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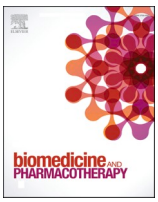

Cannabinoid type-2 receptors modulate terpene induced anxiety-reduction in zebrafish

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ABSTRACT

Terpenes are the most extensive and varied group of naturally occurring compounds mostly found in plants, including cannabis, and have an array of potential therapeutic benefits for pathological conditions. The endocannabinoid system can potently modulate anxiety in humans, rodents, and zebrafish. The 'entourage effect' suggests terpenes may target cannabinoid CB_1 and CB_2 receptors, among others, but this requires further investigation. In this study we first tested for anxiety-altering effects of the predominant 'Super-Class' terpenes, bisabolol (0.001%, 0.0015%, and 0.002%) and terpinolene (TPL; 0.01%, 0.05%, and 0.1%), in zebrafish with the open field test. Bisabolol did not have an effect on zebrafish behaviour or locomotion. However, TPL caused a significant increase in time spent in the inner zone and decrease in time spent in the outer zone of the arena indicating an anxiolytic (anxiety decreasing) effect. Next, we assessed whether CB_1 and CB_2 receptor antagonists, rimonabant and AM630 (6-Iodopravadoline) respectively, could eliminate or reduce the anxiolytic effects of TPL (0.1%) and β-caryophyllene (BCP; 4%), another super-class terpene previously shown to be anxiolytic in zebrafish. Rimonabant and AM630 were administered prior to terpene exposure and compared to controls and fish exposed to only the terpenes. AM630, but not rimonabant, eliminated the anxiolytic effects of both BCP and TPL. AM630 modulated locomotion on its own, which was potentiated by terpenes. These findings suggest the behavioural effects of TPL and BCP on zebrafish anxiety-like behaviour are mediated by a selective preference for CB_2 receptor sites. Furthermore, the CB_2 pathways mediating the anxiolytic response are likely different from those altering locomotion.

1. Introduction

Terpenes are the essential oils of plants and flowers that make up their distinct aromas, flavours, and pigments [1]. They are volatile lipophilic isoprenoids made of gaseous hydrocarbons [2,3] that freely cross cell membranes and have varying adaptive functions and ecological roles, such as repelling predators, attracting pollinators [4], thermal protection, and signalling functions [2,3]. Over 20,000 terpenes have been identified making them one of the most extensive and varied groups of naturally occurring compounds [3]. Terpenes found in the cannabis plant have been of particular interest in experimental research due to the growing popularity of *Cannabis sativa* phytocannabinoids, Δ^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), and their various therapeutic benefits and potential medicinal effects [5]. Terpenes may interact with phytocannabinoids, altering their pharmaceutical qualities, and may contribute to the anxiety-reducing (anxiolytic), antibacterial, anti-inflammatory, and sedative properties of cannabis [6]. This potential interaction is commonly referred to as the 'entourage effect' [1,7,8] which suggests that the functional activity of phytocannabinoids on endocannabinoid receptors is modulated by the presence of terpenes released from the same glandular trichomes [9,10]. The major therapeutic constituents in cannabis, THC and CBD, are known for their action at CB_1 and CB_2 receptors [11]. THC primarily acts on presynaptic CB_1 receptors which mediate an inhibitory effect on neurotransmitter release. This can create a cascading effect on neuronal activity, including an increase in transmitter release from surrounding acetylcholinergic, glutamatergic, and dopaminergic neurons [11]. This mechanism is thought to underly the therapeutic properties of cannabis [12]. Recent studies exploring the effects of the terpene compounds produced in the *Cannabis sativa* plant, such as, limonene, myrcene,

Abbreviations: TPL, (terpinolene); BCP, (beta-caryophyllene); THC, (Δ9- tetrahydrocannabinol); CBD, (cannabidiol); DMSO, (dimethyl sulfoxide).

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pinene and β-caryophyllene (BCP), have demonstrated anxiolytic and sedative properties similar to THC and CBD [13–15]. Taken together, terpene compounds targeting CB_1 and CB_2 receptors may have important implications for medicinal and therapeutic uses [16] and require further investigation.

Zebrafish (*Danio rerio*) have become an established model for studying human development, diseases, genetics, toxicology, and pharmacological effects on physiology and behaviour [17–20]. Zebrafish have a high physiological and genetic homology to humans and share similar brain and central nervous system morphology [21]. Structurally, zebrafish have the same major brain divisions and spinal cord, as well as the major neurotransmitter systems, GABA, glutamate, dopamine, norepinephrine, serotonin, histamine, and acetylcholine [17, 22–24], and express all the major endocannabinoid-related genes [25–27]. Along with their conserved neurochemical structures, zebrafish display robust drug-evoked phenotypic behaviours which many behavioural paradigms have been validated to quantify and measure [19,28]. Furthermore, zebrafish are a valid model in anxiety research as they share an evolutionarily conserved stress response with humans. The zebrafish hypothalamus-pituitary-interrenal (HPI) axis is similar to the human hypothalamus-pituitary-adrenal (HPA) axis, and both species secrete cortisol in response to stress with similar physiological effects [29]. Several zebrafish behavioural tests, such as the open field test, the novel tank dive test, the light dark test, and shoaling test have been validated to reliably identify anxiolytic behavioural biomarkers using a variety of known anxiolytic drugs [18,30–42]. These tests are designed to examine the zebrafish's natural tendency to respond to specific environmental stimuli resulting in proactive defense behaviours (escape and avoidance) such as thigmotaxis (wall-hugging) and geotaxis (bottom-dwelling) [43], neophobia (avoidance of novelty) [44], and scototaxis (light-avoidance) [45]. This study utilized the open field test which quantifies zebrafish anxiety as time spent in specified zones of the testing arena that are representative of species-typical stress responses to a novel environment [18,46,47]. This study also analyzed locomotor behaviours, swimming velocity and immobility, to examine changes in activity levels.

Recent research on the effects of cannabis terpenes using zebrafish behaviour models suggest several super-class terpenes, such as linalool, limonene, myrcene, β-caryophyllene, and α-pinene, to have anxiolytic and sedative properties [13,14]. Given that the endocannabinoid system has been shown to potently modulate anxiety in humans, rodents, and zebrafish [28,48], anxiety-like behaviour is an appropriate biomarker to measure the potential action of cannabis terpenes on CB_1 and CB_2 receptors. The super-class terpenes used in this study, β-caryophyllene (BCP), terpinolene (TPL), and bisabolol, have an established anxiety-reducing effect.

BCP is a bicyclic sesquiterpene most commonly found in oregano, black pepper, and clove, and is predominant among the active constituents in the *Cannabis sativa* plant [1,49,50]. Terpenes are classified according to the number of pairs of isoprenes they are made up of. Sesquiterpenes contain three pairs of isoprenes [7] and are much larger compounds than monoterpenes and are more stable in comparison [3]. BCP has demonstrated a range of medicinal effects including anticancer [51–53], antioxidant [54], and anti-inflammatory [55] properties. Recent studies have cited the potential pharmacological properties of BCP in the treatment of anxiety and depression disorders [15]. For example, Bahi et al. [15] found significant anxiolytic effects of BCP in the elevated plus maze and open field test, and anti-depressant effects in the marble burying test. Similarly, Machado et al. [50] and Rabbani et al. [56] demonstrated BCP to have anxiolytic effects similar to benzodiazepine compounds in the elevated plus maze. Galdino et al. [57] also found anxiolytic effects of BCP with mice in the elevated plus maze, as well as the light dark test.

BCP's mechanism of action has not been clearly defined, however, it can bind to the THC-site on the CB_2 receptor in mammals[2,49,58]. When examining the possible mechanism of action, Galdino et al. [57] found $GABA_A$ and $5\text{-}HT_{1A}$ receptor antagonists did not block the anxiolytic effects of BCP. Using molecular docking simulations to identify naturally occurring CB_2 receptor agonists, Gertsch [2] found BCP selectively binds to CB_2 receptors as a full agonist. However, these results could not be replicated in more recent studies. For example, Santiago et al. [10] found BCP did not directly activate CB_1 or CB_2 receptors or modulate the functional activity of THC at CB_1 or CB_2 receptors using rodent cell cultures transfected with human CB_1 and CB_2 receptors. Similarly, Finlay et al. [9] used radiogland binding assays to detect the action of several super-class terpenes on CB_1 and CB_2 receptors. Although they found BCP to be the only terpene used in their study to have an effect, only a modest effect on CB_2 receptors was detected which did not alter binding enough to consider the effect significant, nor did BCP modulate binding of THC or CBD in their study. However, AM630, an established CB_2 receptor antagonist has been shown to eliminate or block the effects of BCP in animal models. For example, the effects of BCP in the Bahi et al. [15] study mentioned previously were eliminated with inverse CB_2 receptor agonist AM630. Additionally, BCP-reduced alcohol consumption was eliminated when mice were administered AM630 prior to BCP administration [59]. Similarly, BCP-inhibited nicotine self-administration, and motivation to seek nicotine was reversed when AM630 was administered prior to BCP administration [60]. Cannabis compounds targeting CB_2 receptors, free from the adverse CB_1-mediated psychotropic effects, may be beneficial relative to commonly used anxiolytic and anti-depressant drugs [15].

Terpinolene is a monocyclic monoterpene most commonly found in *Citrus* [61] and pine (*Pinus*) [62]. While there is a lack of sufficient evidence of terpinolene's anxiolytic effect or mechanism of action, previous studies have demonstrated terpinolene's pharmacological properties to include sedative [61], antifungal, and antioxidant activity [63]. In studies with mice, TPL was shown to decrease anxiety behaviour in the open field test [61]. Ito and Ito [61] reported its sedative properties were similar to chlorpromazine and a result of antagonistic action in dopaminergic, noradrenergic, and serotonergic neurons. There is also evidence that terpinolene has an anti-inflammatory and antinociceptive effect by inhibiting activity at $5HT_{2A}$ serotonin receptors [64]. A computationally predicted analysis of molecular targets (*in silico*) suggest terpinolene may also selectively bind to CB_2 receptors in the endocannabinoid system which requires further investigation [63].

Bisabolol is a monocyclic sesquiterpene found in plants such as German chamomile (*Matricaria chamomilla*) [65] and sage (*Salvia runcinate*) [66]. Bisabolol-rich oils, such as essential oils extracted from chamomile, have frequently been used as anti-inflammatory agents to treat skin conditions such as eczema and dermatitis by inhibiting leukotriene synthesis [67]. Additionally, chamomile tea has often been used as a natural calming agent [68]. Although the anti-inflammatory effects of bisabolol have been well studied, its effect on anxiety and sedation are relatively unexplored. Chamomile may have meaningful antidepressant as well as anxiolytic activity in humans [69,70] and may relieve some symptoms of ADHD [71]. Studies with bisabolol have found an antinociceptive and sedative effect on mice [72] and an anxiolytic-like effects mediated by $GABA_A$ benzodiazepine receptor interactions [68]. A recent study utilizing a controlled in-vitro heterologous expression system to quantify terpene activation of CB_1 receptors found bisabolol to have no effect alone or when administered with THC [73]. While multiple therapeutic properties of bisabolol have been identified, more research is required to determine its specific behavioural and neuromodulatory effects on anxiety related disorders and their underlying mechanisms.

The purpose of this study was twofold: to test for anxiolytic and sedative effects of BCP, TPL, and bisabolol in zebrafish, and to investigate whether any potential behavioural alterations could be attributed to the terpene binding at endocannabinoid receptor sites. To determine whether the potential anxiolytic properties of the terpenes are mediated by action at endocannabinoid receptor sites, we pretreated fish with either a CB_1 or CB_2 receptor antagonist, then examined anxiety-like

behaviour and locomotion in an open field test [18,46,47]. Rimonabant is a first generation CB_1 receptor antagonist/ inverse agonist. with a high affinity for CB_1 receptors [74]. Rimonabant has consistent antagonizing effects at CB_1 receptors in zebrafish models [48,75] without altering zebrafish behaviour when administered alone [76]. For example, Ruhl et al. [77] found rimonabant reversed the effects of THC in a discriminant learning task where the CB_1 receptor agonist (THC) impaired learning behaviour. AM630 is a CB_2 selective ligand that acts as an inverse agonist at CB_2 receptor sites and as a weak partial antagonist at CB_1 receptors [78]. AM630 can block the effect of phytocannabinoid THC [79] and CB_2 receptor agonist JWH133 [80]. Few studies have examined the effects of AM630 using zebrafish models, however Dahlén et al. [81] found AM630 to eliminate the effects of THC-induced behavioural stereotypy while having no significant effect on zebrafish behaviour .

2. Methods

2.1. Animals and housing

Adult wild-type zebrafish of mixed sex were obtained from MacEwan University's in-house breeding facility (female = 134, male = 150, undetermined = 21). All fish were bred between July 2022 and January 2023. Experiments were conducted between November 2022 and March 2023. All zebrafish were housed in a Tecniplast ZebTEC multilinking system (Tecniplast Group, Toronto ON, Canada) in either 3 or 10-liter polycarbonate tanks. Housing facility water consisted of reverse osmosis water buffered with non-iodized salt, sodium bicarbonate, acetic acid and was treated with an automated water exchange and 5-stage filtration system. Housing facility water was continuously recirculated and filtered through 50 μm of mechanical and activated carbon and UV irradiated. Temperature was maintained at 26–28 °C and pH was maintained at 6.5–8.0. Zebrafish were on a 14-hour light/dark cycle

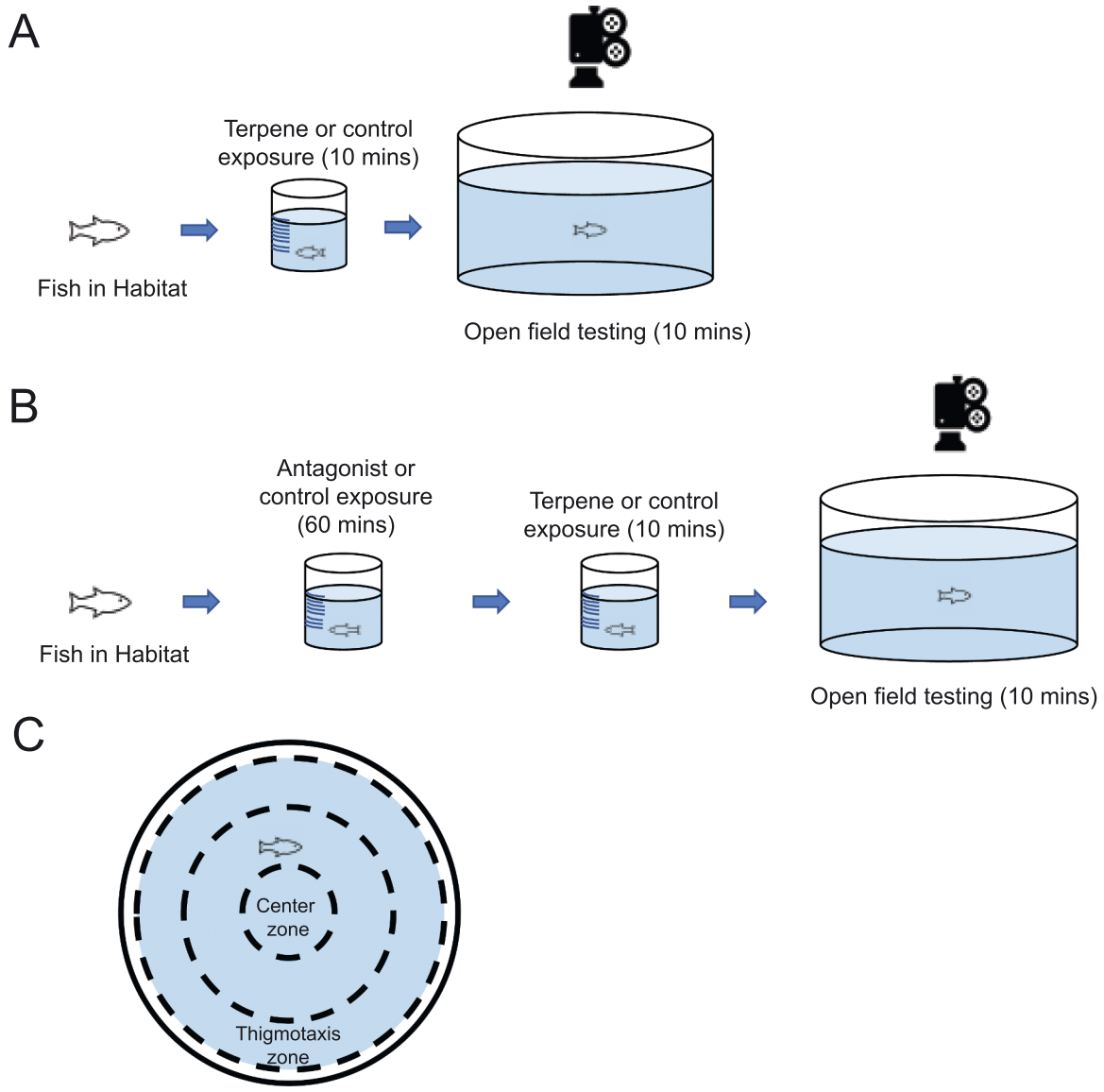

Fig. 1. Treatment administration and behavioural testing. (**A**) Phase 1: To establish an effective dose of terpenes on zebrafish behaviour, individual zebrafish were placed in a terpene or control solution for 10 mins then transferred to the testing arena where behaviour was tracked and recorded for 10 mins. (**B**) Phase 2: To test the effects of rimonabant and AM630, zebrafish were placed in an antagonist or control solution for 60 mins, then transferred to a terpene or control solution for 10 mins, then transferred to the testing arena where behaviour was tracked and recorded for 10 mins. (**C**) The open field testing arena had a 27 cm diameter, 11.5 cm height, and water depth of 5 cm. The annular zones of the arena created in Noldus Ethovision XT consisted of 3 concentric zones each 4.5 cm wide. The outermost circle is the thigmotaxis zone, followed by the transition zone, and the innermost circle is the center zone.

from 7:00 AM to 9:00 PM and were fed twice daily with Gemma Micro 300 fish pellets (GEMMA Micro, Maine, USA).

2.2. Behavioural testing

All behavioural testing protocols used in this study were based on a previous study conducted by Johnson and colleagues [14]. *Experiment 1 – Acute exposure*: In phase 1 of this study, individual zebrafish were administered either bisabolol or TPL at one of three concentrations, or a control solution for 10 min. Zebrafish were then transferred to the testing arena where behaviour was tracked and recorded for an additional 10 min (Fig. 1A). *Experiment 2 – Cannabinoid receptor antagonism*: In phase 2 of this study, the effects of terpenes BCP and TPL were challenged with cannabinoid receptor antagonists. TPL and BCP, or a control solution, were administered for 10 min after a 60 min administration of either rimonabant or AM630, then transferred into the testing arena where behaviour was tracked and recorded for 10 min (Fig. 1B). Control trials were interspersed throughout testing days. The open field arena was 27 cm in diameter, 11.5 cm tall, and filled with a water level of 5 cm. Prior to each trial the testing arena was filled with habitat water to prevent build-up of waste, potential alarm cues, drug residue spill off from the fish, and to maintain water temperature. After each trial, zebrafish were placed into a beaker to be sexed then returned to a habitat tank and fed. The water temperature in the habitat tank of experimental zebrafish, dosing beakers, and testing arena was maintained at 26–28 °C with seedling heat mats (Hydrofarm Horticultural Products, Petaluma CA). Luminance in the testing room was ~32 cd/m^3 (cal SPOT photometer; Cooke Corp. CA, USA). Lights were covered with opaque plexiglass to eliminate any reflection on the housing facility water in the testing arena. Zebrafish swimming behaviour was recorded by a Basler GenICam acA1300–60gc Area Scan video camera (Basler Inc., USA) suspended 1 m above the testing arena. All zebrafish were tested in an identical manner and swimming behaviour was tracked and recorded using Noldus EthoVision XT ® tracking software (v. 11.0, Noldus, Wageningen, NL).

2.2.1. The open field exploration arena

The open field exploration test measured both anxiety-like and locomotor behaviour by calculating the cumulative time spent in each of the arena zones (s), swimming velocity (cm/s), and cumulative time spent immobile (s). The arena was divided into 3 virtual zones created within EthoVision: the thigmotaxis (outer) zone, the transition zone, and the center (inner) zone (Fig. 1C). Fish were individually netted into the testing arena halfway between the center and thigmotaxis zones. Immobility was determined at a 5% threshold, whereby, a fish would be considered immobile if tracking software detected less than a 5% change in the pixels of the body of the fish [13].

2.3. Drug administration

All zebrafish were administered treatments via in-water immersion according to Dahlén et al. [81]. All treatment concentrations were determined from previous studies [14,81] or pilot testing. On testing days, zebrafish were transferred by netting into a 3-liter habitat tank and were habituated in the testing room for a minimum of 15 min prior to treatment administration. The habituation tanks and testing arena were fully surrounded by white corrugated plastic to reduce extraneous visual stimuli. After habituation, Phase 1 zebrafish were netted into a 600 mL dosing beaker containing 400 mL of one of three doses of bisabolol (0.001%, 0.0015%, and 0.002%), TPL (0.01%, 0.05%, and 0.1%), or a control solution for 10 min. Phase 2 zebrafish were netted into a dosing beaker containing rimonabant (10 μM), AM630 (3.5 μM), or a control solution for 60 min then transferred by net to another dosing beaker containing either TPL (0.1%) or BCP (4%) for 10 min. After terpene administration, individual zebrafish were immediately netted and placed into the open field testing arena for 10 min. All terpenes and antagonists were purchased from Sigma-Aldrich (Ontario, Canada) and solutions were made fresh in-house prior to experimentation on testing days. Terpene solutions were stirred vigorously and left to dissolve for a minimum of 25 min prior to administration. All terpenes and antagonists were mixed into 400 mL of habitat water. Control solutions consisted of 400 mL of habitat water. Solution pH was monitored before and after the addition of treatment compounds and maintained within 6.8–7.5 pH. Zebrafish were administered treatments individually in 600 mL dosing beakers surrounded by white corrugated plastic to reduce any behavioural alterations due to visual conspecific cues [82]. Individual zebrafish were randomly assigned to either a control group or to one of the treatment conditions. All zebrafish were experimentally naïve and were not fed prior to drug administration or experimentation. To check for any significant sex interactions on drug effects, sex differences were analyzed between males and females across all treatment groups. There were no significant sex differences among anxiety or locomotor variables, with the exception of the BCP + rimonabant group where males exhibited less immobility than females. Refer to Supplementary Methods for more information.

2.3.1. Bisabolol

In phase 1 testing, bisabolol (≥95%) was administered in 0.001% (n = 15), 0.0015% (n = 15), and 0.002% (n = 15) concentrations. Bisabolol concentrations were based on pilot testing (data not shown).

2.3.2. Terpinolene

In phase 1 testing, TPL (≥93%) was administered in 0.01% (n = 20), 0.05% (n = 20), and 0.1% (n = 20) concentrations. TPL concentrations were based on pilot testing (data not shown). For phase 2 testing, TPL was administered in 0.1% concentrations (n = 15) after the administration of CB receptor antagonists.

2.3.3. β-caryophyllene

In phase 2 testing, BCP (≥80%) was administered in 4% concentrations (n = 15) dissolved in 0.1% ethanol (EtOH) after the administration of CB receptor antagonists. BCP concentration was based on a previous study [14]. EtOH (0.1%) was also added to the control solution (n = 15). A control group comparison showed 0.1% EtOH had no significant effects on zebrafish swimming behaviour when compared to the non-EtOH control group (Supplementary Methods).

2.3.4. Rimonabant

An in-house stock solution of rimonabant (≥98%) containing 0.1% dimethyl sulfoxide (DMSO; 5.40 mL) was administered in 0.1% concentrations (10 μM; n = 15). In phase 2 testing, zebrafish were dosed in rimonabant or a control solution for 60 min prior to terpene administration. Rimonabant concentration was determined by previous pilot testing (data not shown).

2.3.5. AM630

An in-house stock solution of AM630 (≥90%) containing 0.1% DMSO (2.83 mL) was administered in 0.1% concentrations (3.5 μM; n = 15). In Phase 2 testing, zebrafish were dosed in AM630 or a control solution for 60 min prior to terpene administration. AM630 concentration was based on a previous study [81].

2.3.6. DMSO

A second control group was added to test whether the DMSO vehicle had a behavioural effect on zebrafish anxiety variables and locomotion. DMSO was added to 400 mL of habitat water in a 0.1% concentration (n = 15). Zebrafish were dosed in the DMSO solution for 60 min and behaviour was compared to the control group to detect any significant behavioural differences between the two groups. No significant differences were observed. DMSO concentration was based on a previous study [81]. DMSO control data is represented in Fig. 4 and is available in supplementary materials.

2.4. Statistical analysis

All data were analyzed using GraphPad Prism Software (Version 9.1.2; GraphPad, San Diego, CA, USA) to determine whether any significant differences in anxiety-like or locomotor behaviours existed between the treatment groups and the control group. If a significant difference was detected, a multiple comparison was used to determine which treatment groups were significantly different than the control group. Data were assessed for normality using the D'Agostino-Pearson omnibus normality test and assessed for homogeneity using Bartlett's test for equal variances. Parametric data was analyzed using an ordinary one-way ANOVA followed by post-hoc Dunnett's multiple comparison test. Non-parametric data was analyzed using a Kruskal-Wallis with post-hoc Dunn's multiple comparison test. Data with unequal variance was analyzed using the Brown-Forsythe ANOVA. Data were excluded from analyses if the full data was not acquired by tracking software for the total time each fish spent in the arena. A missed sample indicates that EthoVision was unable to analyze a given frame from the video. This can occur when the camera does not provide the expected number of frames per second, or if there is a poor connection to the camera. If the missing sample occurs at the beginning of the trial, EthoVision is not able to interpolate the missing data and the beginning of the trial will not be recorded. Due to missing samples, the following data were omitted: *Acute exposure.* TPL control group n = 1. *Cannabinoid receptor antagonism.* BCP group n = 2, RIM + BCP group n = 1, and AM630 + BCP group n = 2. In the ethanol control group comparison, data were analyzed using a two-tailed Mann-Whitney test. Sex differences were analyzed using a two sample t-test. An alpha level of $p < .05$ and a 95% confidence interval was used to indicate statistical significance. All values are presented as mean ± standard error in

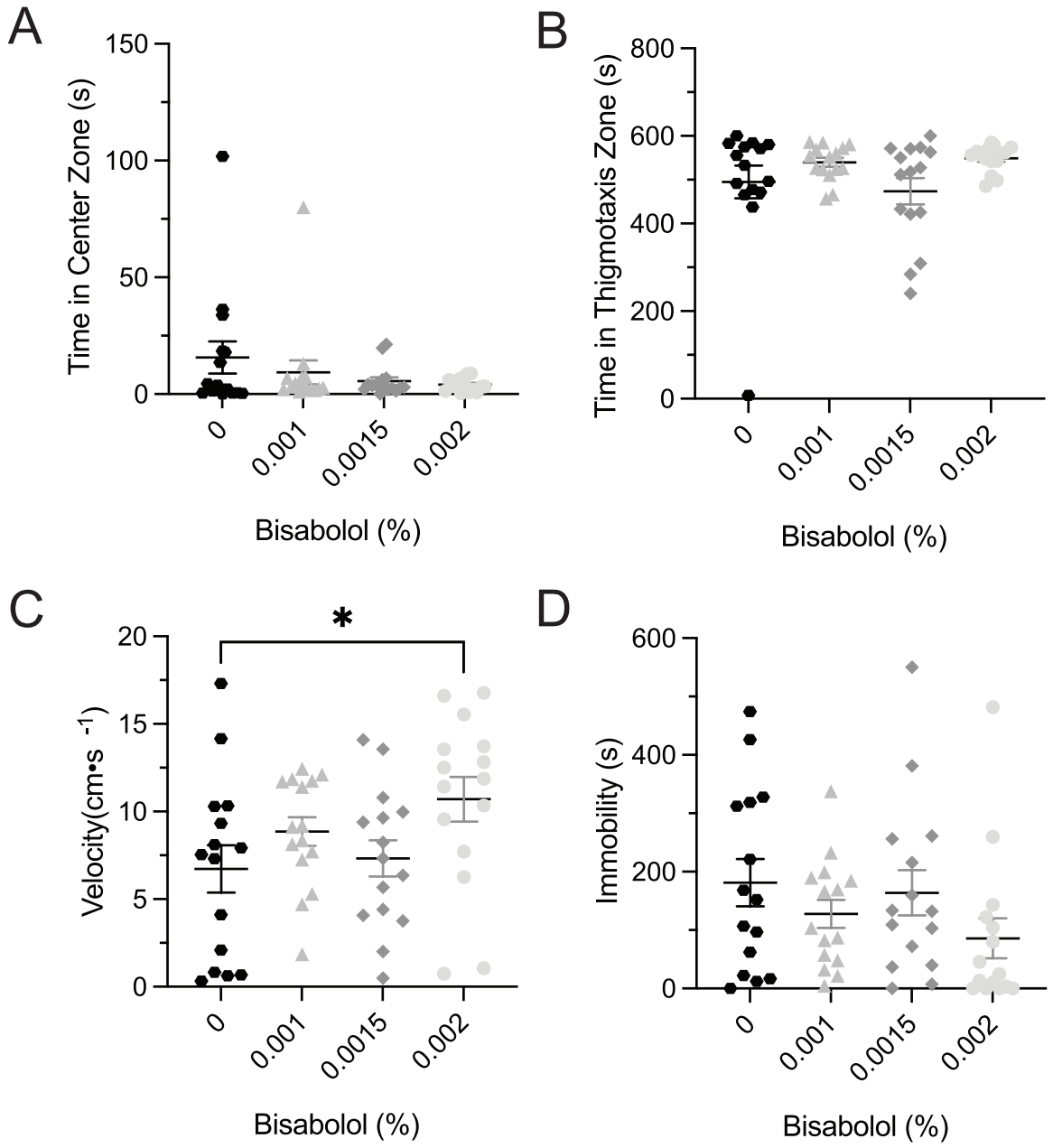

Fig. 2. The effects of bisabolol on anxiety-like and locomotor behaviour in the open field test. (**A**) Bisabolol had no effect on time spent in the center zone ($F(3, 28.02) = 1.4$, $p = .2636$) or (**B**) time spent in the thigmotaxis zone ($F(3, 30.56) = 2.094$, $p = .1216$). (**C**) Bisabolol increased swimming velocity in the 0.002% group ($F(3, 56) = 2.431$, $p = .0746$) but (**D**) had no effect on time spent immobile ($H(4) = 6.355$, $p = .0955$). All data are presented as mean ± S.E.M. Significant differences are indicated by * ($p < 0.05$).

measurement (S.E.M.).

2.5. Ethics statement

All experiments were approved by the MacEwan University Animal Ethics Board (AREB) under protocol number 101853 in compliance with the Canadian Council for Animal Care (CCAC) experimental guidelines. This study was carried out in compliance with ARRIVE guidelines for animal research.

3. Results

3.1. Bisabolol

Bisabolol had no significant effect on time spent in the center zone across all treatment groups when compared to the control group ($F(3, 28.02) = 1.4$, $p = .2636$; Fig. 2A). Bisabolol also had no significant effect on time spent in the thigmotaxis zone across treatment groups when compared to the control ($F(3, 30.56) = 2.094$, $p = .1216$; Fig. 2B).

Bisabolol had a significant effect on velocity between groups ($F(3, 56) = 2.431$, $p = .0746$). There was a significant increase in velocity between the 0.002% group (10.70 ± 1.3 cm/s, $p = .0443$, n = 15) and the control group (6.728 ± 1.3 cm/s, n = 15; Fig. 2C). Bisabolol had no significant effect on immobility across treatment groups when compared to control ($H(4) = 6.355$, $p = .0955$; Fig. 2D).

3.2. Terpinolene

Terpinolene had a significant effect on the duration of time spent in the center zone between groups ($F(3, 44.85) = 5.184$, $p = .0037$). There was a significant increase in time spent in the center zone in the 0.1% group (98.20 ± 24.3 s, $p = .0243$, n = 20) when compared to the control group (22.85 ± 9.9 s, n = 19; Fig. 3A). Terpinolene also had a significant effect on time spent in the thigmotaxis zone ($F(3, 61.20) = 12.84$, $p < .0001$). There was a significant increase in time spent in the transition zone in the 0.1% group (263.3 ± 27.2 s, $p < .0001$, n = 20) when compared to the control group (472.3 ± 27.8 s, n = 19; Fig. 3B).

Terpinolene had a significant effect on velocity between groups ($F(3, 75) = 13.64$, $p < .0001$). There was a significant decrease in velocity in the 0.1% (4.38 ± 0.5 cm/s, $p < .0001$, n = 20) and 0.05% group (5.29 ± 0.7 cm/s, $p < .0001$, n = 20) when compared to the control group (10.29 ± 0.9 cm/s, n = 19; Fig. 3C). Terpinolene also had a significant

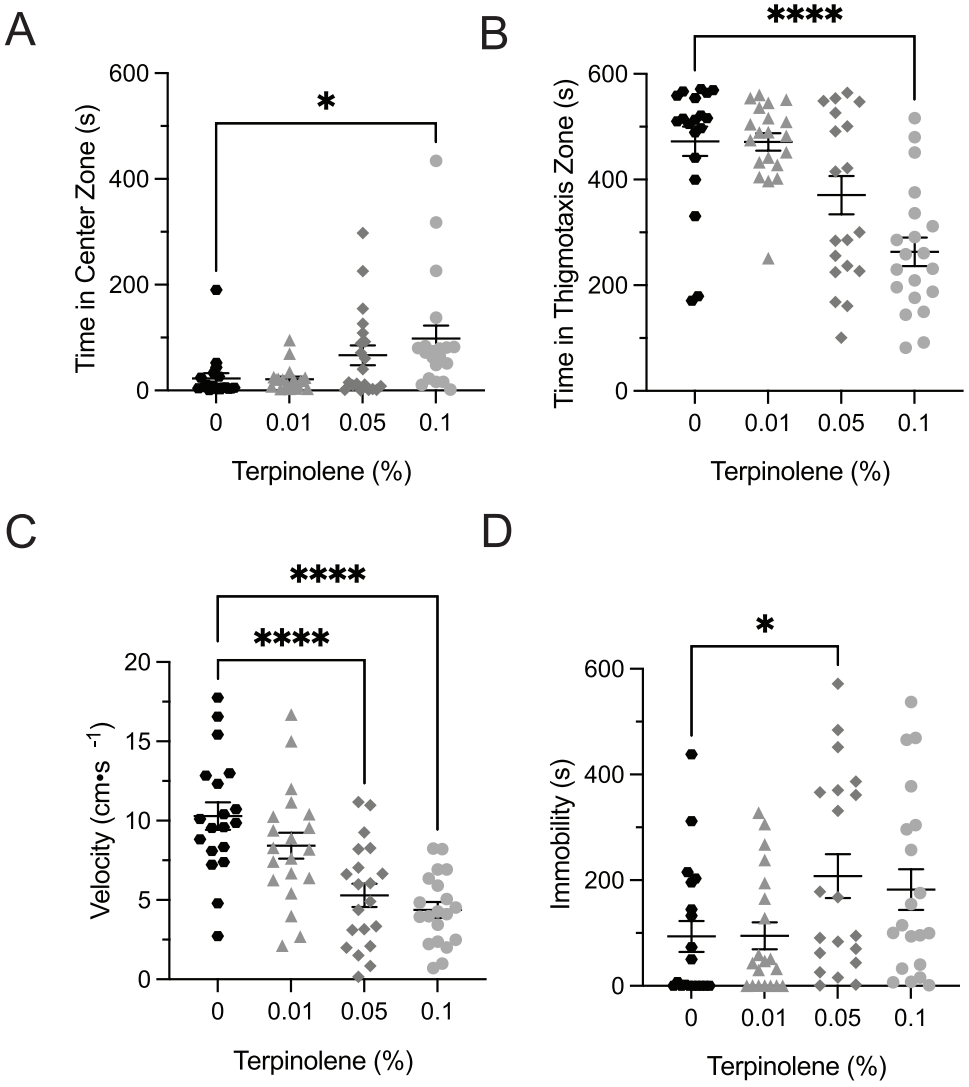

Fig. 3. The effects of terpinolene on anxiety-like and locomotor behaviour in the open field test. (**A**) Terpinolene increased time spent in the center zone in the 0.1% group ($F(3, 44.85) = 5.184$, $p = .0037$) and (**B**) time spent in the thigmotaxis zone in the 0.1% group ($F(3, 61.20) = 12.84$, $p < .0001$). (**C**) Terpinolene decreased swimming velocity in the 0.05% and 0.1% group ($F(3, 75) = 13.64$, $p < .0001$). (**D**) Terpinolene also increased time spent immobile in the 0.05% group ($H(4) = 10.96$, $p = .0120$). All data are presented as mean ± S.E.M. Significant differences are indicated by * ($p < 0.05$) and * ** * ($p < .0001$).

effect on duration of time spent immobile between groups ($H(4) = 10.96$, $p = .0120$). There was a significant increase in immobility in the 0.05% (207.9 ± 41.6 s, $p = .0250$, n = 20) when compared to the control group (93.55 ± 29.4 s, n = 19; Fig. 3D).

3.3. β-caryophyllene and rimonabant

Rimonabant did not significantly modulate the effect of BCP on time spent in the center zone of the arena. BCP administered alone and after the administration of rimonabant significantly increased the cumulative duration of time zebrafish spent in the center of the arena ($F(4, 17.3) = 6.852$, $p = .0017$). There was a significant difference between the BCP group (45.43 ± 11.6 s, $p = .0457$, n = 13) and the BCP + rimonabant group (120.5 ± 36.6 s, $p = .0397$, n = 14) when compared to the control group (10.93 ± 2.5, n = 15). No significant differences were observed when rimonabant was administered alone (26.14 ± 5.2, $p = .0601$, n = 15; Fig. 4A).

Rimonabant also did not modulate the effect of BCP on time spent in the thigmotaxis zone. BCP administered alone and after the administration of rimonabant significantly decreased the cumulative duration of time spent in the thigmotaxis zone ($H(5) = 20.9$, $p = .0003$). There was a significant difference between the BCP group (356 ± 32.8 s, $p = .0081$, n = 13) and the BCP + rimonabant group (322.9 ± 36.4 s, $p = .0015$, n = 14) when compared to the control group (487.2 ± 22 s, n = 15). No significant differences were observed when rimonabant was administered alone (447 ± 28.5 s, $p > .9999$, n = 15; Fig. 4B).

Rimonabant did not modulate the effect of BCP on swimming velocity. BCP administered alone or following the administration of rimonabant significantly decreased velocity ($F(4, 67) = 7.355$, $p < .0001$). There was a significant difference between the BCP group (6.17 ± 0.7 cm/s, $p = .0125$, n = 13) and the BCP + rimonabant group (6.4 ± 0.8 cm/s, $p = .0187$, n = 14) when compared to the control group (9.7 ± 0.6 cm/s, n = 15). No significant differences were observed when rimonabant was administered alone (8.3 ± 0.8 cm/s, $p = .5641$, n = 15; Fig. 4C). BCP, rimonabant, nor BCP + rimonabant had a significant effect on the cumulative duration of time spent immobile when compared to the control group ($F(4, 46.93) = 1.644$, $p = .1791$; Fig. 4D).

3.4. β-caryophyllene and AM630

AM630 eliminated the effect of BCP on time spent in the center zone of the arena. While BCP had a significant effect on time spent in the center zone, these effects were eliminated following the administration of AM630 ($F(4, 44.71) = 2.681$, $p = .0435$). There was a significant increase in time spent in the center zone in the BCP group (45.43 ± 11.6 s, $p = .0457$, n = 13) but not in the BCP + AM630 group (27.45 ± 7.8 s, $p = .2215$, n = 13) or AM630 administered alone (28.5 ± 12.2 s, $p = .5237$, n = 15) when compared to the control group (10.93 ± 2.5 s, n = 15; Fig. 4E).

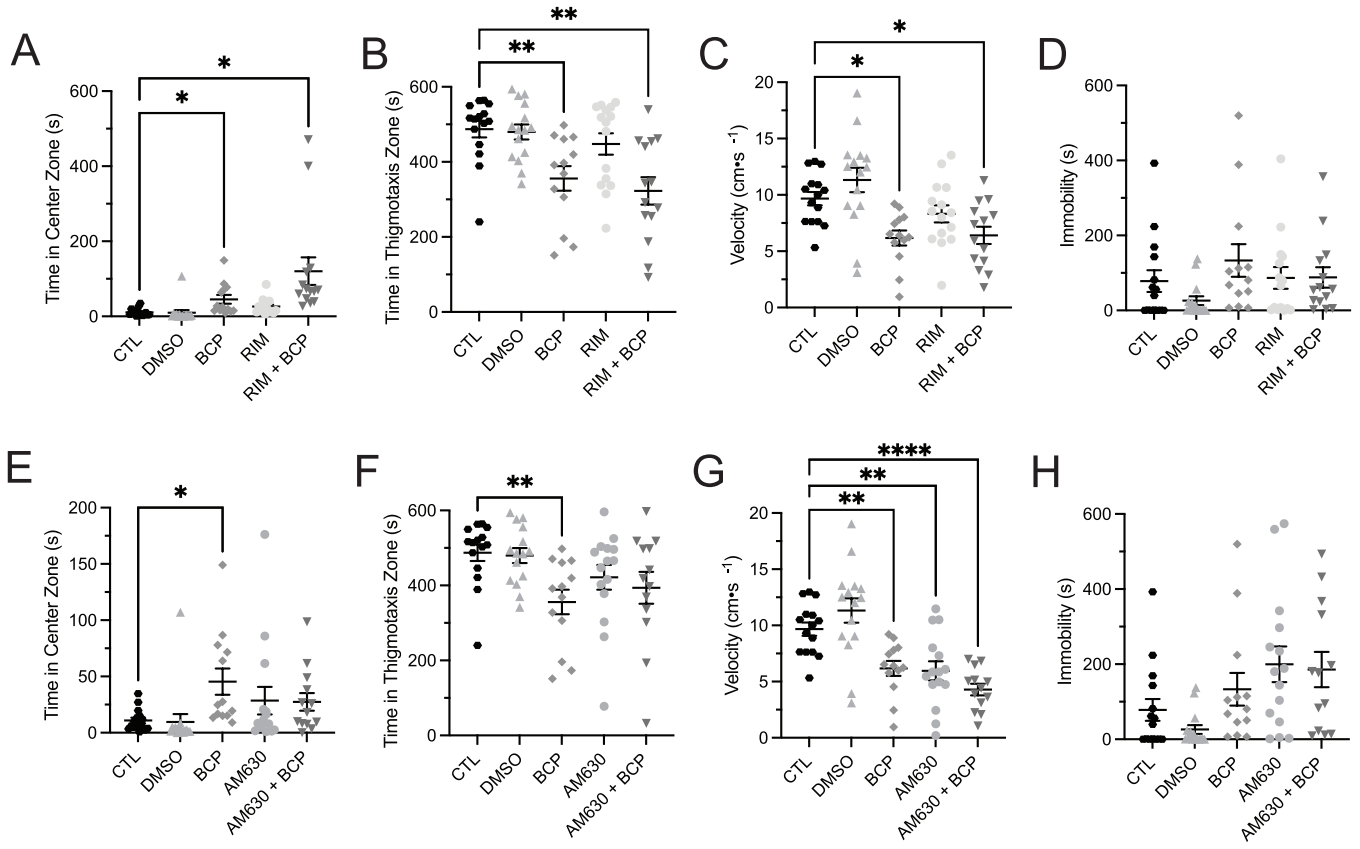

Fig. 4. The effects of rimonabant and AM630 on BCP-reduced anxiety-like and locomotor behaviour in the open field test. (**A**) BCP increased time spent in the center zone. Rimonabant did not modulate the effect of BCP on time spent in the center zone ($F(4, 17.26) = 6.852$, $p = .0017$). (**B**) BCP decreased time spent in the thigmotaxis zone. Rimonabant did not modulate the effect of BCP on time spent in the thigmotaxis zone ($H(5) = 20.9$, $p = .0003$). (**C**) BCP decreased swimming velocity. Rimonabant did not modulate the effect of BCP on swimming velocity ($F(4, 67) = 7.355$, $p < .0001$). (**D**) BCP nor rimonabant had an effect on immobility ($F(4, 46.93) = 1.644$, $p = .1791$). (**E**) BCP increased time spent in center zone. AM630 eliminated the effect of BCP on time spent in the center zone ($F(4, 44.71) = 2.681$, $p = .0435$). (**F**) BCP decreased time spent in thigmotaxis zone. AM630 eliminated the effect of BCP on time spent in thigmotaxis zone ($H(5) = 13.8$, $p = .0080$). (**G**) BCP decreased swimming velocity. AM630 did not eliminate the effects of BCP on swimming velocity. When administered alone, AM630 decreased swimming velocity ($F(4, 50.84) = 14.11$, $p < .0001$). (**H**) BCP nor AM630 had an effect on immobility ($F(4, 48.61) = 3.816$, $p = .0089$). All data are presented as mean ± S.E.M. Significant differences are indicated by * ($p < .05$), * * ($p < .01$), and * ** * ($p < .0001$).

AM630 also modulated the effects of BCP on the cumulative time spent in the thigmotaxis zone. BCP had a significant effect on time spent in the thigmotaxis zone, however these effects were eliminated following the administration of AM630 ($H(5) = 13.8$, $p = .0080$). There was a significant decrease in time spent in the thigmotaxis zone in the BCP group (356 ± 32.8 s, $p = .0028$, n = 13) but not in the BCP + AM630 group (393.8 ± 42.4 s, $p = .1337$, n = 13) or AM630 administered alone (421.9 ± 32.9 s, $p = .2902$, n = 15) when compared to the control group (487.2 ± 22 s, n = 15; Fig. 4F).

AM630 did not modify the effect of BCP on swimming velocity. BCP significantly decreased velocity, and this effect was not eliminated after the administration of AM630 ($F(4, 50.84) = 14.11$, $p < .0001$). There was a significant decrease in velocity in the BCP group (6.2 ± 0.7 cm/s, $p = .0023$, n = 13) and BCP + AM630 group (4.3 ± 0.5 cm/s, $p < .0001$, n = 13) when compared to the control group (9.7 ± 0.6 cm/s, n = 15). AM630 also significantly decreased swimming velocity when administered alone (5.9 ± 0.8 cm/s, $p = .0054$, n = 15; Fig. 4G). BCP, AM630, nor BCP + AM630 had a significant effect on the cumulative duration of time spent immobile when compared to the control group ($F(4, 48.61) = 3.816$, $p = .0089$; Fig. 4H).

3.5. Terpinolene and rimonabant

Rimonabant did not modulate the effects of terpinolene on the duration of time spent in the center zone ($F(3, 21.51) = 12.26$, $p < .0001$). There was a significant increase in time spent in the center zone between the terpinolene group (140.2 ± 28.5 s, $p = .0026$, n = 15) and terpinolene + rimonabant group (83 ± 12.8 s, $p = .0007$, n = 15) when compared to the control group (20.5 ± 5.1 s, n = 15). There was no significant difference from the control when rimonabant was administered alone (26 ± 5.2 s, $p = .8236$, n = 15; Fig. 5A).

Rimonabant also did not modulate the effects of terpinolene on time spent in the thigmotaxis zone ($F(3, 38.3) = 14.32$, $p < .0001$). There was a significant difference between the terpinolene (271.7 ± 43.1 s, $p = .0001$, n = 15) and the terpinolene + rimonabant group (337.5 ± 27.5 s, $p < .0001$, n = 15) when compared to the control group (526.3 ± 12.8 s, n = 15). No significant differences were observed when rimonabant was administered alone (448 ± 28.5 s, $p = .0609$, n = 15; Fig. 5B).

Rimonabant also did not modulate the effect of terpinolene on velocity ($H(4) = 19$, $p = .0003$). There was a significant decrease in velocity between the terpinolene group (4.7 ± 0.9 cm/s, $p = .0020$, n = 15) and the terpinolene + rimonabant group (4.5 ± 0.8 cm/s, $p = .0017$, n = 15) when compared to the control group (10.07 ± 1.3 cm/s, n = 15). No significant differences were observed when rimonabant was administered alone (8.3 ± 0.8 cm/s, $p > .9999$, n = 15; Fig. 5C). There were no significant differences in immobility between the control and any treatment groups ($H(4) = 7.178$, $p = .0664$; Fig. 5D).

3.6. Terpinolene and AM630

AM630 did modulate the effects of terpinolene on the duration of time spent in the center zone of the arena ($F(3, 24.55) = 11.68$,

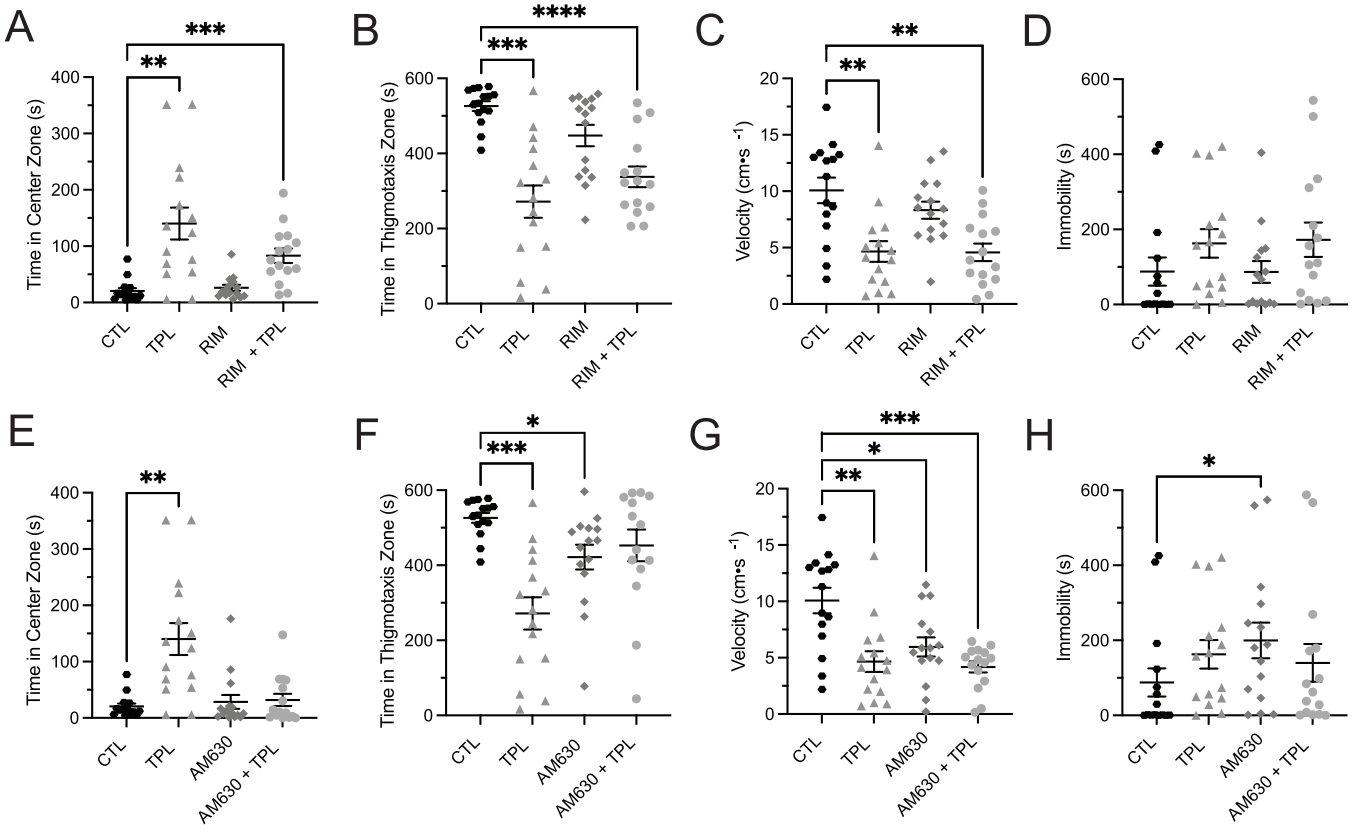

Fig. 5. The effects of rimonabant and AM630 on terpinolene-reduced anxiety-like and locomotor behaviour in the open field test. (A) Rimonabant did not modulate the effect of terpinolene on time spent in the center zone ($F(3, 21.5) = 12.26$, $p < .0001$) or (B) time spent in the thigmotaxis zone ($F(3, 38.3) = 14.32$, $p < .0001$). (C) Rimonabant also did not modulate the effect of terpinolene on swimming velocity ($H(4) = 19$, $p = .0003$). (D) There was no effect on immobility across treatment groups ($H(4) = 7.178$, $p = .0664$). (E) AM630 eliminated the effect of terpinolene on time spent in the center zone ($F(3, 24.55) = 11.68$, $p < .0001$) and (F) time spent in the thigmotaxis zone. AM630 administered alone also had an effect on time spent in the thigmotaxis zone ($F(3, 42.74) = 9.376$, $p < .0001$). (G) AM630 did not modulate the effect of terpinolene on swimming velocity and also had an effect when administered alone ($F(3, 45.6) = 9.347$, $p < .0001$). (H) While terpinolene administered before and after AM630 had no effect on immobility, AM630 increased immobility when administered alone ($H(4) = 6.871$, $p = .0761$). All data are presented as mean ± S.E.M. Significant differences are indicated by * ($p < .05$), * * ($p < .01$), * ** ($p < .001$), and * ** * ($p < .0001$).

$p < .0001$). While terpinolene had a significant effect on time spent in the center zone, these effects were eliminated following the administration of AM630. There was a significant increase in time spent in the center zone in the terpinolene group (140.2 ± 28.5 s, $p = .0026$, n = 15) but not in the AM630 (28.5 ± 12.2 s, $p = .9060$, n = 15) or terpinolene + AM630 group (32 ± 10.8 s, $p = .7125$, n = 15) when compared to the control group (20.5 ± 5.1 s, n = 15; Fig. 5E).

AM630 also modulated the effects of terpinolene on time spent in the thigmotaxis zone ($F(3, 42.74) = 9.376$, $p < .000$). While terpinolene had a significant effect on the duration of time spent in the thigmotaxis zone, these effects were eliminated following the administration of AM630. There was a significant decrease in time spent in the thigmotaxis zone in the terpinolene group (271.7 ± 43.1 s, $p = .0001$, n = 15) but not in the terpinolene + AM630 group (452.9 ± 42.2 s, $p = .2945$, n = 15) when compared to the control group (526.3 ± 12.8 s, n = 15). A weak but significant difference was also observed when AM630 was administered alone (421.9 ± 32.9 s, $p = .0249$, n = 15; Fig. 5F).

AM630 did not modulate the effect of terpinolene on swimming velocity. While terpinolene significantly decreased average velocity, this effect was not eliminated with the pretreatment of AM630 ($F(3, 45.6) = 9.347$, $p < .0001$). There was a significant difference between the terpinolene group (4.65 ± 0.9 cm/s, $p = .0027$, n = 15) and the terpinolene + AM630 group (4.18 ± 0.5 cm/s, $p = .0004$, n = 15) when compared to the control group (10.07 ± 1.1 cm/s, n = 15). AM630 also had a weak but significant effect when administered alone (5.9 ± 0.8 cm/s, $p = .0211$, n = 15; Fig. 5G). AM630 did not modulate the effect of terpinolene on immobility ($H(4) = 6.871$, $p = .0761$). Neither terpinolene (162.8 ± 38 s, $p = .1463$, n = 15) or terpinolene + AM630 (140 ± 50.3 s, $p = .7312$, n = 15) had a significant effect on time spent immobile when compared to the control group (87.83 ± 37.4 s, n = 15). However, when administered alone, AM630 significantly increased time spent immobile (199.7 ± 47.4 s, $p = .0433$, n = 15; Fig. 5H).

4. Discussion

The goals of this study were to first investigate the anxiolytic and locomotor effects of super-class cannabis terpenes on zebrafish anxiety-like behaviour, then assess whether the putative effects are mediated by the selective binding to CB_1 and/or CB_2 receptors. In the first phase of this study, TPL had an anxiolytic effect on zebrafish behaviour, whereas bisabolol had no significant effects. In the second phase of this study, TPL and BCP were administered after the administration of rimonabant, AM630, or a control solution. Both TPL and BCP reduced zebrafish anxiety-like behaviour in the open field test when zebrafish were pretreated with a control solution. Zebrafish pretreated with rimonabant did not show any different behavioural responses than without rimonabant, however, AM630 eliminated the anxiolytic effects of both TPL and BCP. These results indicate that TPL and BCP have an anxiolytic effect on zebrafish behaviour which may be mediated by CB_2 receptors and not CB_1 receptors.

4.1. Anxiolytic neuromodulatory effects via CB_2 receptor activation

The open field test is a zebrafish paradigm adapted from rat and mouse models to measure locomotor and anxiety-like behaviour [83]. The open field test was adapted to assess anxiety-related behaviour in zebrafish and has since been validated as a consistent measure in zebrafish research and is widely used in zebrafish behavioural studies [13,14,37–39]. This test quantifies zebrafish anxiety-like behaviour by calculating the cumulative duration of time spent in specified zones of the arena (thigmotaxis and inner) [18,47,84]. Time spent in the thigmotaxis zone, or nearest the outer walls of the arena, demonstrates escape or centrophobic behaviour which is indicative of heightened anxiety in zebrafish [46]. The duration of time spent in the inner zone is indicative of exploratory behaviour which is associated with a decrease in anxiety-like behaviour [18]. At the highest concentration, both TPL and BCP caused behavioural alterations contrary to the typical stress-response zebrafish exhibit when exposed to a novel environment [18,33,46,47]. In both 10 min and 60 min exposure trials, zebrafish treated with each terpene spent significantly more time exploring the exposed center area of the arena and less time hugging the outer walls of the arena, suggesting an anxiolytic effect. Furthermore, neither rimonabant nor AM630 significantly altered zebrafish anxiety-like behaviour when administered alone. However, when zebrafish were pretreated with antagonists prior to terpene administration, AM630 eliminated the anxiolytic effects of TPL and BCP on time spent in the inner and outer zones of the arena while rimonabant caused no behavioural alterations. This suggests that the anxiolytic effect of the terpenes may be a result of selective binding to CB_2 receptors in the endocannabinoid system.

CB_2 receptors are heterotrimeric g-protein coupled receptors containing three subunits (alpha, beta, and gamma) that modulate cellular function by causing a second messenger cascade [85]. When the CB_2 receptor is activated by endogenous cannabinoids (endocannabinoids), adenylyl cyclase activity is inhibited through Gi/Go_α subunits [86]. The inhibition of adenylyl cyclase inhibits the production of cyclic AMP (cAMP) from adenosine triphosphate (ATP) resulting in a decrease in the activity of cAMP-dependent protein kinase A (PKA) [87]. The cAMP/PKA signaling pathway is known to regulate stress responses in mammals and is associated with the HPA and autonomic nervous system [88]. Increased cAMP signalling is known to be involved in anxiety-like phenotypes resulting from increased PKA activity [88]. Therefore, the activation of alpha subunits on the CB_2 receptor by TPL and BCP may trigger a signaling cascade that inhibits the zebrafish stress response when introduced to a novel environment. Further testing could yield valuable insights into their medicinal potential for the development of alternative treatments for a variety of pathological disorders.

4.2. Alterations in locomotor behaviour

TPL and BCP significantly reduced swimming velocity, consistent with a sedative effect of other terpenes [13,14]. Unlike the anxiolytic effects, this was not eliminated by rimonabant or AM630. No effect of TPL or BCP on zebrafish immobility was observed, and while rimonabant had no effect on locomotor variables, AM630 appeared to reduce locomotor activity. Taken together, this suggests that the neural pathways that mediate sedation and anxiolysis are differentially regulated by the interaction of terpenes and CB_2 receptors. For example, AM630 decreased swimming activity of zebrafish larvae but not CB_2-*KO* larvae [89], suggesting that the effects of AM630 on locomotion are CB_2 receptor specific [89]. Furthermore, endocannabinoids bind to CB_2 receptor subunits that are distinct from those occupied by AM630 [86] which may explain the differential effects on anxiety and locomotor variables observed in this study. For example, AM630 has been shown to significantly alter both swimming velocity and immobility, as well as play a significant role in the development of zebrafish locomotor systems [90,91]. Therefore, the CB_2 pathways mediating anxiolytic responses are likely different from those altering locomotion, however, this requires further mechanistic evaluation. Another scenario, which is not mutually exclusive, is that the locomotor effects are more sensitive to terpenes than the anxiolytic effects. TPL significantly decreased velocity at 0.05% and 0.1%, but only had an effect on anxiety-like behaviour at 0.1% (Fig. 3).

Some behavioural research in zebrafish attempts to attribute changes in locomotion to altered anxiety-like behaviour [92]. However, recent studies have shown that these locomotor behaviours may not be a reliable proxy of anxiety-like behaviour and are contingent upon the type of test used [42]. For example, increased swimming velocity may correspond to escape behaviour associated with heightened anxiety, or boldness behaviour associated with decreased anxiety. Similarly, increased immobility may suggest a freezing response associated with anxiety, fear, or alarm responses, or an inhibitory response associated with sedation and impairment [42,93]. Thus, as demonstrated

previously [42], and shown here with different responses across variables with the same concentrations of terpenes, it is necessary to analyze locomotor variables as independent of anxiety-like behaviour variables.

In summary, we have provided direct evidence that CB_2 receptors regulate the anxiolytic effects of BCP and TPL. This confirms a cooperative effect of some terpenes with cannabinoids, which helps to explain the complex effect of cannabis on behaviour.

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CRediT authorship contribution statement

Trevor Hamilton: Conceptualization, Methodology, Resources, Visualization, Supervision, Project administration, Writing – review & editing, Funding acquisition. **Andrea Johnson**: Investigation, Formal analysis, Writing – original draft preparation, Writing – review & editing, Visualization. **James Hudson**: Investigation. **Ryan Verbitsky**: Investigation. **Rachel Dean**: Investigation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors declare that the data supporting the findings of this study are available within the paper [and its supplementary information files] and are available upon request.

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Author contributions

A.J. carried out experiments, data analysis and drafted the manuscript. R.V., J. H., and. R.D. carried out data analysis and experiments. T. J.H. conceived of the study and participated in research coordination and manuscript editing. All authors read and approved the final manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115760.

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