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From the Dinner Pot to Smoking Pot; How a Better Understanding of Cannabidiol in Crayfish could Alleviate Anxiety and Modulate Hunger

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List of Abbreviations

Abbreviation	Definition
5-HT, 5HT	Serotonin
CBD	Cannabidiol
DALY	Disability-Adjusted Life Year
EPSP	Excitatory Postsynaptic Potential
GAD	General Anxiety Disorder
LG	Lateral Giant (Neuron)
LSAS	Liebowitz Social Anxiety Scale
NE	Norepinephrine
NMJ	Neuromuscular Junction
PET	Positron Emission Tomography
PTSD	Post-Traumatic Stress Disorder
SAD	Social Anxiety Disorder
SNP	Simple Nucleotide Polymorphism
SSRIs	Selective Serotonin Reuptake Inhibitors
TSQM	Treatment Satisfaction Questionnaire for Medications
VH	Van Harreveld (Saline Solution)

Abstract

Anxiety affects approximately 1/3 of the US population and presents in many different forms, ranging from social to panic disorders. It also presents with high comorbidity for other mental disorders. One treatment is Selective Serotonin Reuptake Inhibitors (SSRIs) which allow for increased activation of serotonin (5-HT) receptors. SSRIs come with an extensive list of side effects, which can fail to maintain quality of life. Cannabidiol (CBD) is a cannabis derived compound which has been shown to decrease anxiety by activation of multiple subtype 5-HT amine receptors. CBD has few side effects, is not psychoactive, and exhibits anti-psychotic properties. The current understanding of CBD's mechanisms is limited specifically in invertebrates, where to date limited published research involves behavior and cannabinoids. Decapod crustaceans, such as crayfish, have emerged as a novel approach to studying drugs of abuse. Within the neural structures of the crayfish tails are 5-HT receptors that control tail-flips, a withdraw reflex when placed into a fight. Serotonin has also been linked to aggression and decision making for engaging in fights with other crayfish. Additionally, evidence currently suggests CB1 receptors are present at neuromuscular junctions (NMJ) and may have an impact on motility. For this thesis, crayfish were administered either CBD, 5-HT, or a vehicle control. Analysis of motility by percent of time moving or rest, amount of food consumed, and aggression in paired fights were conducted. No statistical significance was found for CBD influencing motility and hunger. However, the duration of fights significantly increased when injected with CBD and when paired with 5-HT injected crayfish. This evidence supports the main hypothesis that CBD increases serotonin receptor activity in crayfish as seen with SSRIs, thus could be of use in treating anxiety.

Hypothesis

The primary goal of this study was to provide evidence that CBD increases 5-HT receptor activity in crayfish tail neural tissue resulting in decreased tail-flip behavior. This same escape reflex has been shown to be decreased with use of SSRIs. Additionally, there are serotonin receptors lining the digestive system in crayfish that could be influenced by CBD. Finally, cannabinoid receptors have been found in the NMJ of crayfish thus suggesting that CBD may increase or decrease the motility of crayfish. The ultimate question at hand was if CBD is an adequate candidate for replacing SSRIs when gauged by serotonin activation in crayfish. Hunger and motility behaviors could also be changed by CBD and were thus explored.

Specific Aims

The most prevalent mental disorder in the US is anxiety, affecting 33.7% of the population at one point in their life. Generalized Anxiety Disorders (GAD), Social Anxiety Disorders (SAD), agoraphobias, panic disorders, and specific phobias make up what is generalized into anxiety. One concerning component of anxiety is the high comorbidity for other mental disorders. One method of treatment is Selective Serotonin Reuptake Inhibitors (SSRIs) which allow for increased activation of serotonin (5-HT) receptors. However, SSRIs come with an extensive list of side effects, some of which fail to maintain quality of life. Cannabidiol (CBD) is a cannabis derived compound which has been shown to decrease anxiety by activation of multiple subtype 5-HT amine receptors. Unlike SSRIs, CBD has few side effects. Additionally, it is not psychoactive and even has anti-psychotic properties. CB1 receptors can have bound CBD where it acts as an allosteric inhibitor of anandamide resulting in decreased drive for food. The current understanding of CBD's mechanisms is limited specifically in invertebrates where to date no published articles involve behavior and cannabinoids. Decapod

crustaceans, specifically crayfish, have emerged as a novel approach to studying drugs of abuse. Crayfish tails contain 5-HT receptors that control tail-flips, a withdrawal reflex to rapidly retreat. One specific serotonin linked behavior is aggression and decision making for engaging in fights with other crayfish. Additionally, evidence currently suggests CB1 receptors are present at neuromuscular junctions (NMJ) and may have an impact on motility. Preliminary studies were conducted to evaluate any change CBD has on the NMJ.

Aim 1: Observe and measure fights of equal sized crayfish via time spent engaged in aggressive behavior and fight duration. Crayfish administered with CBD and 5-HT or agonist will be paired by weight and placed into fights. The correlation between time and behavior will indicate how CBD and SSRIs compare within simple neural structures.

Aim 2: Observe and measure amount of food consumed. CBD and 5-HT or agonist will be administered. The quantitative consumption will indicate how CBD and SSRI compare on hunger.

Aim 3: Examine motility by time spent resting verses moving. CBD and saline control injections will be completed in crayfish. The resulting ability to move will be examined to determine if CBD has an influence on the neuromuscular junction.

CBD has been shown to influence the same receptors as serotonin that produce anxiety. Over a third of the population will experience an anxiety disorder. The current SSRIs used to return functionality to impacted individual leaves them exposed to equally unpleasant side effects. A new treatment may be found through CBD which offers potentially comparable relief without the

tradeoff of current medications. Treatment with CBD in crayfish should produce similar results of those injected with serotonin.

Introduction and Background

Anxiety Economics and Prevalence

The economic and epidemiological survey of mental disorder can best be described as common and costly. Mental illnesses, including anxiety are ranked in the top five global exorbitant conditions. The out of pocket costs for treatment were found to be 23.1% in 1996, and 25.0% in 2006. In addition, mental disorders saw a rise in total costs from $35.2 billion to $57.5 billion between 1996 to 2006. This correlated with the total number of cases increasing from 19.3 million to 36.2 million in the same time frame(1). Estimates in the year 2010 indicate that the cost is now around US $2.5 trillion and will increase to $6.0 trillion by 2030. In addition to direct costs of treatment for an illness one must consider the indirect expenses of an illness such as the Disability-Adjusted Life Year (DALY). Mental illnesses have a larger impact on the DALY then other chronic conditions such as diabetes (2,3). Despite having high cost and prevalence associated with mental disorders, the general public places higher budgetary priority on somatic illness such as cancer and diabetes (4). Multiple economic and personal burdens are associated with mental disorders and there is a need for more awareness (5). The most prevalent mental disorder in the US was found to be anxiety of any type ranging from 18.0% (6) to 38.0% (7). This includes general anxiety disorders (GAD), social or separation anxiety disorders (SAD), agoraphobias, specific phobias, social phobias, panic disorders, and post-traumatic stress disorders (PTSD). Anxiety was found to be comorbid with other mental illnesses like depression and bipolar disorders as well as physical disabilities such as asthma or diabetes (3,8).

Anxiety Pathology

The pathological cause of mental illness is a complex network of disruptions in normal brain activity. One such area of interest for depression and anxiety is the group of serotonin amine receptor family along with norepinephrine (NE) turnover (Reviewed by Ressler and Nemeroff 2000). 5-HT receptors are found throughout the central nervous system such as the pathways of the basal ganglia, hippocampus, hypothalamus and spinal cord through the dorsal raphe. Serotonin receptors include 12 different subtypes, such as the 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_3, and 5-HT_4 families (10–12). For this study, the action of the 5-HT1A receptor as related to anxiety was investigated. 5-HT_{1A} abnormalities have been well documented for their presence in patients with depression and anxiety. One example is the analysis of suicide victim who experienced depression. The dysregulation of 5-HT_{1A} in the victims indicated a direct correlation to amine-based receptor malfunctions and decreased receptor prevalence. In addition, positron emission tomography (PET) of comorbid patients for panic disorders and depression revealed significant alterations in the 5-HT_{1A} receptor prevalence compared to controls (13). A similar PET scan study using males with SAD indicated a deceased binding potential for the 5-HT_{1A} receptors (14). One explanation for the dysregulation could genetic predisposition. Genetic examination concluded that up regulation of auto receptors within the dorsal raphe from simple nucleotide polymorphism (SNP) could result in the decreased firing rate (15,16). Evidence also suggests that 5-HT_{1A} plays a key role in development of emotional behaviors. This was shown with fine tuning of the 5-HT_{1A} heteroreceptors and auto receptors using knockout mice models (16–18). To summarize, PET scans, genetic predisposition, pharmacological studies and post mortem analysis all concluded that 5-HT_{1A} has a crucial role in anxiety. This is postulated to be due to a decreased binding potential in the pathological state for anxiety and depression (19). But

the actual role of serotonin and 5-HT_{1A} activation is still under debate. Serotonin dysregulation has also been linked to neurodegenerative disease like Parkinson's where it presents with depression (11) and tremors (20). The current treatment for pathological 5-HT receptor abnormalities is with Selective Serotonin Reuptake Inhibitors (SSRIs). The current theory for SSRI treatment is that additional 5-HT is available for activating 5-HT_{1A}. This results in a less pathological state for treatment of anxiety and depression disorders (21).

Anxiolytic Medication and Side Effects

One option for treatment of anxiety disorders is use of SSRI compounds, such as paroxetine, fluvoxamine, sertraline, fluoxetine, and escitalopram. SSRIs have been shown to be effective in both anxiety and depression and are often utilized when disease states presents individually or in comorbidity (22). Early studies on paroxetine, fluvoxamine, sertraline and fluoxetine and citalopram concluded the statistical significance in decreased social anxiety based on the Liebowitz Social Anxiety Scale (LSAS)(23), while later summaries of the studies show additional evidence that included escitalopram (24). The primary mode of action of SSRIs is to produce additional synaptic 5-HT, thus increasing the activation of the serotonin receptors (25). However, the hypothesized activation of various subtypes of receptors in the body could be the source of multiple side effects (10). These include alterations in sexual function leading to delayed ejaculation, decreased libido, and anorgasmia in up to 60% of patients. Gastrointestinal systems can include nausea, diarrhea, dry mouth, constipation, and anorexia. The central nervous system can be impacted to produce anxiety, insomnia, sedation, and nightmares. This can include extrapyramidal symptoms like akathisia (constant movement or motor restlessness). Approximately twenty five percent of patients on SSRIs have reported insomnia or somnolence. Additional adverse effects include bleeding by inhibition of platelet function resulting in bruising and epistaxis. Hyponatremia can occur but is rare. Serotonin syndrome can arise from hyperstimulation of the 5-HT receptors if SSRIs are used with monoamine oxidase inhibitors, pentazocine or L-tryptophan. This overstimulation leads to nausea, diarrhea, restlessness, extreme agitation, hyperreflexia, autonomic instability, myoclonus, hyperthermia, rigidity, delirium, seizures and status epilepticus. In extreme instances this can lead to cardiovascular collapse, coma and death. The withdrawal from use of SSRIs can lead to discontinuation

syndrome, characterized by dizziness, nausea, weakness, insomnia, anxiety, irritability and headache. These symptoms dissipated over time, usually a few weeks. Tapering off SSRIs before complete discontinuation helps alleviate withdrawal symptoms(26). The prevalence of these side effects is well documented. In one cohort study of 584 patients actively on SSRIs the top most side effects included sleepiness during the day (21%), dry mouth (22%), profuse sweating (20%), sexual dysfunction (19%), dizziness (12%). Sleepiness, restlessness, muscle spasm, twitching, nausea, constipation, and diarrhea were reported below at or below 10% of cases (27). An additional study conducted using a drug safety monitoring service utilized a Treatment Satisfaction Questionnaire for Medications (TSQM) to collect patient data for those on SSRIs of citalopram, escitalopram, fluoxetine, paroxetine, and sertraline. Of the approximate 700 patients surveyed, 38% indicated that they experienced one or more side effect, while 229 listed the adverse effect of sexual dysfunction, sleepiness, and weight gain as the most crucial. The symptoms were also ranked by how bothersome they presented which indicated that 7% found them extremely bothersome, 19% very bothersome, 40% somewhat bothersome, 29% little bothersome, and 5% not at all bothersome (28). One of the earlier studies on SSRI use for 75-105 days examined 401 patients via phone interviews to conclude that 344 (86%) reported experience at a minimum one side effect, while 219 (55%) indicated that at least 1 or more of the side effects was bothersome. Sexual dysfunction and drowsiness tied at 17% for most common bothersome. Those who experienced a side effect in the first two weeks indicated that at the end of the study blurred vision (85%) and sexual dysfunction (83%) remained. These results show how the bothersome side effects are persistent after three months of treatment (29). One hypothesis for the range of side effects centers on SSRIs causing inhibition of dopamine neurotransmitter release either by direct interaction or by modulation of serotonin receptors for

dopamine. This was concluded by examination of clinical reports on side effect occurrences and instances where stimulation of dopamine releasing treatments counteracted SSRI side effects (30). Due to the high out-of-pocket cost of treatment, increased prevalence, and well documented side effect, alternative treatments for anxiety have been pursued. One such treatment with potential anxiolytic effect is cannabidiol (CBD), a phytocannabinoid derived the plant cannabis sativa (31).

CBD and Endocannabinoids

Cannabidiol or CBD has emerged as a novel compound for its various interaction with multiple receptors in the human body. These receptors include CB1, CB2, PPARγ, and 5-HT1. The correlated physiological response is weight loss, insulin sensitivity, reduced atherosclerosis, and anxiolysis respectively. Additionally, CBD has been found to lower high glucose levels and cell inflammation due to its antioxidant, anti-inflammatory properties (32), relief of Aβ-Induced neuroinflammation to promote hippocampal neurogenesis as related to Alzheimer's disease (33), protective properties against myocardial ischemic reperfusion injuries (34), and treatment potential for acute and anticipatory nausea (35). Cannabinoids in medicine is not unheard as documentation indicates that cannabis was used in China over a thousand of years ago (36). The recent rediscovery of its benefits has led to a surge into marijuana medical research. Over the past thirty years extensive research has been done on cannabis receptors within the human body. This led to discovery of endogenous cannabinoids used in the human body that specifically act on the hunger drive mechanism (37). The safety limit for CBD was found through animal models and human trials to be safe at elevated levels beyond the useful range (38). CBD has long been known to counteract the effects of Δ9-THC, the active component of cannabis that produces the sensation of feeling high as well as paranoia and anxiety. This was shown in a study that used human subjects who consumed both compounds and were surveyed for anxiety and paranoia (39). Extending past this, CBD has been shown to be non-psychoactive and even display anti-psychotic properties through both human trials and animal studies (40). Investigation into the antianxiety effects of CBD have been promising. The use SSRIs was compared against CBD to determine the potency of anxiolysis. Elevated plus-maze evaluations of anxiety with CBD in rats concluded with increased time spent on the open arms (41). Similarly, Vogel conflict tests with

rats came to the same conclusion that CBD had alleviated anxiety behavior as observed by increased punished licks (42). The use of CBD in humans produced a U-shaped curve for decreased anxiety similar to those found with SSRIs when subjects were placed into public speaking scenarios (43). In addition, a dampened emotional response was not seen with treatment of CBD. A study of 38 healthy individuals concluded that CBD did not numb emotional responses to negative social stimuli nor social rejection which indicates that behavior changes are not seen with use of CBD (44). The specific physiological reason that CBD has antianxiety properties has been thought to be from the effect on 5-HT receptors. This was demonstrated by administrations of CBD to the dorsolateral periaqueductal of rats followed by observations on elevated plus-maze and Vogel conflict test. Use of an antagonist to 5-HT1a stopped the effects from the CBD(45). Alternatively, the co-presence of 5-HT3a and CB1 in the interneurons of the hippocampus and dentate gyrus was found through situ hybridization histochemistry and could lead to avenues for research. Additional studies are required to further determine the extent that these receptors play on anxiety (46). To better understand the social and behavior effects of CBD examination in other biological systems could provide further insight. A recent approach to pharmacology has been to use invertebrates as models for physiology. Recent studies have shown evidence for CB1 receptors at the neuromuscular junction(NMJ) of crayfish from antibody-selective immunofluorescent staining microscopy (47) and pharmacological activation measured via excitatory post synaptic potentials electrical amplitude (48). In addition to the cannabinoid receptors, 5-HT receptors are also found within crayfish. The approach to using SSRIs within crayfish has been shown to dramatically change the outcome of their behaviors as serotonin receptors play a crucial role in aggression and social ranking.

Crayfish Physiological Model

Decapod crustaceans, specifically crayfish, have emerged as a novel physiological model for studying pharmacological properties. Some of the most dramatic example include studies involving drugs of abuse where the behavior changes were most notable. (49–52). Crayfish have been found to express serotonin and serotonin receptors within various tissues(53,54). Genetic studies concluded that 5-HT1A and 5-HT2 were conserved compared with *Procambarus clarkii* (southern swamp crayfish) and *Panulirus interruptus* (lobster) in structure and function (55). Direct injections of serotonin into the brains of crayfish induced an avoidance behavior and decreased movement when placed into a light-dark maze (56). This provides insight into how 5-HT plays a role in the CNS of crayfish and produces anxiety like behaviors. However, within the peripheral nervous system the effects of serotonin are vastly different. Serotonin has been found to alter aggressive behaviors and cause changes in social ranking (57). This change is due to unidentified subtype receptors being found within the tail neural circuits that controls tail-flip escape behavior (58). Specifically, the Lateral Giant (LG) neurons receives input from mechanosensory of the abdomen (59). With the use of the pharmacological 5-HT neurotoxin 5,7-Dihydroxytryptamine the LG neuron was shown to be directly modulated by 5-HT (60). The excitatory postsynaptic potential (EPSP) for the LG neuron has been shown to be inhibited by excessive serotonin in both monosynaptic(α) and di-synaptic(β) connections (61,62). Serotonin has also been shown to depolarize the LG neuron allowing for less resistance to distal dendrites (63). A study conducted using 5-HT_{1A} and 5-HT_2 receptor agonists, 1-(3-chlorophenyl) piperazine dichloride (m-CPP Cl2) and α-methylserotonin meleate (α-CH3 5-HT maleate) respectively, concluded that social isolation, subordination and dominance played a key role in how the EPSP changes from serotonin. The specific receptor subtypes identified from the study

were 5-HT_1 and 5-HT_2 like receptors that act in inhibition and excitation, respectively, when serotonin is present (64). With the connection that tail flip behavior was associated with 5-HT, the next logical step examined if SSRIs could decrease the retreating muscular reflex from the LG nerve. Injections of 5-HT directly into equally sized crayfish increased their fighting durations and intensity favoring aggression. Preliminary studies using acute injects of fluoxetine (Prozac) indicated that SSRIs had negligible behavioral changes, but this was expected as with humans the same result have been found. Notable changes with SSRIs require extended treatment times (65). Additional studies with fluoxetine postulated that during elevated levels serotonin is taken up by the LG neuron and released during times of fights. This would account for the fact the various concentrations of 5-HT change fight characteristics but the use of acute SSRIs had little to no effect (66). However, acute use of fluoxetine was shown to increase the amplitude of the ESPS at the neuromuscular junction indicating an excitatory effect, while chronic use had no effect. This was postulated to be caused by changes made to glutamate neurotransmitter release, potentially causing reuptake inhibition as with serotonin (67). This would account for the fact that other naturally occurring anxiolytics have been shown to play a role in glutamate related neural activity. One such example is Hypericum perforatum from St John's Wort(68). This would also account for the observed action of endocannabinoids on the crayfish neuromuscular junction. Initial use of a cannabinoid like pharmacological agent indicated a glutamatergic decrease in EPSP amplitude in crayfish at the NMJ (48), while this was later identified as CB1 receptors from immunofluorescence and microscopy (47). Further studies using CB1 receptor agonist found that the EPSP was decreased at the NMJ in crayfish (69). Currently, there no published studies that look at crayfish behavior with injections of endocannabinoids, including cannabidiol. Since CBD has been shown to act as an allosteric

inhibitor of CB1 receptors this could play a role in motility and was addressed in this study as a potential confounding variable for behavior analysis. Initial trials were conducted to measure the degree that CBD can alter movement.

Methods and Materials

Animals

Male and female intermolt (form I) crayfish, *Faxonius propinquus*, with fully intact appendages were socially and physically isolated in a flow-through holding tank. Crayfish will also be kept at a constant temperature (23°C) and light:dark cycle (14hr:10hr) and crayfish were isolated for a minimum of one week prior to experimentation to reduce the effects of prior social experience for fight trial. Crayfish were size-matched within 90% for carapace length (from rostrum to beginning of abdomen) and weight to reduce size influences on fights (70). Each crayfish, regardless of treatment, were used only once during this study. Crayfish were marked with white correction fluid on the carapace for later identification during behavioral analysis for fights (Bergman lab). For hunger analysis crayfish were deprived of food for a minimum of two days prior to study. Crayfish used for the motility trials were not isolated or starved prior to injections.

Drug Selection

To determine the effects of CBD on aggression, food seeking, and neural activity the use of Van Harreveld's (VH) saline solution was employed as a vehicle control. The standard control was 5-HT. For all trials 5-HT was injected at a concentration of 5ug/g, a concentration sufficient to cause postural changes (71). The control (Van Harreveld's solution) consisted of 12 g of NaCl, 0.4 g of KCl, 2 g of $CaCl_2$, 0.5 g of $MgCl_2$, 0.2 g of $NaHCO_3$ per 1 L of H_2O with a pH of 7.4 (72). CBD trials for hunger were administered at a dose of 2.0 μg/g, 5 μg/g, and 10 μg/g. For fight analysis only 2.0 μg/g of CBD was injected, and 10 μg/g for motility studies. These concentration were chosen from previous rodent studies (42).

Drug administration and preparation

Crayfish weight was used to determine proper amount of drug required for each animal. Crayfish were then randomly assigned to an experimental group. Syringe injections were completed through the dorsal portion of the tail between abdomen plates into the cardiac sinus. A petroleum jelly (Vaseline) was used to help seal the wound and prevent leakage. Crayfish which weighted above 5g received injections of 1.0 ml while smaller crayfish received 0.5 ml injects. The compound concentration injected relative to the size remained the same. For the CBD injections, initial stock of 1.0mg/ml ampule suspended in methanol was dried over air until CBD globules remained. The CBD was reconstituted with VH Saline at a concentration of 0.2mg/ml by heating to 70 degrees Celsius while stirring with a magnetic stir bar.

Behavior observations

Aim 1: Aggressive interaction protocol

For fight experiments, crayfish were tested in pairs consisting of animals that differed by no more than 10% in body weight and 10% in carapace length and were the same sex. After injection of solution the crayfish were placed in the separate compartments of the fight arena and allowed to acclimate for 15 min. The fight arena is made of Plexiglas (20 × 20 × 14 cm) and is divided into halves, separated by opaque retractable walls. The arena holds 10L of dechlorinated water (filled to a depth of 4 cm from the top of the tank). After acclimation, the divider was removed, allowing two crayfish to interact. An opaque curtain surrounds the fight tank to prevent external distractions. The interacting animals were video recorded for 15 to 30 minutes. After the fight the crayfish were returned to isolation tanks and not reused in future trials (Bergman lab).

Aim 1: Fight Analysis

All fights were digitally recorded from a camera positioned one meter above the test arena. For each encounter, recordings of the winners and losers of each fight was determined as

well as the temporal mechanics of the fight (Table 1). Temporal mechanics include time to different fight intensities and duration of the initial encounter. All interactions were analyzed by examining the behavior of both participants while each individual receives a unique ethogram value. In addition, the percent of time spent in interactions was analyzed.

The identities of initiating and winning animals are to be recorded for each interaction. The crayfish that first engages an opponent with either a meral spread (Claws raised) or physically contact was deemed the initiator. The dominant crayfish is determined by the animal that pursues its opponent (i.e. the loser) as it retreats or tail-flips away, or if the two crayfish adopt body postures indicating dominance. Dominant crayfish tend to exhibit high body postures, extended tails and pointing or raised claws, whereas subordinate crayfish tend to exhibit lowered body position and tails curled under the body (70). Prior studies indicate that 5-HT can induce an “aggressive posture" that resembles a meral spread – a common display of dominant animals (73). Yet Tierney and Mangiamele (2001) note that the aggressive meral spread posture could involve either an elevated or depressed posture, so when observing postural effects alone status roles will not necessarily be assigned.

Table 1: Crayfish Ethogram Codes (Used to score fight intensity levels)

Intensity Level	Description
-2	Tailflip away from opponent or fast retreat
-1	Retreat by slowly backing away from opponent
0	Visually ignore opponent with no response or threat display
1	Approach without a threat display; claws raised
2	Initial claw use by boxing, pushing and/or touching
3	Active claw use by grabbing and/or holding opponent

Aim 2: Hunger and food-seeking

To determine the effects CBD has on food-seeking behavior and hunger, each pre-starved crayfish was injected with 2ug/g, 5 ug/g, 10ug/g CBD, VH saline control or 5ug/g 5-HT and placed into a small isolated tank approximately 10 inches by 10 inches and allowed five minutes to acclimate. The weight of a small portion of a tilapia filet was taken prior to being placed into the insolation tank. After 10 minutes, if the fila piece remained intact it was removed, dried, and weighted again. The difference in initial and final weights were used a percent indicator for how much was consumed.

Aim 3: Motility

To assess the degree that CBD plays on the NMJ, crayfish were injected with saline control Van Harreveld's solution or 10ug/g CBD and placed an isolation tank with opaque curtain to decrease external distractions. The animals were video recorded to observed total distance explored in the tank for the initial 12 minutes. The crayfish was then returned to isolation tanks and not reused for further trials.

Results

Statistics

All statistical analysis was completed using SPSS 25 provided by Grand Valley State University. A significance level of 0.05 was used for hypothesis testing outcomes.

Motility

The descriptive statistics for the motility trials can be found in Table 2. From this data Figure 1 displays the mean percent of time resting while Figure 2 is the mean percent of time moving. Table 3 displays the group statistics for number of samples based on compound. Note how the VH saline controls had a large standard deviation compared to the CBD. These results were analyses with a two-sample T-test of means(Table 4) which yielded no significant difference. A Mann-Whitney U Test was performed and no significance was found using a non-parametric testing method as well.

Table 2 Descriptive Statistics for Motility

	Compound			Statistic	Std. Error
Percent Time Resting	Blank VH Saline	Mean		50.5000	11.01741
		95% Confidence Interval for Mean	Lower Bound	22.1788	
			Upper Bound	78.8212	
		5% Trimmed Mean		50.2037	
		Median		48.5000	
		Variance		728.300	
		Std. Deviation		26.98703	
		Minimum		20.67	
		Maximum		85.67	
		Range		65.00	
		Interquartile Range		51.50	
		Skewness		.188	.845
		Kurtosis		-2.258	1.741
	10 ug/mg CBD	Mean		52.0202	3.35792
			Lower Bound	44.0799	

		95% Confidence Interval for Mean	Upper Bound	59.9604	
		5% Trimmed Mean		52.3743	
		Median		52.8333	
		Variance		90.205	
		Std. Deviation		9.49763	
		Minimum		31.67	
		Maximum		66.00	
		Range		34.33	
		Interquartile Range		3.95	
		Skewness		-1.254	.752
		Kurtosis		3.948	1.481
Percent Time Moving	Blank VH Saline	Mean		49.5000	11.01741
		95% Confidence Interval for Mean	Lower Bound	21.1788	
			Upper Bound	77.8212	
		5% Trimmed Mean		49.7963	
		Median		51.5000	
		Variance		728.300	
		Std. Deviation		26.98703	
		Minimum		14.33	
		Maximum		79.33	
		Range		65.00	
		Interquartile Range		51.50	
		Skewness		-.188	.845
		Kurtosis		-2.258	1.741
	10 ug/mg CBD	Mean		47.9798	3.35792
		95% Confidence Interval for Mean	Lower Bound	40.0396	
			Upper Bound	55.9201	
		5% Trimmed Mean		47.6257	
		Median		47.1667	
		Variance		90.205	
		Std. Deviation		9.49763	
		Minimum		34.00	
		Maximum		68.33	
		Range		34.33	
		Interquartile Range		3.95	
		Skewness		1.254	.752
		Kurtosis		3.948	1.481

Figure 1 Mean Percent of Time Resting Based on Compound

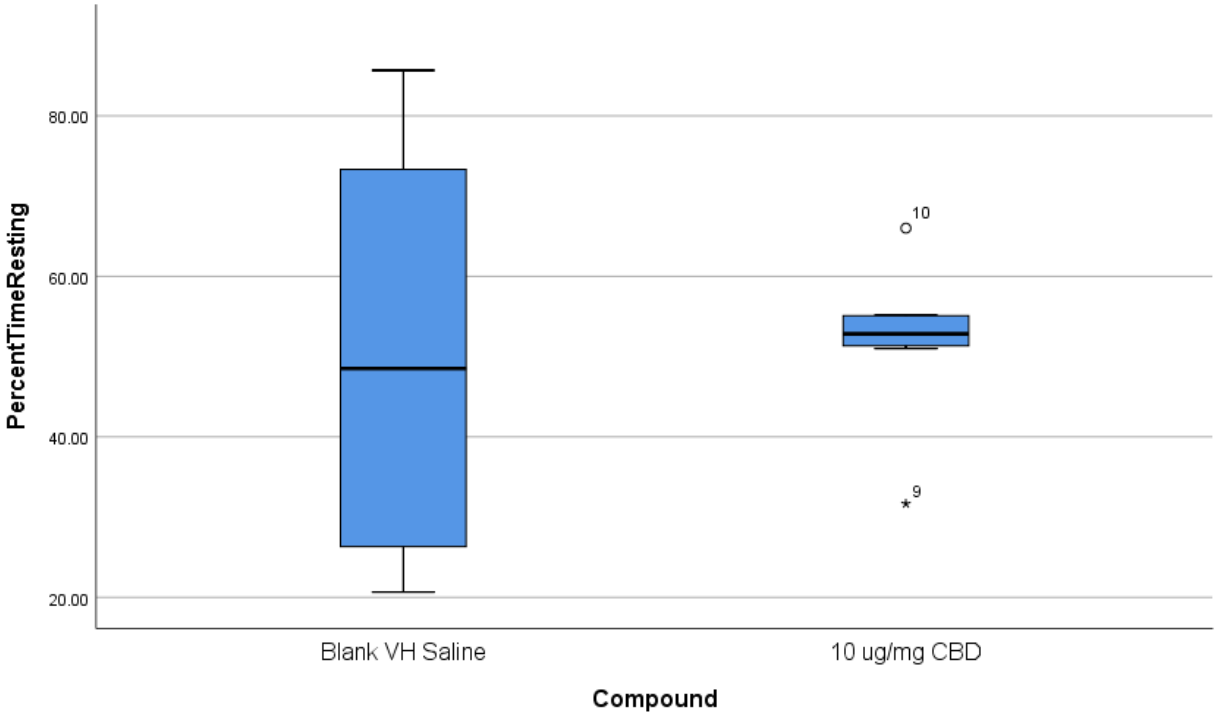


Figure 2 Mean Percent of Time Spent Moving Based on Compound

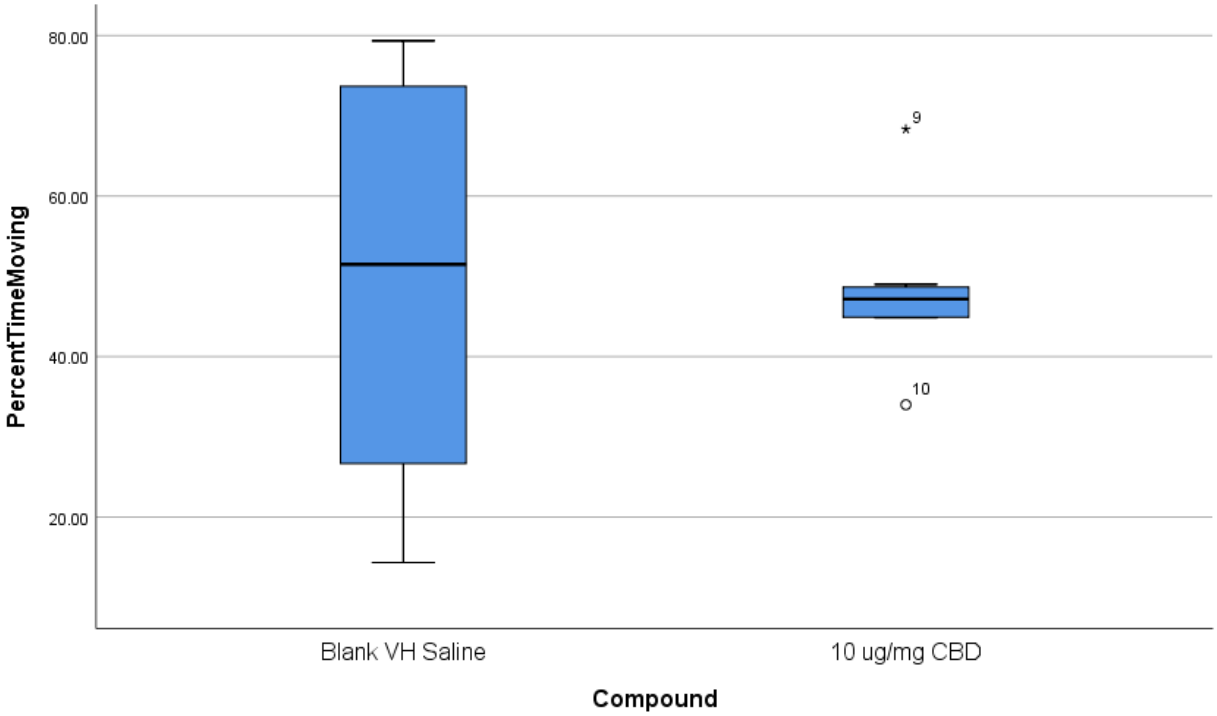


Table 3 Motility Statistics Summary

	Compound	N	Mean	Std. Deviation	Std. Error Mean
Percent Time Resting	Blank VH Saline	6	50.5000	26.98703	11.01741
	10 ug/mg CBD	8	52.0202	9.49763	3.35792
Percent Time Moving	Blank VH Saline	6	49.5000	26.98703	11.01741
	10 ug/mg CBD	8	47.9798	9.49763	3.35792

Table 4 Independent Samples Test For Percent Time Resting and Moving

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Percent Time Resting	Equal variances assumed	17.580	.001	-.149	12	.884	-1.52016	10.19098	-23.72440	20.68408
	Equal variances not assumed			-.132	5.935	.899	-1.52016	11.51777	-29.77754	26.73722
Percent Time Moving	Equal variances assumed	17.580	.001	.149	12	.884	1.52016	10.19098	-20.68408	23.72440
	Equal variances not assumed			.132	5.935	.899	1.52016	11.51777	-26.73722	29.77754

Fights

The descriptive statistics can be found in Table 5 for the two areas of interest for fight analysis. The values analyzed included the mean fight score by ethogram, and the percent of time spent fighting. Boxplots were generated for the mean fight scores based on compound (Figure 3) and the mean percent of time spent fighting by compound injected (Figure 4). Table 6 redisplays the descriptive statistics for the ANOVA analysis completed in Table 7. Significance was found with a pvalue of 0.004 for percent of time fighting being different between injection types. No significance was found for total fights in the first twelve minutes(additional area investigated) and mean fight ethogram score. Table 8 displays the post-hoc analysis which indicates that significance found for mean percent of time fighting was higher for both the CBD and 5HT when compared to Blank VH saline control injections. All other areas held no significance. A summary of the number of trials completed for analysis was generated(Table 9). Additional analysis was completed to examine how the effects of pair-wise matching by compound injected might change the means. Table 10 includes the number of samples used while Table 11 details the descriptive statistics for the pair-wise analysis. Boxplots for Mean Fight Score(Figure 5) showed less variance as compared to the Mean Percent Time Fighting(Figure 6) based on the compound pairing type specifically for the CBD-5-HT pairings. ANOVA analysis was completed (Table 12). Significance was found that Mean Percent of Time Fighting was not the same for all groups analyzed. Post-Hoc analysis in Table 13 shows that the CBD-5HT injections pairs had significantly longer fight times compared to Blank-Blank, Blank-CBD, and Blank-5HT injection pairings. Due to the low number of subjects a non-parametric test was conducted and yielded similar results to the ANOVA analysis. Figure 7 displays the hypothesis test summary for pair-wise analysis of Mean Fight Score and Mean Percent of Time Fighting. Significance was found for the Mean Percent of Time Fighting but not for the Mean Fight Scores by group. Boxplot

summary and table of counts for the Kruskal-Wallis non-parametric testing can be found in Figure 8. A table of adjusted significance is displayed in Figure 9. Statistical significance was found that the Blank-Blank compared to CBD-5-HT pairing fights for Mean Percent of Time Fighting were not the same. Finally Figure 10 displays the non-parametric box plots and summary for fight analysis based on ethogram which yielded no statistical significance. The comparison of blank/saline-blank/saline to CBD-5HT yielded statistically different distribution of mean percent time spent fighting. This correlates with the results found from the parametric testing.

Table 5 Descriptive Statistics for Paired Fight Analysis for Individual Injection Type

	Compound			Statistic	Std. Error
Mean Fight Score	Blank	Mean		.656685	.0897042
		95% Confidence Interval for Mean	Lower Bound	.462891	
			Upper Bound	.850479	
		5% Trimmed Mean		.669979	
		Median		.721154	
		Variance		.113	
		Std. Deviation		.3356425	
		Minimum		.0000	
		Maximum		1.0741	
		Range		1.0741	
		Interquartile Range		.3214	
		Skewness		-1.016	.597
		Kurtosis		.474	1.154
	CBD	Mean		.761996	.1101632
		95% Confidence Interval for Mean	Lower Bound	.501502	
			Upper Bound	1.022491	
		5% Trimmed Mean		.794031	
		Median		.862534	
		Variance		.097	

		Std. Deviation		.3115887	
		Minimum		.0000	
		Maximum		.9474	
		Range		.9474	
		Interquartile Range		.1054	
		Skewness		-2.697	.752
		Kurtosis		7.442	1.481
	5HT	Mean		.720250	.0998248
		95% Confidence Interval for Mean	Lower Bound	.484202	
			Upper Bound	.956298	
		5% Trimmed Mean		.718091	
		Median		.723636	
		Variance		.080	
		Std. Deviation		.2823471	
		Minimum		.2222	
		Maximum		1.2571	
		Range		1.0349	
		Interquartile Range		.1613	
		Skewness		.256	.752
		Kurtosis		2.914	1.481
Percent Time Fighting	Blank	Mean		16.381926	2.5435482
		95% Confidence Interval for Mean	Lower Bound	10.886924	
			Upper Bound	21.876927	
		5% Trimmed Mean		16.187434	
		Median		17.049001	
		Variance		90.575	
		Std. Deviation		9.5170859	
		Minimum		2.0000	
		Maximum		34.2647	
		Range		32.2647	
		Interquartile Range		13.3939	
		Skewness		.394	.597
		Kurtosis		-.394	1.154
	CBD	Mean		38.122512	7.6897197
		95% Confidence Interval for Mean	Lower Bound	19.939214	
			Upper Bound	56.305809	
		5% Trimmed Mean		38.120920	
		Median		33.529412	

		Variance		473.054	
		Std. Deviation		21.7498117	
		Minimum		8.6667	
		Maximum		67.6070	
		Range		58.9403	
		Interquartile Range		43.3895	
		Skewness		.143	.752
		Kurtosis		-1.184	1.481
	5HT	Mean		39.327267	7.3418015
		95% Confidence Interval for Mean	Lower Bound	21.966665	
			Upper Bound	56.687869	
		5% Trimmed Mean		39.494039	
		Median		35.368127	
		Variance		431.216	
		Std. Deviation		20.7657506	
		Minimum		8.0943	
		Maximum		67.5584	
		Range		59.4641	
		Interquartile Range		38.1281	
		Skewness		.100	.752
		Kurtosis		-.885	1.481

Figure 3 Mean Fight Score by Ethogram based on Compound Used

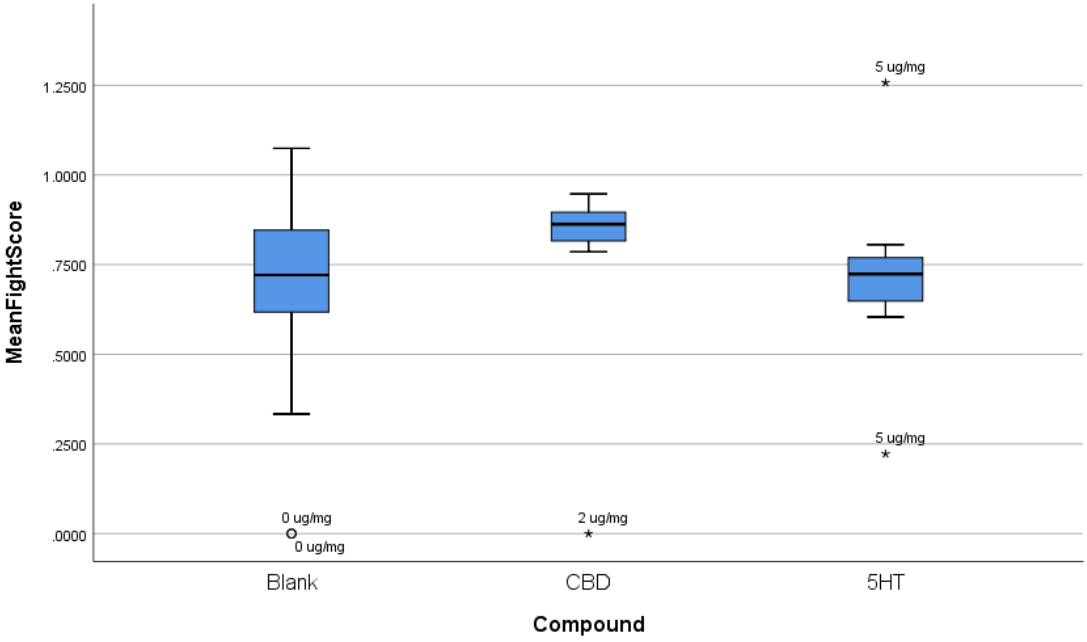


Figure 4 Mean Percent of Time Fighting based on Compound Used

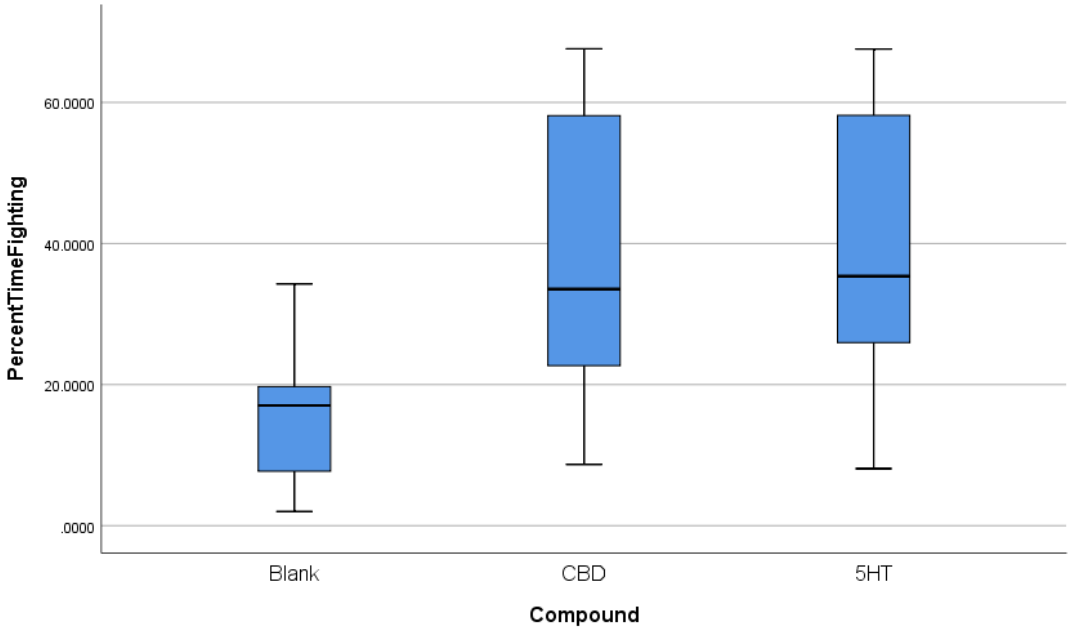


Table 6 Descriptive For Fight Analysis based on Compound

	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean	Minimum	Maximum

					Lower Bound	Upper Bound		
Percent Time Fighting	Blank	16.381926	9.5170859	2.5435482	10.886924	21.876927	2.0000	34.2647
	CBD	38.122512	21.749817	7.6897197	19.939214	56.305809	8.6667	67.6070
	5HT	39.327267	20.765756	7.3418015	21.966665	56.687869	8.0943	67.5584
	Total	28.298173	19.687729	3.5944713	20.946654	35.649692	2.0000	67.6070
Total Fights In First 12 mins	Blank	4.00	2.287	.611	2.68	5.32	1	8
	CBD	3.50	1.414	.500	2.32	4.68	2	6
	5HT	4.88	2.588	.915	2.71	7.04	2	9
	Total	4.10	2.171	.396	3.29	4.91	1	9
Mean Fight Score	Blank	.656685	.3356425	.0897042	.462891	.850479	.0000	1.0741
	CBD	.761996	.3115887	.1101632	.501502	1.022491	.0000	.9474
	5HT	.720250	.2823471	.0998248	.484202	.956298	.2222	1.2571
	Total	.701719	.3086335	.0563485	.586473	.816964	.0000	1.2571

Table 7 ANOVA Significance for Fight Analysis based on Compound

		Sum of Squares	df	Mean Square	F	Sig.
Percent Time Fighting	Between Groups	3733.226	2	1866.613	6.713	.004
	Within Groups	7507.369	27	278.051		
	Total	11240.595	29			
Total Fights In First 12mins	Between Groups	7.825	2	3.913	.820	.451
	Within Groups	128.875	27	4.773		
	Total	136.700	29			
Mean Fight Score	Between Groups	.060	2	.030	.301	.743
	Within Groups	2.702	27	.100		
	Total	2.762	29			

Table 8 Multiple Comparisons for Fight Analysis based on Compound

Tukey HSD

Dependent Variable	(I) CompoundNumeric	(J) CompoundNumeric	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Percent Time Fighting	Blank	CBD	-21.7405859*	7.3903384	.018	-40.064322	-3.416850
		5HT	-22.9453412*	7.3903384	.012	-41.269077	-4.621605
	CBD	Blank	21.7405859*	7.3903384	.018	3.416850	40.064322
		5HT	-1.2047553	8.3374262	.989	-21.876717	19.467207
	5HT	Blank	22.9453412*	7.3903384	.012	4.621605	41.269077
		CBD	1.2047553	8.3374262	.989	-19.467207	21.876717
Total Fights In First 12 mins	Blank	CBD	.500	.968	.864	-1.90	2.90
		5HT	-.875	.968	.643	-3.28	1.53
	CBD	Blank	-.500	.968	.864	-2.90	1.90
		5HT	-1.375	1.092	.430	-4.08	1.33
	5HT	Blank	.875	.968	.643	-1.53	3.28
		CBD	1.375	1.092	.430	-1.33	4.08
Mean Fight Score	Blank	CBD	-.1053115	.1402095	.736	-.452949	.242326
		5HT	-.0635653	.1402095	.893	-.411203	.284073
	CBD	Blank	.1053115	.1402095	.736	-.242326	.452949
		5HT	.0417462	.1581776	.962	-.350442	.433935
	5HT	Blank	.0635653	.1402095	.893	-.284073	.411203
		CBD	-.0417462	.1581776	.962	-.433935	.350442

*. The mean difference is significant at the 0.05 level.

Table 9 Case Processing Summary for Fight Analysis based on Compound Used

		Cases					
		Valid		Missing		Total	
	Compound	N	Percent	N	Percent	N	Percent
Mean Fight Score	Blank	14	100.0%	0	0.0%	14	100.0%
	CBD	8	100.0%	0	0.0%	8	100.0%
	5HT	8	100.0%	0	0.0%	8	100.0%
Percent Time Fighting	Blank	14	100.0%	0	0.0%	14	100.0%
	CBD	8	100.0%	0	0.0%	8	100.0%
	5HT	8	100.0%	0	0.0%	8	100.0%

Table 10 Case Processing Summary for Fight Analysis when Examined by Fight Compound Pair Match Type

		Cases					
		Valid		Missing		Total	
	FightBasedOnPairing	N	Percent	N	Percent	N	Percent
Mean Fight Score	Blank-Blank	8	100.0%	0	0.0%	8	100.0%
	Blank-CBD	6	100.0%	0	0.0%	6	100.0%
	Blank-5HT	6	100.0%	0	0.0%	6	100.0%
	CBD-5HT	10	100.0%	0	0.0%	10	100.0%
Percent Time Fighting	Blank-Blank	8	100.0%	0	0.0%	8	100.0%
	Blank-CBD	6	100.0%	0	0.0%	6	100.0%
	Blank-5HT	6	100.0%	0	0.0%	6	100.0%
	CBD-5HT	10	100.0%	0	0.0%	10	100.0%

Table 11 Descriptive Statistics for Fight Analysis by Compound Pair Match Type

	FightBasedOnPairing			Statistic	Std. Error
Mean Fight Score	Blank-Blank	Mean		.821476	.0825685
		95% Confidence Interval for Mean	Lower Bound	.626232	
			Upper Bound	1.016719	
		5% Trimmed Mean		.834561	
		Median		.836120	
		Variance		.055	
		Std. Deviation		.2335389	
		Minimum		.3333	

		Maximum	1.0741	
		Range	.7407	
		Interquartile Range	.2775	
		Skewness	-1.288	.752
		Kurtosis	2.503	1.481
	Blank-CBD	Mean	.663156	.1385670
		95% Confidence Interval for Mean	Lower Bound	.306958
			Upper Bound	1.019354
		5% Trimmed Mean	.685914	
		Median	.769231	
		Variance	.115	
		Std. Deviation	.3394186	
		Minimum	.0000	
		Maximum	.9167	
		Range	.9167	
		Interquartile Range	.3720	
		Skewness	-2.009	.845
		Kurtosis	4.302	1.741
	Blank-5HT	Mean	.675463	.1651737
		95% Confidence Interval for Mean	Lower Bound	.250871
			Upper Bound	1.100056
		5% Trimmed Mean	.680673	
		Median	.686217	
		Variance	.164	
		Std. Deviation	.4045914	
		Minimum	.0000	
		Maximum	1.2571	
		Range	1.2571	
		Interquartile Range	.4552	
		Skewness	-.495	.845
		Kurtosis	2.187	1.741
	CBD-5HT	Mean	.644804	.0957962
		95% Confidence Interval for Mean	Lower Bound	.428098
			Upper Bound	.861510
		5% Trimmed Mean	.663817	
		Median	.726667	
		Variance	.092	
		Std. Deviation	.3029340	

		Minimum		.0000	
		Maximum		.9474	
		Range		.9474	
		Interquartile Range		.3610	
		Skewness		-1.442	.687
		Kurtosis		1.334	1.334
Percent Time Fighting	Blank-Blank	Mean		13.547899	1.9583838
		95% Confidence Interval for Mean	Lower Bound	8.917057	
			Upper Bound	18.178741	
		5% Trimmed Mean		13.714009	
		Median		16.745061	
		Variance		30.682	
		Std. Deviation		5.5391459	
		Minimum		5.7385	
		Maximum		18.3673	
		Range		12.6289	
		Interquartile Range		10.6705	
		Skewness		-.637	.752
		Kurtosis		-2.042	1.481
	Blank-CBD	Mean		23.910131	5.9498709
		95% Confidence Interval for Mean	Lower Bound	8.615501	
			Upper Bound	39.204761	
		5% Trimmed Mean		24.552106	
		Median		32.352941	
		Variance		212.406	
		Std. Deviation		14.5741478	
		Minimum		2.0000	
		Maximum		34.2647	
		Range		32.2647	
		Interquartile Range		26.9338	
		Skewness		-1.040	.845
		Kurtosis		-1.340	1.741
	Blank-5HT	Mean		18.869403	3.6548650
		95% Confidence Interval for Mean	Lower Bound	9.474274	
			Upper Bound	28.264533	
		5% Trimmed Mean		18.820056	
		Median		20.542636	
		Variance		80.148	

		Std. Deviation		8.9525543	
		Minimum		8.0943	
		Maximum		30.5328	
		Range		22.4385	
		Interquartile Range		17.8279	
		Skewness		-.213	.845
		Kurtosis		-1.407	1.741
	CBD-5HT	Mean		48.388479	5.9041803
		95% Confidence Interval for Mean	Lower Bound	35.032295	
			Upper Bound	61.744663	
		5% Trimmed Mean		49.232394	
		Median		50.939457	
		Variance		348.593	
		Std. Deviation		18.6706576	
		Minimum		13.9795	
		Maximum		67.6070	
		Range		53.6275	
		Interquartile Range		31.7335	
		Skewness		-.557	.687
		Kurtosis		-.783	1.334

Figure 5 Mean Fight Score by Ethogram Based on Compound Pair Match Type

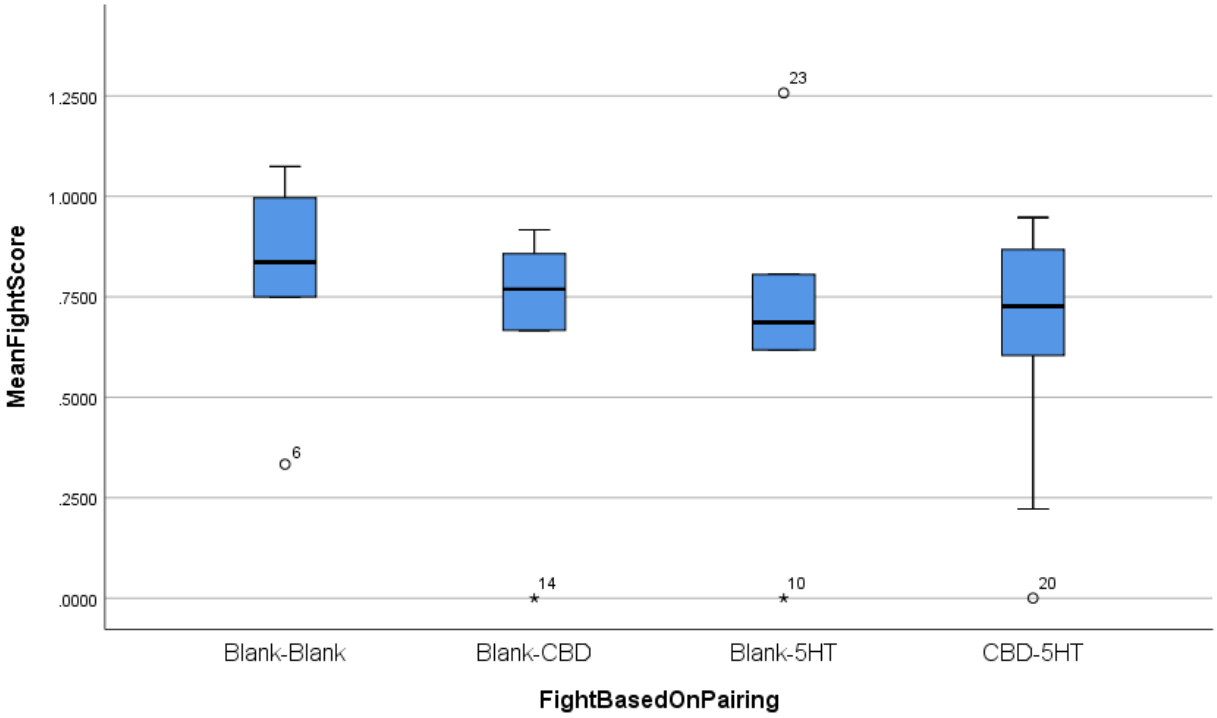


Figure 6 Mean Percent Time Fighting based on Compound Pair Match Type

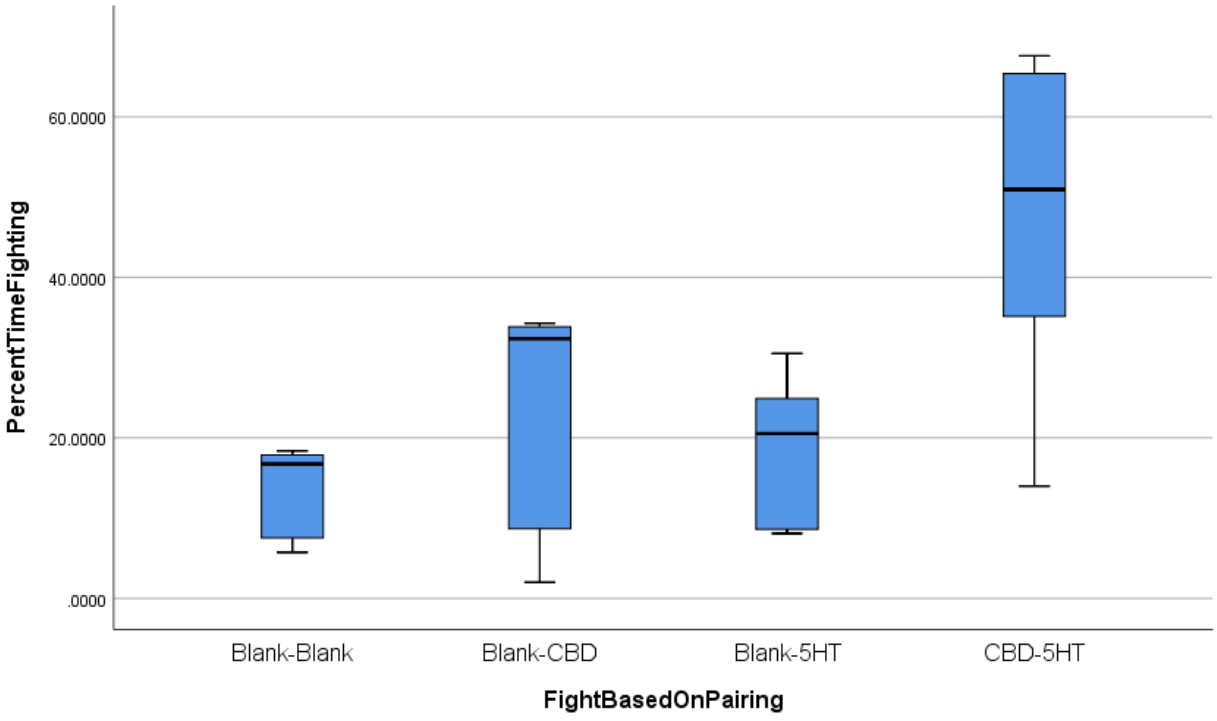


Table 12 ANOVA Results for Fights based on Compound Pair Match Type

		Sum of Squares	df	Mean Square	F	Sig.
Mean Fight Score	Between Groups	.160	3	.053	.533	.663
	Within Groups	2.602	26	.100		
	Total	2.762	29			
Percent Time Fighting	Between Groups	6425.708	3	2141.903	11.566	.000
	Within Groups	4814.886	26	185.188		
	Total	11240.595	29			

Table 13 Multiple Comparisons for Fights based on Compound Pair Match Type

Tukey HSD

Dependent Variable	(I) FightBased OnPairing	(J) FightBased OnPairing	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Mean Fight Score	Blank-Blank	Blank-CBD	.1583193	.1708547	.791	-.310390	.627029
		Blank-5HT	.1460124	.1708547	.828	-.322697	.614722
		CBD-5HT	.1766719	.1500634	.646	-.235000	.588344
	Blank-CBD	Blank-Blank	-.1583193	.1708547	.791	-.627029	.310390
		Blank-5HT	-.0123070	.1826514	1.000	-.513378	.488764
		CBD-5HT	.0183526	.1633684	.999	-.429819	.466524
	Blank-5HT	Blank-Blank	-.1460124	.1708547	.828	-.614722	.322697
		Blank-CBD	.0123070	.1826514	1.000	-.488764	.513378
		CBD-5HT	.0306595	.1633684	.998	-.417512	.478831
	CBD-5HT	Blank-Blank	-.1766719	.1500634	.646	-.588344	.235000
		Blank-CBD	-.0183526	.1633684	.999	-.466524	.429819
		Blank-5HT	-.0306595	.1633684	.998	-.478831	.417512
Percent Time Fighting	Blank-Blank	Blank-CBD	-10.3622318	7.3493636	.505	-30.523887	9.799423
		Blank-5HT	-5.3215042	7.3493636	.887	-25.483159	14.840151
		CBD-5HT	-34.8405803*	6.4550200	.000	-52.548765	-17.132396
	Blank-CBD	Blank-Blank	10.3622318	7.3493636	.505	-9.799423	30.523887
		Blank-5HT	5.0407276	7.8568002	.918	-16.512988	26.594444
		CBD-5HT	-24.4783485*	7.0273357	.009	-43.756578	-5.200119
	Blank-5HT	Blank-Blank	5.3215042	7.3493636	.887	-14.840151	25.483159

		Blank-CBD	-5.0407276	7.8568002	.918	-26.594444	16.512988
		CBD-5HT	-29.5190761*	7.0273357	.001	-48.797306	-10.240846
	CBD-5HT	Blank-Blank	34.8405803*	6.4550200	.000	17.132396	52.548765
		Blank-CBD	24.4783485*	7.0273357	.009	5.200119	43.756578
		Blank-5HT	29.5190761*	7.0273357	.001	10.240846	48.797306

*. The mean difference is significant at the 0.05 level.

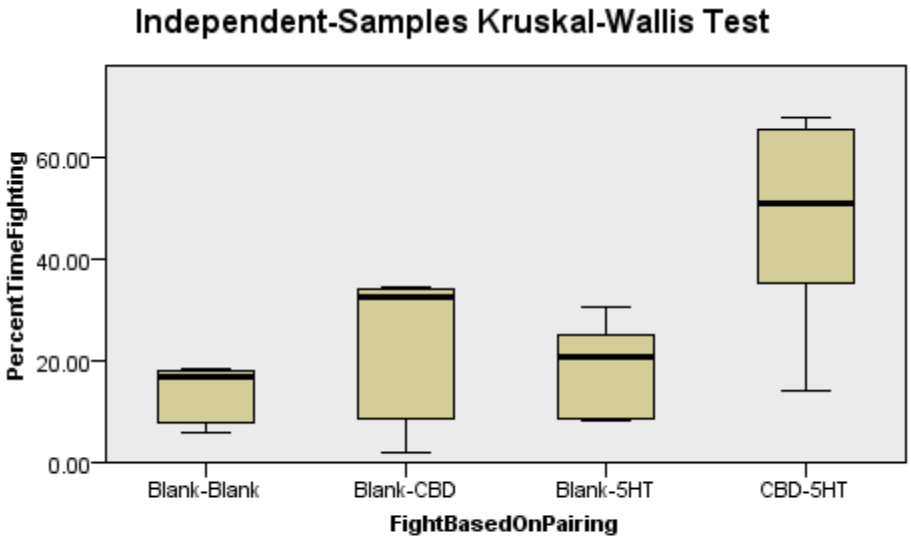
Figure 7 Non-parametric Analysis of Time Spent Fighting By Pair Match Type

Hypothesis Test Summary

	Null Hypothesis	Test	Sig.	Decision
1	The distribution of PercentTimeFighting is the same across categories of FightBasedOnPairing.	Independent-Samples Kruskal-Wallis Test	.001	Reject the null hypothesis.
2	The distribution of MeanFightScore is the same across categories of FightBasedOnPairing.	Independent-Samples Kruskal-Wallis Test	.431	Retain the null hypothesis.

Asymptotic significances are displayed. The significance level is .05.

Figure 8 Non-parametric Boxplots for Pair-based Time Spent Fighting by Pair Match Type



Total N	30
Test Statistic	15.585
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.001

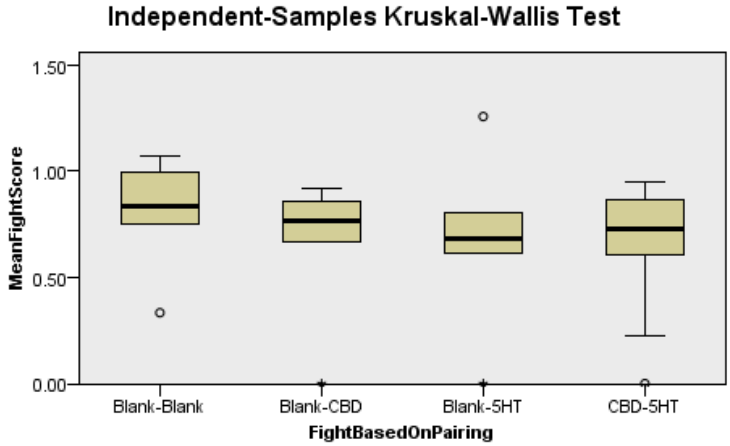
1. The test statistic is adjusted for ties.

Figure 9 Pairwise Comparisons of Mean Percent of Time Fighting and Hypothesis Table for Non-parametric Testing by Pair Match Type

Sample1-Sample2	Test Statistic	Std. Error	Std. Test Statistic	Sig.	Adj.Sig.
Blank-Blank-Blank-5HT	-4.167	4.753	-.877	.381	1.000
Blank-Blank-Blank-CBD	-7.000	4.753	-1.473	.141	.845
Blank-Blank-CBD-5HT	-15.800	4.174	-3.785	.000	.001
Blank-5HT-Blank-CBD	2.833	5.081	.558	.577	1.000
Blank-5HT-CBD-5HT	-11.633	4.545	-2.560	.010	.063
Blank-CBD-CBD-5HT	-8.800	4.545	-1.936	.053	.317

Each row tests the null hypothesis that the Sample 1 and Sample 2 distributions are the same.
Asymptotic significances (2-sided tests) are displayed. The significance level is .05.
Significance values have been adjusted by the Bonferroni correction for multiple tests.

Figure 10 Non-Parametric Box Plots and Summary for Fight Analysis based on Ethogram Score for Pair Match Type



Total N	30
Test Statistic	2.752
Degrees of Freedom	3
Asymptotic Sig. (2-sided test)	.431

1. The test statistic is adjusted for ties.
2. Multiple comparisons are not performed because the overall test does not show significant differences across samples.

Hunger

The number analyzed for each compound type is summarized in table 14. A table of descriptive statistics(Table 15) for mean percent of food consumed based on the compound injected and concentration was generated. A boxplots was generated for the mean percent of food consumed based on compound and concentration(Figure 11). ANOVA analysis was completed (Table 16) and yielded no statistical significance for variance in percent of food consumed. The results for statistical comparison based on compound were used to create a means plot(Figure 12). No significance was found between groups for percent of food consumed.

Table 14 Case Processing Summary for Percent Food Consumed by Compound and Concentration

		Cases					
		Valid		Missing		Total	
	Compound	N	Percent	N	Percent	N	Percent
Percent Food Consumed	Blank	16	100.0%	0	0.0%	16	100.0%
	CBD 2ug/mg	12	100.0%	0	0.0%	12	100.0%
	CBD 5ug/mg	16	100.0%	0	0.0%	16	100.0%
	5HT 5ug/mg	16	100.0%	0	0.0%	16	100.0%
	CBD 10ug/mg	16	100.0%	0	0.0%	16	100.0%

Table 15 Descriptive Statistics for Mean Percent Food Consumed by Compound and Concentration

	Compound			Statistic	Std. Error
Percent Food Consumed	Blank	Mean		.0406	.02046
		95% Confidence Interval for Mean	Lower Bound	-.0030	
			Upper Bound	.0842	
		5% Trimmed Mean		.0318	
		Median		.0000	
		Variance		.007	
		Std. Deviation		.08185	
		Minimum		.00	
		Maximum		.24	
		Range		.24	
		Interquartile Range		.04	
		Skewness		2.011	.564
		Kurtosis		2.809	1.091
	CBD 2ug/mg	Mean		.0200	.00870
		95% Confidence Interval for Mean	Lower Bound	.0008	
			Upper Bound	.0392	
		5% Trimmed Mean		.0178	
		Median		.0000	
		Variance		.001	
		Std. Deviation		.03015	
		Minimum		.00	
		Maximum		.08	
		Range		.08	
		Interquartile Range		.05	
		Skewness		1.242	.637
		Kurtosis		.001	1.232
	CBD 5ug/mg	Mean		.0219	.01152
		95% Confidence Interval for Mean	Lower Bound	-.0027	
			Upper Bound	.0464	
		5% Trimmed Mean		.0143	
		Median		.0000	
		Variance		.002	
		Std. Deviation		.04608	

		Minimum		.00	
		Maximum		.18	
		Range		.18	
		Interquartile Range		.02	
		Skewness		3.064	.564
		Kurtosis		10.219	1.091
	5HT 5ug/mg	Mean		.0163	.00826
		95% Confidence Interval for Mean	Lower Bound	-.0014	
			Upper Bound	.0339	
		5% Trimmed Mean		.0114	
		Median		.0000	
		Variance		.001	
		Std. Deviation		.03304	
		Minimum		.00	
		Maximum		.12	
		Range		.12	
		Interquartile Range		.02	
		Skewness		2.596	.564
		Kurtosis		6.734	1.091
	CBD 10ug/mg	Mean		.0050	.00438
		95% Confidence Interval for Mean	Lower Bound	-.0043	
			Upper Bound	.0143	
		5% Trimmed Mean		.0017	
		Median		.0000	
		Variance		.000	
		Std. Deviation		.01751	
		Minimum		.00	
		Maximum		.07	
		Range		.07	
		Interquartile Range		.00	
		Skewness		3.873	.564
		Kurtosis		15.213	1.091

Figure 11 Mean Percent Food Consumed base on Compound and Concentration Injected

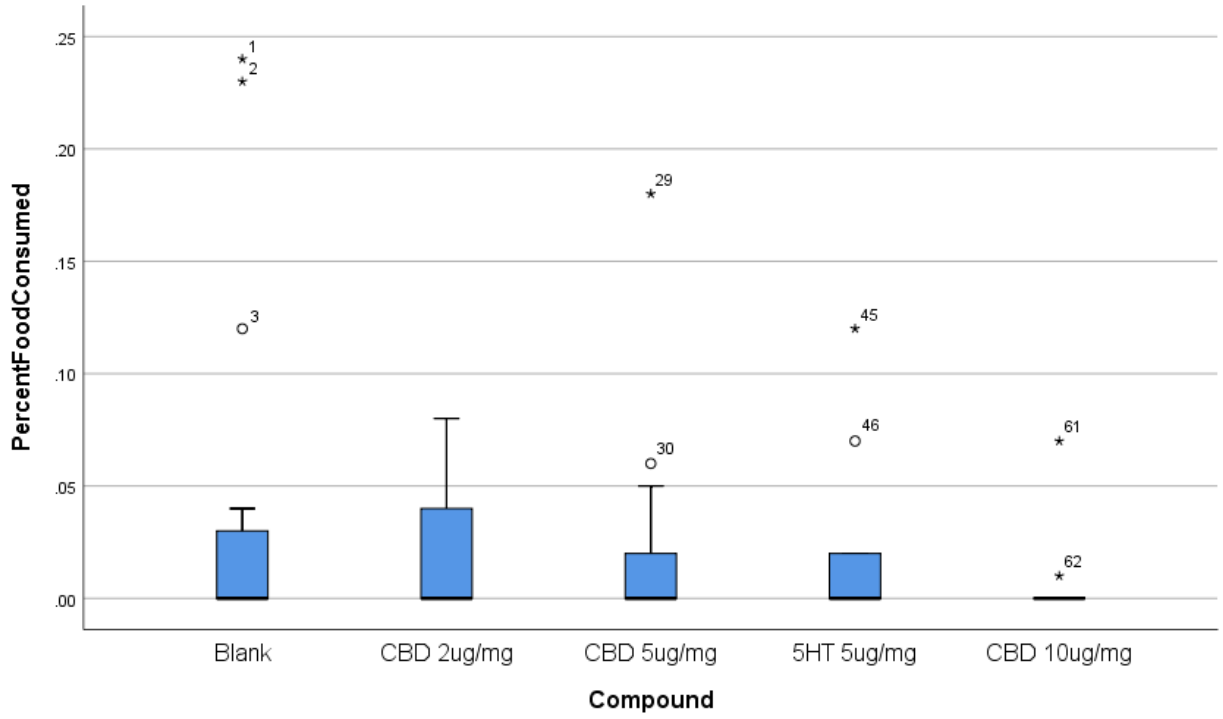


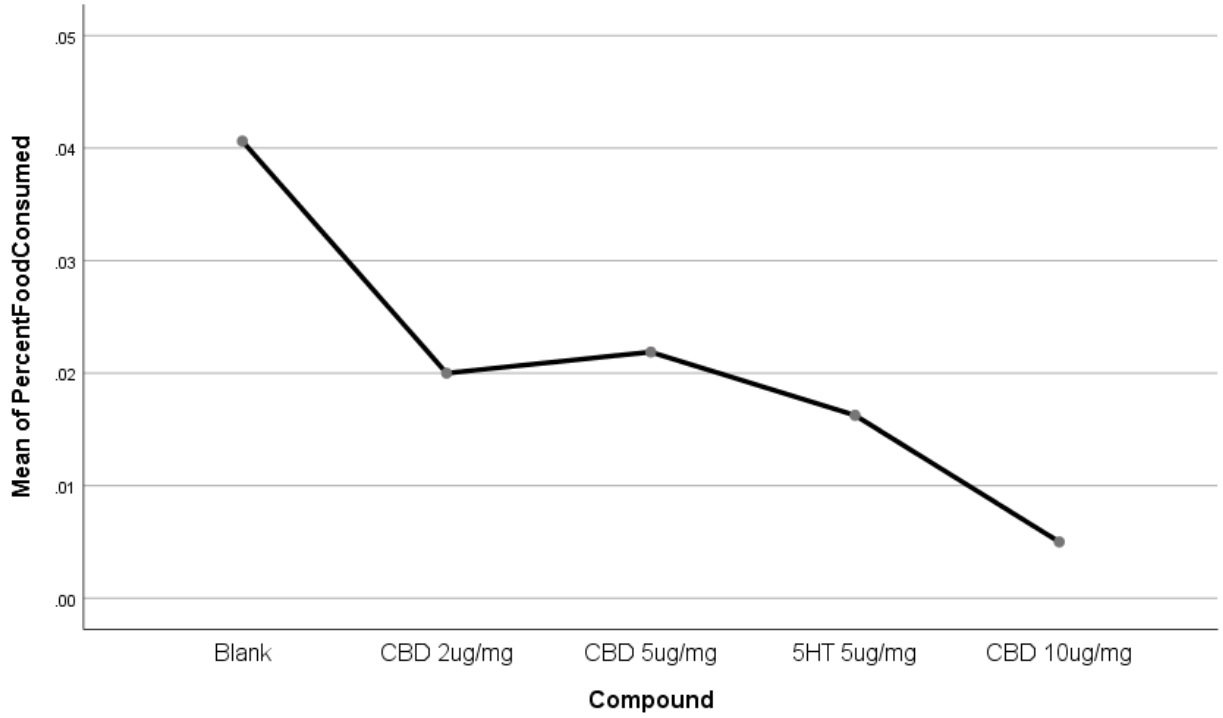
Table 16 Multiple Comparisons For Mean Percent of Food Consumed based on Compound Used

ANOVA

PercentFoodConsumed

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.011	4	.003	1.156	.337
Within Groups	.163	71	.002		
Total	.174	75			

Figure 12 Means Plot for Percent Food Consumed based on Compound and Concentration



Discussion

Motility

The purpose of examining motility was to determine if injections of CBD would influence the outcomes from fights by hindering movement. Part of the complex social interaction during a fight involves both postural changes and raising the claws into a meral spread (73). Immunofluorescence tagging of CB1 receptors in crayfish have been identified via microscopy (47). In addition, EPSP amplitudes were decreased at the NMJ after cannabidiol-like agent was used, suggesting that CBD would down regulate AP frequency at the muscular junction (67,69). Our research demonstrated a general decrease in movement after CBD injections compared to VH saline controls (i.e. blanks). However, no statistical significance was found overall indicating that movement was not inhibited or promoted from injections of CBD when compared against VH saline controls. The number of trials was relatively small though, with only 6 VH saline injections and 8 CBD injections completed. A larger sample may help to indicate overall reductions in motility. In addition, only one high concentrated dose of 10ug/g was administered due to time constraints brought on by COVID-19. Future trials could be completed using a range of doses. Perhaps a better indicator of influence of the NMJ would be trials based on treadmill activity or food seeking behavior where more time would be spent purposefully in movement rather than passively exploring. For the purpose of this project, the goal from motility analysis was to determine if CBD interacted with the NMJ enough to influence the interaction for dominance with competing crayfish. Based on the above results, the change in movement was not significant enough to inhibit or influence the outcome of a fight at the doses administered.

Fights

The two main ways fight interactions were analyzed involved a mean behavioral ethogram score of aggression (Table 1), and mean percent of time spent fighting. Serotonin has been shown to directly influence tail-flip response in crayfish (58). The decreased EPSP at the Lateral Giant (LG) neuron via increased synaptic 5-HT directly influenced behavior (61,62). CBD has been known to act on 5-HT receptors in mammalian models (74). It was postulated that if CBD interacted with the LG neuron of the tail tissue influencing fights that the measured value for the ethogram score would increase. However, this was not observed when compared against all other groups. Video analysis of fights included ethogram ranking, tail-flip counts, percent of time interacting in a fight, total number of interactions during the initial 12 minutes of recording, and if a crayfish died during the fight. Only two areas, mean ethogram rank and mean percent of time fighting based on pairs, were noted for any significance of frequency and were pursued for statistical significance. No significance was observed for the ethogram ranking using parametric and non-parametric tests. However, the analysis of mean percent time fighting based on injection pairing types yielded significance for both parametric and non-parametric tests. Specifically VH saline control-control pairs compared to CBD-5-HT pairs indicated that the means were statistically different with a p-value of 0.001 using Kruskal-Wallis analysis. Blank-Saline control pairs had a mean of 13.54% compared to 48.39% for CBD-5HT pairs. This influence is postulated to be from CBD binding to the 5-HT1 receptors on the LG neuron acting as a agonist, thus inhibiting the EPSP for tail-flip retreat behavior and increasing aggressive posturing and meral spread. The CBD could act two fold by both activating the 5-HT_{1A} receptors as well as increasing synaptic serotonin that would otherwise bind to the respective receptors. The significance found for CBD influencing fights was concluded with a concentration of 2 ug/g as

compared to the 5ug/g 5-HT. If the U-shaped curved observed with most pharmacological agents is applied to CBD than the optimal concentration could be different from what was used for testing. This means that the significant difference observed could be amplified with the proper dosage. General observation of the fights indicated that 5-HT produced aggressive fight behaviors such as curved tail postures, meral spread, and rapid pursuit of opponents. CBD produced a passive, responsive behavior to pursuers where fights were not sought out as quickly as with 5-HT. A meral spread (raised claws) and curved posture were still produced when opponents approached CBD crayfish. However, once engaged in a fight CBD crayfish appeared to retreat less frequently than VH saline injected crayfish. This would explain the relative similarity in ethogram ranking and the increase in percent of time spent engaged in combat. These observations were anecdotal and therefore not accurately measured behaviors and the validity remains unknown. Review of raw data indicated that the number of ethogram rankings of 1 (pursuing opponent) were scattered and no clear pattern emerged using this method. Additional video analysis could be conducted with a reconstructed ethogram to observe only pursuit behavior. The fights were completed with a low number of trials as three of the four main injection types were conducted with only 6 paired fights. Additional pairings would increase the power of the analysis. These pairings could include CBD-CBD and 5-HT-5-HT compound matching to better assess the fight outcomes. For this study, the significance found for mean percent of time fighting builds on previous postulations that CBD would influence the tail-flip response to retreat from fights and indicates a relationship between cannabinoids and crayfish behavior.

Hunger

Food seeking behavior was addressed extensively with a range of CBD concentrations. The purpose was to explore the possibility that cannabinoids could influence various receptors throughout the nervous system of crayfish, including the 5-HT subtypes found throughout the eye-stalks and central nervous system (55). These receptors line the digestive system of crayfish as well and could result in alterations in hunger if stimulated. However, after extensive analysis at multiple concentrations statistical significance was not found for CBD injections changing the amount of food consumed as compared to blank-saline controls or 5-HT injections. This may be due to the injury incurred during the injection process, as all animals exhibited the same lack of hunger despite being deprived of food prior to testing. To properly address the potential that injury influences the drive for food, alternative drug delivery methods could be employed, such as the placement of a capillary tube and/or using an injection pump after the injection site heals. In addition, a Y-maze could be utilized to further address a drive to pursue food over an extended period of time. For the purpose of this analysis, no observed behavioral changes occurred after injections of CBD to influence amount of food consumed.

Conclusion

To date, no trials have been published that involved cannabinoids and behavior analysis of crayfish. This project is the first step towards understanding how the simpler nervous system of crayfish utilizes cannabidiol with serotonin receptors. While no significance was found for CBD influencing motility and hunger, it was determined that there is a significant difference in mean time spent fighting when paired. This significance is postulated to be from activation of 5-HT_{1A} receptors. These receptors in humans are influenced by SSRIs to treat a variety of mental disorders such as anxiety and depression. SSRIs come with extensive side effects and that can partially decrease the quality of life and alter behaviors. The side effects from CBD are minimal when compared to SSRIs. However the effects of CBD, specifically in simple neurological models, has not been well studied. Yet, cannabis has gained wide-legalization across the US resulting in an increase in consumption of cannabinoids such as CBD. Further studies in crayfish and elsewhere should be completed to support the proper uses and dangers of cannabidiol within an unregulated market.

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