionally, the total distance moved during the last light and dark periods was calculated. To focus on the anxiety response to illumination changes, only the distance traveled during the first half of each period was included in the analysis. Results were expressed as mean ± standard deviation. Statistical significance was determined using the Welch Test and Games-Howell Post Hoc Tests. Differences between the mean adjusted distances in the light and dark periods for each treatment group were evaluated with paired sample t-tests, with a p-value of less than 0.05 considered statistically significant.

## 3. Results

Figure 1 demonstrates that healthy zebrafish (egg water control) exhibit significantly higher locomotion in the dark compared to the light, with a p-value of 0.003 (Table 1). This pattern of increased activity in the dark was also observed in the DMSO control and caffeine groups, with p-values of 0.011 and 0.014, respectively (Table 1). Caffeine and alprazolam displayed effects consistent with anxiogenic and anxiolytic properties, respectively, though these effects were not statistically significant overall. Notably, caffeine significantly reduced swim distance in the dark (p = 0.032), indicating a potential anxiolytic effect in this condition. Caffeine led to decreased swim distance in both light and dark periods (Figure 2), while alprazolam increased swim distance only during the light period, suggesting a potential anxiogenic effect during this phase.

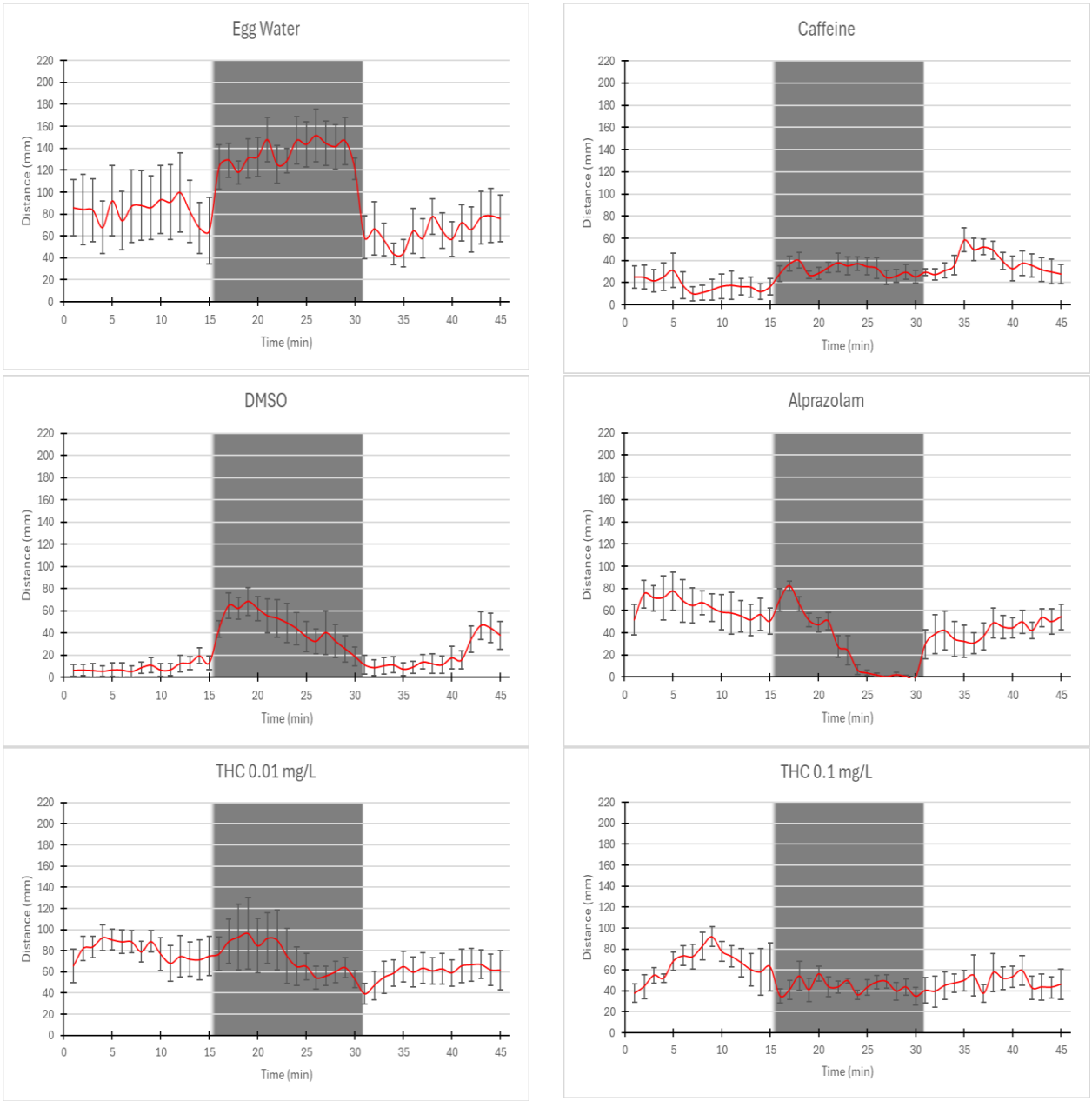


**Figure 1** Mean Distance Moved (mm) Per Minute by Zebrafish Larvae in Treatments (n = 6).

Regarding THC, the lower dose (0.01 mg/L) appeared to have an anxiolytic effect in the dark, while the higher dose (0.1 mg/L) seemed to have an anxiogenic effect. However, these differences were not statistically significant (Figure 3). The results suggest that while THC may modulate anxiety-like behavior in zebrafish, the effects are not as pronounced or consistent as those observed with caffeine or alprazolam. Overall, the data support the presence of significant

differences in locomotion related to light and dark conditions and hint at the potential for THC to influence anxiety-like behavior, though further research is needed to confirm these effects.

**Table 1** Mean distance ± SD moved by larvae in the different treatment groups for the first halves of the dark period and the final light period and the related p-values for the statistical tests (n = 6).

| **Treatment Groups** | **Mean distance ± SD travelled in each period (cm)** | | **Paired Sample t-test p-values** | **Games-Howell p-values** | |
|---|---|---|---|---|---|
| | Dark | Light | | Dark | Light |
| EW Control | 113.9 ± 44.7 | 50.6 ± 51.2 | 0.003* | - | - |
| DMSO Control | 40.9 ± 17.1 | 7.2 ± 11.5 | 0.011* | 0.072[a] | 0.487[a] |
| Caffeine | 23.2 ± 7.1 | 28.4 ± 9.3 | 0.014* | 0.032*[a] | 0.923[a] |
| Alprazolam | 39.4 ± 8.5 | 24.4 ± 23.5 | 0.230 | 1.000[b] | 0.678[b] |
| 0.01 mg/L THC | 62.2 ± 44.3 | 38.8 ± 22.8 | 0.066 | 0.900[b] | 0.145[b] |
| 0.1 mg/L THC | 31.5 ± 12.6 | 31.4 ± 18.1 | 0.996 | 0.920[b] | 0.188[b] |

EW – egg water; SD – standard deviation; [a] Compared to EW control in statistical test ; [b] Compared to DMSO control in statistical test' * Statistically significant

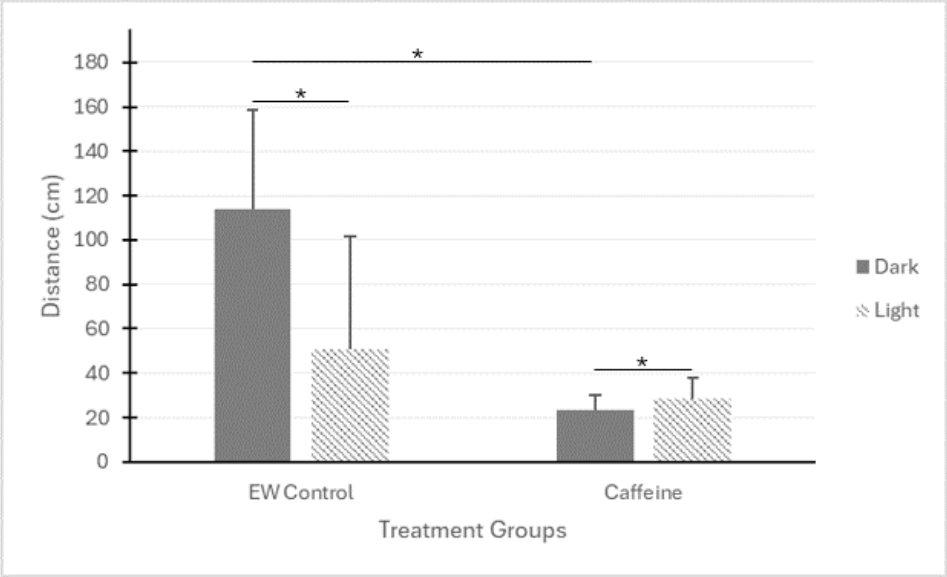


**Figure 2** Mean Distance moved (cm) in the First Half of the Final Dark and Light Periods for the Treatments Delivered in Egg Water, EW (n = 6). *Statistically significant, $p < 0.05$

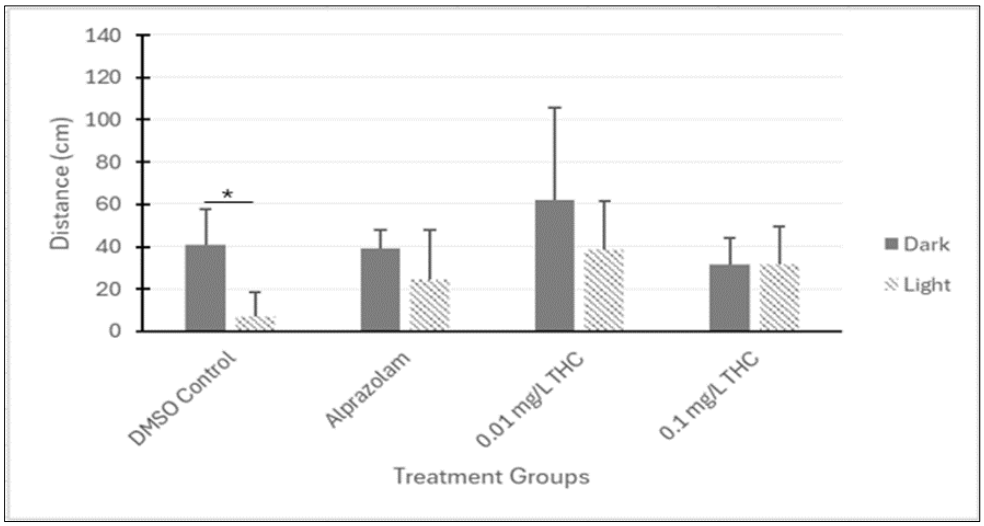


**Figure 3** Mean Distance moved (cm) in the First Half of the Final Dark and Light Periods for Treatments Delivered in DMSO (n =6). *Statistically significant, $p < 0.05$

## 4. Discussion

*Behavior of Healthy Zebrafish Larvae in the Light-Dark Assay:* The zebrafish light-dark transition test is a well-established method for assessing anxiety-like behavior [16, 21]. This assay simulates sudden changes in illumination, which triggers a fear response in zebrafish, leading to increased locomotion in the dark. This pattern is evident in the control groups (egg water and DMSO) as shown in Figure 1, with significant differences in movement between the light and dark periods ($p = 0.003$ and 0.011 for egg water and DMSO controls, respectively, Table 1). The zebrafish's preference for darker environments, known as scototaxis [14], is an evolutionary adaptation for survival. In the dark, zebrafish are better concealed from potential predators, which reduces their fear and encourages exploratory behavior. This natural tendency to seek darker areas helps them avoid detection by predators [22]. Consequently, healthy zebrafish exhibit high activity levels when they are not under threat, often engaging in behaviors such as foraging for food [23]. This increased locomotion in the dark reflects their reduced anxiety and increased exploratory behavior when they feel secure. An abrupt transition from darkness to light reduces the distance traveled by zebrafish, as observed in the control groups shown in Figure 1. Zebrafish perceive the sudden exposure to a bright environment as a potential threat due to increased visibility to predators. Consequently, they reduce their activity to minimize the risk of being detected.

*Effect of Anxiolytic and Anxiogenic Agents on Larval Zebrafish in the Light-Dark Assay:* The observed changes in locomotion indicate that zebrafish reduce their activity when in an anxious state and increase it when stress-free. This was evident in larvae exposed to 100 mg/L caffeine, a compound known for its anxiogenic effects (Figure 1). Caffeine-treated larvae exhibited reduced movement in both light and dark periods compared to the egg water control larvae (Figure 2). This finding aligns with research by Maeda et al. [24]. Caffeine-treated larvae also traveled further in the light than in the dark ($p = 0.014$, Table 1), which deviates from the expected response to illumination changes.

In contrast, alprazolam, an anxiolytic agent, led to increased distance traveled during the light period compared to the DMSO control, although this result was not statistically significant (Figure 3). Additionally, there was no significant difference in the distance traveled during the dark period between alprazolam and DMSO-treated larvae.

*Effect of THC on Larval Zebrafish Behavior in the Light-Dark Assay:* For THC concentrations, it was anticipated that higher doses would reduce zebrafish locomotion, given that increased THC is typically associated with anxiogenic effects [3, 25]. This expectation was partially supported by the results shown in Figure 1, where zebrafish exposed to the lower THC dose (0.01 mg/L) exhibited greater locomotion throughout the experiment compared to those receiving the higher dose (0.1 mg/L). Specifically, in the dark period, the mean total swim distance increased for the 0.01 mg/L THC group compared to the DMSO control, while it decreased for the 0.1 mg/L THC group (Table 1, Figure 3). However, these differences were not statistically significant.

During the light phase, both THC doses led to increased movement, but again, the results were not statistically significant. This may suggest that the anxiolytic properties of THC are more pronounced in the context of higher anxiety typically associated with light environments. Although both THC concentrations showed higher locomotion in the dark,

these observations were not statistically significant ($p = 0.066$ for the lower dose and $p = 0.996$ for the higher dose, Table 1).

Additionally, larvae treated with 0.01 mg/L THC exhibited higher overall locomotion compared to those treated with alprazolam, though this difference was not significant. This finding could imply that THC at the lower dose might be more effective than alprazolam, highlighting a potential area for further investigation.

## 5. Conclusion

This study suggests that THC may influence anxiety-like behavior in zebrafish and exhibits a biphasic effect; however, these findings were not statistically significant. The lack of significant results may be attributed to the small sample size, which limits the generalizability of the conclusions. Future research should employ automated tracking systems for zebrafish movement to streamline data analysis and accommodate larger sample sizes. Additionally, exploring a broader range of THC doses could provide more definitive evidence on the relationship between THC concentration and its anxiolytic effects.

## Compliance with ethical standards

### *Acknowledgments*

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### *Disclosure of conflict of interest*

The authors declare that there is no conflict of interest.

### *Statement of ethical approval*

Ethical approval was granted by the Mona Campus Research Ethics Committee (Ref: CREC-AN.004,2022/2023).

### *Authors' Contribution*

- Shelby Kyra Kerr: Data collection, analysis and manuscript drafting
- Mohammad Kutub Ali: Research supervisor and coordinator, manuscript reviewer

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