PTSD-like Behaviors in Zebrafish Models:

Cannabidiol's Interaction with Fundamental Biological Components of PTSD

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This honors thesis was prepared under the direction of the candidate's honors thesis advisor, Dr. Matthew Blankenship, Department of Psychology at Western Illinois University, and it has been approved by the members of the candidate's thesis committee

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Fear Reduction in Zebrafish Models :

Cannibidiol's Interaction with Fundamental Components of PTSD

As of 2024, the political climate on a global scale has been littered with many ongoing, growing, feared, and stagnant conflicts. Many of which have been going on (and affecting millions) for years to no apparent avail. When reflecting on current conflicts, more typical notions of war and combat may come to mind, such as one group combatting another. While these notions of conflict still prove highly applicable towards areas of today's political atmosphere and day-to-day life, as reinforced by observable and ongoing wars such as the multi-year Russian invasion of Ukraine or the increasing Israeli-Palestinian conflict, alternative and unconventional forms of conflict have been also appearing and widening the scope of those affected. Within the States particularly, the unfortunate sharp rise of mass shootings (shooting that results in the injury or death of four or more people), gun violence, and domestic violence has gripped the nation, affecting countless U.S. citizens. Thus, spearheading war-like violence into the homes of almost all levels of American society and generating what some regard as an epidemic of death within the US (Donnelly et al., 2023). Furthermore, the US witnessed more than 650 mass shootings in the year of 2023 alone (Gun Violence Archive, 2023). Within 2017, youth in the US alone faced a prevalence of over 5,000 deaths due to interpersonal violence, with 88.9% of those deaths being caused by a firearm. Within the same year, an additional 519,000 youth injuries were reported, all of which required medical attention and were caused by similar acts of violence (Rivara et al., 2019). Adults within the US face over 16,000 homicides and 1.6 injuries requiring medical attention similarly caused by acts of violence annually (Sumner et al., 2015). Within the home, families observed a sharp increase of domestic violence during the 2019 Covid pandemic (Boserup et al., 2020). These proximally closer occurrences of violence have effectively brought war-like conflict into the home and day-to-day life, even being observed on a personal level with the recent tragedy of the 2023 mass shooting occurring right off campus at Western Illinois University. Additionally, the mainstream violence being observed frequently on the forefront of virtually all of American society has widespread implications on not just physical health, but also carries significant emotional and psychological risks such as higher risks of posttraumatic stress disorder, substance abuse, and perpetuating violence.

With the concept of conflict and war slowly changing as the mere experience and sight of it increases, the scope and impact of those affected has ultimately been multiplied. Where conflicts used to be thought of as infrequent but glorious conflicts in which those who volunteer go to heroically fight in for the benefit of freedom, reality strays from this line of thinking by eliminating the infrequent and foreign aspects of conflict. Especially when war-like violence has breached the threshold of the home, almost everybody in American society now lives with some level of risk and the notion that reality rarely contains organized “wars” but rather multiple ongoing cycles of violence able to be seen on every level of society. Ultimately allowing war-like violence to become more frequent, personal, perpetual, and desensitizing to its negative effects. The unfortunate aspect of these events is that one particular demographic is continually caught within these cycles of violence, playing pivotal yet perpetuating roles within the more global or large-scale conflicts all while constantly reaping the psychologically damaging aspects of such involvements and enduring the additional conflicts that affect life at home. This particular demographic being the soldiers and enlisted personnel who endure the frontend of combat.

On the field, soldiers experience a specific psychopathological effect, combat stress reaction, which is brought about by the severe and highly intense scenes/events endured on the battlefield. Combat stress reaction (CSR) involves intense fear of bodily harm or death, which is often followed by extreme fatigue, diminished bodily function and response, altered emotional response, and depression. Due to the constant high stress of active duty, many military personnel commonly experience high rates of CSR. Unfortunately, this highly intense fear reaction is correlated with an increased chance of post-traumatic stress disorder by 660%, inherently pinning CSR as a significant precursor for post-traumatic stress disorder within soldiers (Solomon & Mikulincer, 2006).

Post-traumatic stress disorder (PTSD) shares similar traits with CSR and can be seen as a direct successor of CSR. Furthermore, PTSD itself is characterized by the re-experiencing of negative symptoms that coincided with an experienced or observed life-threatening or traumatic experience. The DSM-5 lists five criterion clusters for PTSD, in which a symptom to some degree from each cluster must be present for a potential diagnosis. These criterion clusters include: (1) a previous direct or indirect exposure to a threatening situation, (2) a persistent re-experiencing of the situation, (3) a present proactive avoidance/fear of related stimuli, (4) presence of negative thoughts appearing or worsening after the experience, and (5) trauma related arousal and reactivity that began or worsened after the trauma (American Psychiatric Association, 2023). PTSD also deals a considerable amount with the brain's memory functioning, affecting various aspects of memory-related functions due to these intense experiences. As a result, symptoms of PTSD also include lack of memory control, distressing emotions tied with memory recollections of those experiences, unwanted memory recalls, and memory blindness or an inability to recall (Verfaellie & Vasterling, 2009). PTSD is also correlated with a increased rate of suicidal ideation in those that experience the condition. Unfortunately, PTSD is not always diagnosed and commonly goes unseen. This is alarming as in the midst of a mental health epidemic, our nation faces a nationwide average of 40,000 civillian suicides and over 8000 military suicides per year (Castro & Kintzle, 2014). Furthermore, those who do manage to get treatment do not always see the program through to the end, generating a high dropout rate associated with PTSD treatment (Szafranski et al., 2017). This is concerning as this dropout rate is also coupled with an increasing incidence rate for PTSD especially in military personnel, as a study reveals a 400% minimum increase in incident rates for PTSD in active service members across the branches from 2001 to 2017 (Judkins et al., 2020).

Predictively, these high rates could possibly be reduced if further emphasis was put on PTSD treatment as current options lack either full development or research, as only one psychotherapeutic method having enough evidence to be recommended for practice by the Institute of Medicine, being CBT exposure therapy. Options are also limited from a pharmacological approach as well, as compounds like propranolol, prazosin, ketamine, and methylenedioxymethamphetamine being some of the only viable compounds applicable to treat PTSD, all of which have varying success rates and immense negative side effects (Cukor et al., 2009). Additionally, those affected with PTSD face heavy financial burdens while seeking treatment. Studies have shown that civilians pay an average of $18,640 per individual while military personnel pay an average of $25,684 per individual to treat PTSD. (Davis et al., 2022). All these factors have made a call for exploring more accessible, cost-efficient, and effective avenues for PTSD treatment. Fortunately, one compound has met all of these requirements; cannabidiol.

Cannabidiol (CBD) is one of the naturally occurring phytocannabinoids derived from the cannabis sativa plant that has gained massive popularity both recreationally and scientifically over time. The usage of CBD can be dated back hundreds of years to early Asia where it was sought as an herbal medicine (Crocq, 2020). In the modern world, CBD is sought for a variety of implications including pain relief, stress reduction, sedation, and anti-inflammation. Thankfully, through the discovery of the human endocannabinoid system (ECS) and cannabinoid receptors, these medicinal effects of various cannabinoids including CBD have been able to be empirically studied. For instance, CBD is primarily involved in the indirect modulation of CB1 receptors and partial agonism of CB2 receptors. CBD's indirect influence on the CB1 receptor mitigates anxiety and stress while promoting neuroprotection and pain reduction. Agonism on the CB2 receptors helps CBD contribute to anti-inflammation, immune system regulation, and enhancing pain reduction. Although CBD gains its effective anxiolytic properties primarily through agonism of the 5HT1A serotonin subtype receptors. Furthermore, these effects are further reinforced by CBD's modulation and partial agonism of adenosine receptors, particularly A1 and A2A receptors respectively. This enhances CBD's effects of anti-inflammation, pain reduction, and sleep promotion alongside inducing vasodilation within the body's cardiovascular system. CBD also acts as an agonist to the TRPA1 receptors which further elicits beneficial pain reducing and anti-inflammatory effects. Another key interaction pertaining to CBD is the inhibition of the orphan G-protein-coupled receptor GPR55 (Nazario et al, 2015; White, 2019). The inhibition of GPR55 specifically allows CBD to provide antineoplastic benefits by hindering the growth and movement of possible cancer cells and anti-seizure effects due to GPR55's role in neuronal excitability (Gray & Whalley, 2020; Seltzer et al, 2020).

Given CBD's extensive interactions with key receptors in regulating stress, anxiety, sleep, and pain, it is evident that CBD offers a potential approach in treating symptoms commonly associated with PTSD. Additionally, CBD's holistic effects coupled with the readiness of availability and comparatively cheap cost assert CBD as a viable treatment alternative for PTSD. Many human correlational studies have yielded positive Thus, the aim of this study is to examine CBD's effect on rudimentary biological components of PTSD related to anxiety and stress, in hopes to guide future research in adopting CBD as a treatment alternative for PTSD. Through the use of zebrafish (Danio Rerio) as animal models and an unpredictable-stress-schedule to elicit PTSD-like states within the zebrafish, it is hypothesized that the induction of CBD will reduce the frequency of PTSD-like behaviors within zebrafish. It is to note that the findings of this study alone does not solely assert CBD as a potential treatment of PTSD, but warrant and guide further clinical research that could.

Method

Subjects

Although no animal model can fully replicate the human experience, zebrafish have been utilized and proven to be viable models within many other studies that aim to observe the effects of a wide variety of drugs and psychotropic compounds (Neelkantan et al., 2013). Zebrafish have shown high physiological and genetic homology to humans as they contain fully characterized genomes that contain many functional protein domains almost completely identical to those of humans, along with robust behavioral phenotypes that make them suitable cross-species models. (Evan & Alan, 2016). Zebrafish also contain well developed monoaminergic, glutamatergic, opioid, cholinergic, and endocannabinoid neurotransmitter systems which allow them to experience the relevant psychotropic actions of various compounds, like cannabidiol. Zebrafish also engage in social interactions with other zebrafish in a manner similar to how humans fundamentally interact with other humans. This allows zebrafish to be viable models for in vivo observations, making more social-specific behaviors such as avoidance, isolation, fear, and social grouping behaviors able to be observed. Not to mention that these behaviors can also be observed while under the influence of psychotropic compounds.

One critical aspect to iron out is how PTSD will be observed within the zebrafish, as one cannot inherently give an organism like a zebrafish a complex disorder that requires conscious social interaction and context. To work around this, PTSD will be broken down into primary rudimentary components that can be translated “across-species”, or components that can be observed in species that are genetically homogenous to humans. The primary PTSD component studied will be the fear reaction, which will be observed in the zebrafish models through the application of a chronic unpredictable acute stressor. While this component alone does not solely account for PTSD in its entirety, it does play a pivotal role in PTSD’s functioning. In humans, fear reactions correlated with the onset of PTSD (like CSR) are often spawned from the direct threat to one’s own safety or wellbeing, additionally correlating with a sharp increase of acute anxiety and stress. Due to their high homogeneity to humans, this study aims to recreate this happenstance found in humans as zebrafish react in a similar manner when placed in similar threatening circumstances or experiencing stressors (Mohnot, 2009). Additionally, zebrafish exhibit an array of fear-like behaviors in response to these stressors, such as avoidant/erratic locomotion (zig-zagging, increased movement speed and displacement), avoidance of lit areas, freezing, reduced cognitive performance, and perimeter clinging (thigmotaxis) behaviors (Selvaraj et al., 2022). Chronic and unpredictable exposure to these stressors can also create lasting fearful states within zebrafish akin to PTSD (Golla et al., 2020). These fearful states elicited within the zebrafish serve as a viable fear reaction component homogeneous to that of human-based PTSD. Also, the overt reactions zebrafish give in response to chronic and unpredictable stressors allow for the actions of CBD to be easily examined in a behavioral manner, furthering the efficacy of zebrafish as animal models. With this in mind, zebrafish will be adopted as the animal model for this study.

Materials

Zebrafish care and CBD exposure. Healthy zebrafish (danio rerio) embryos were sifted and obtained from an experimentally naive tank at the Western Illinois University Neuroscience Lab. All care and maintenance procedures for the zebrafish were adapted from pre-existing routines within the lab. The collected embryos were experimentally undisturbed and allowed to incubate into larval zebrafish for 10 *dpf*. The CBD utilized within the study was obtained from Your CBD Store located in Macomb, IL. The CBD arrived in a 1000 mg vial that contained CBD in oil-based extract form with ratios of 10 mg CBD per 25 mL. It is to note that pure experimental use CBD was not able to be obtained and the CBD utilized within the study was produced for recreational human consumption and contained impurities other than CBD (such as natural and artificial flavoring). To obtain the desired ratio of 0.15 mg/L CBD and 0.05% DMSO within a conventional litre tank, .375 mL of the CBD extract was vortexed with 0.5 mL DMSO to create a water-soluble test solution able to be administered to the zebrafish. Administration techniques were adapted from previous zebrafish CBD research to optimize solution absorption while minimizing both mortality risk and harm (Pandelides et al., 2020).

Tank apparatus. All zebrafish embryos share a 5 gallon bucket during the incubation period. After this period, the fish are moved to a conventional litre aquarium for the duration of the Chronic Unpredictable Stressor (CUS) block. After the CUS block, each fish is then put in their own individual 125 ml Erlenmeyer flask for identification purposes. Additionally, every holding apparatus was equipped with an air bubbler and light timer to mimic natural environments and daylight cycles. Tank waters were routinely siphoned and replaced with freshly treated regular tank water from the experimentally naive parent tank every 4 days, except during treatment blocks. During the testing block, a light/dark test aquarium containing a non-transparent divider that easily allows test fish to traverse both light and dark compartments is utilized. The light/dark tank is constructed from a 4-inch cross section cut of a PVC pipe that has 7-inches in diameter. Additionally, the light compartment has no lid to allow for the placement and collection of the test fish while the dark compartment contains a light-blocking lid. Above the light/dark testing tank, a video camera mounted to capture an overhead recording of the fish's behaviors within the light compartment.

Procedure

Zebrafish eggs are harvested at the WIU Psychology Neuroscience Lab from an experimentally naive breeding tank. All healthy fertilized eggs are collected via a siphon vacuum and then isolated in a separate container for incubation. Each container is then given hatched brine shrimp which allows the zebrafish eggs to incubate. The eggs are allowed to develop until 10 days-post-fertilization (*dpf*) with no ulterior disturbances other than tank maintenance and feeding. During this time, batches of zebrafish larvae are given a stress condition and treatment condition assignment. Starting on the 11th *dpf*, the larval zebrafish are moved to a conventional tank and undergo a 7-day stress block. During this block, batches with the *stressed* condition assignments undergo a Chronic Unpredictable Stress (CUS) schedule while batches with the *non-stressed* condition assignment remain undisturbed for the remainder of the stress block. The CUS schedule itself spans the total duration of the 7 day stress block, beginning on the 11th *dpf* and ending on the 17th *dpf*. In the schedule, two stimuli are randomly chosen from a predetermined list and administered to *stressed* condition fish for two random 5-minute intervals between 12:00 pm and 5:00 pm. The predetermined list of stressors include: (1) chasing zebrafish around with a pipet, (2) exposing the fish to a strobing 500 lumen LED array, (3) increase in water turbulence (Golla et al. 2020). Additionally, all normal feeding and maintenance routines will be maintained during this time. After the CUS block and beginning on 18 *dpf*, the batch then undergoes a four day treatment block depending on their treatment condition assignment. The treatment conditions include: a test condition (0.15 mg/L CBD + 0.05% dimethylsulfoxide; DMSO), a vehicle-control condition (0.05% DMSO), and a control condition (regular tank water) (Carty et al. 2017). Once the treatment tank has been set up with the batch's corresponding treatment water, fish are then transferred via fine mesh net from the previous stress tank to the treatment tank. The batch remains undisturbed for the duration of the treatment block to allow for adequate ingestion of the treatment water's contents, until 22 *dpf* (Pandelides et al., 2020). Beginning on the 23rd *dpf*, the batch will undergo a testing block. Before testing and still on the 23rd *dpf*, the batch is individualized and separated into holding flasks, with one fish to one flask. Each flask is given an identification label and filled with regular water which is maintained for the duration of the test block. Each fish will individually undergo a light/dark preference test on 24 *dpf*, 26 *dpf*, and 30 *dpf* (following a 1, 3, 7 day testing schedule). At the start of each test, one fish is transferred from their holding flask into the light compartment of the testing tank. After the recording is starting and a timecard is presented in front of the camera, the fish is allowed to freely roam the test tank for 5 minutes undisturbed from any external movements, noises, or stimuli that could affect the fish's behavior during the test. After the 5 minutes, the fish is then transferred back to their respective holding flask until their next test. After the final test and on the 31st *dpf*, each test fish is then released from the study and transferred to a conventional holding tank alongside the other test fish that have completed the study. All regular feed, cleaning, and maintenance routines are upheld for the remainder of the fish's natural expectancy. All recordings of each run of the light/dark test are coded by hand for movement frequencies, time spent engaged in perimeter clinging (observed by setting up ½ inch wide on-screen zones via editing software, recorded in seconds), and time spent in the light chamber (in seconds). Coded data was entered and saved into a Google spreadsheet for further data analysis via IBM SPSS.

Results

No data was able to be collected for the test conditions due to experienced zebrafish mortality rates. Despite this, partial data was able to be collected and processed for the control group. At the end of the 7 day testing block, a total of 12 *stressed* and 35 *non-stressed* data points were collected for the control group. An independent samples t-test was conducted for movement frequency, thigmotaxis behavior, and the amount of time spent in the light chamber to find behavior significance. Of the three behavioral dimensions, only movement frequency was found to have a significant difference in group means, $t(45) = 3.40$, $p < .001$. Additionally, the *stressed* control group had a higher overall average movement frequency compared to *non-stressed* control groups across the testing block (M = 226.25 vs. M = 94.40). For average time spent in thigmotaxis behaviors, the *stressed* control group had a slightly higher overall average time in thigmotaxis compared to the *non-stressed* control group (M = 77.58 seconds vs.

M = 73.42 seconds). For the amount of time spent in the light chamber, the *stressed* control group had a slightly higher overall average time in the light chamber compared to the *non-stressed* control group (M = 256 seconds vs. 246 seconds). Additional tests were conducted to measure the between-subject and within-subject effects. Within the control group, tests of within-subject effects yielded significant results, $F(2, 10) = 5.84$, $p = 0.021$. Despite this, tests for between-subjects effects did not yield significant results, $F(1, 5) = 5.811$, $p = 0.061$.

Discussion

Zebrafish Mortality Rates. While zebrafish serve as suitable animal models for this study and have served as viable models for countless others, zebrafish are correlated with a 50% natural expected mortality rate. This rate can increase for a wide range of reasons both in and out of the scope of the study, such as treatment and stress protocols to uncontrollable external facility conditions. While best attempts were made to keep survival rates as optimal as possible, no data was able to be collected for the experimental and vehicle control groups. Several in-lab protocol and literature reviews were held to evaluate the quality of experimental techniques, but all methods and procedures were conducted identically to those adopted from previous studies. Although, one main difference between this study and the adopted methods is the form of CBD utilized, as other studies had the ability to obtain and utilize pure CBD in crystalline form whereas this study had to utilize recreational oil-based CBD due to budgeting constraints. Additionally, the treatment and stress protocols were optimized to reduce any harm or risk imposed to the fish. Furthermore, all protocols were both reviewed and accepted by the Institutional Animal Care and Use Committee (IACUC) review board at Western Illinois University. While the difference between CBD forms could pose a contributing factor towards the high mortality rate experienced in this study, batches would often experience detrimental mortality rates before reaching the treatment blocks. Additionally, both *stressed* and *non-stressed* assigned groups experienced similar high mortality rates. The mortality rates experienced within this study also differed from other studies included in the literature review, leading to the notion that some external factor played into high mortality rates experienced within this study. This is further supported as all protocols and procedures that deal with the maintenance or interaction with any of the fish within the study were adopted from previous successful studies and pre-existing lab routines. Unfortunately, time constraints disallowed adequate measures to be implemented to empirically assert what was causing the high mortality rates. One potential and unavoidable factor that could have contributed to the high mortality rates is the dated piping system that the laboratory has, as this directly affected the quality of water utilized within the study. Another out-of-scope factor that could have contributed is the notion that the laboratory is only equipped with open-lid tanks which may collect unwanted pollutants that could especially affect the development of larval zebrafish. The latter is predictably exacerbated by the conditions of the lab itself, as some areas of the ceiling and air system respectively remain unclean, even in areas directly above where the study's fish were kept. Inferrably, if access to research purposed CBD and sterile keeping conditions for models were able to be obtained, it would be easier to acquire necessary amounts of data to adequately test this study's hypothesis.

Data Significance. Despite having no useful data for the experimental groups to adequately test the original hypothesis of whether or not the induction of CBD will correlate with a significant effect on the behavioral presence of fear responses in stress condition fish, there is partial data able to be used to test the validity of the chronic unpredictable stress schedule. Moreover, the control group yielded adequate data to observe whether or not the stress schedule had a significant effect on the fish. The independent samples t-test gave that of the three fear avoidance behavior dimensions observed, only the dimension of movement frequency had a significant difference between the *stressed* and *non-stressed* control conditions. While the *stressed* condition had a higher overall mean movement frequency, this does not hold true for every day of the testing block. Referring to figure 1, the *non-stressed* group had a higher average movement frequency during the first test that then sharply reduced in average frequency during the second test and remained consistent during the third test. This contrasts to that of the *stressed* condition which inherently had the inverse situation, as the *stressed* condition had a lower initial movement frequency average that drastically increased during the second round of tests and remained constant throughout the third. Albeit partial data, this could point to the validity of the CUS schedule imposing a significant effect on the fish's behaviors. Moreover these findings suggest the CUS schedule did elicit some degree of a "*stressed*" state within the zebrafish. This is supported by the notion that the *stressed* condition had higher means for all of the fear avoidance behavior dimensions, and with the difference in the average movement frequency having statistical significance. This notion is additionally supported by the within-subject effects test that yielded significant results. While the between-subject effects test yielded insignificant results, this would predictably change if the sampling size were to increase and more fish were able to survive until the testing block.

Conclusion

While the findings yielded may not align with the original goal of this study, they still prove beneficial in the overall field of PTSD research. That is, while the hypothesis of whether or not CBD will reduce the frequency of fear-avoidance behaviors in *stressed* zebrafish was not able to be empirically examined, this study was able to accomplish testing the validity of the chronic unpredictable stress schedule in eliciting *stressed* states akin to PTSD within fish. By confirming the validity of such a schedule, a small but vital step has been made in the field of research pertaining to PTSD and related fear disorders. This is due to the sheer importance of asserting the empirical efficacy of a schedule that can elicit a state within animal models in a manner both robustly observable and similar in function to the human experience. Not only pertaining specifically to PTSD, being able to recreate a state akin to complex disorders within animal models is paramount towards our ability as a society to curate empirically supported treatments. Additionally, the data yielded from this study supports the usage of the chronic unpredictable stressor schedule in eliciting lasting fear states akin to PTSD in zebrafish. This can hopefully guide and provide benefit to future animal model research relating to PTSD and fear related disorders. Furthermore, if this study was able to be replicated at a dedicated zebrafish lab, and with the inclusion of pure research-purposed CBD, an adequate test of this study's hypothesis would predictably be attained.

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Appendix

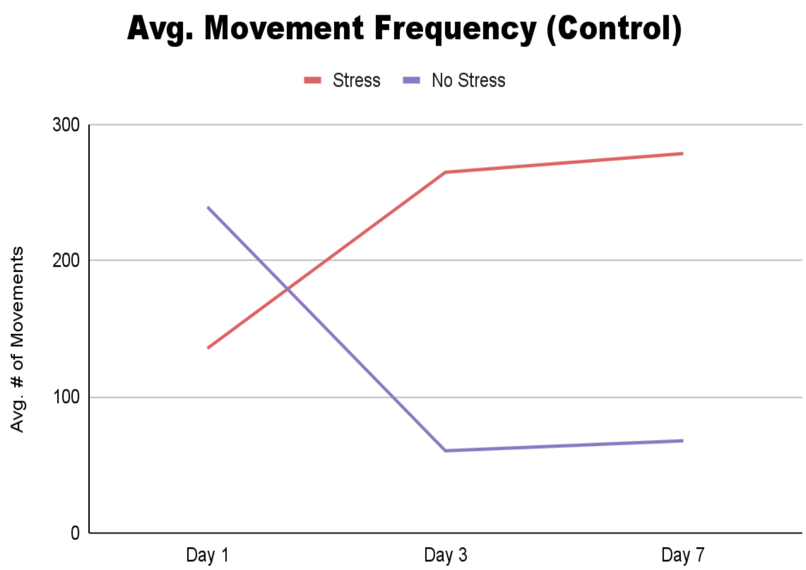

Fig.1: A graph of the average movement frequencies for the control group's *stressed* and *non-stressed* conditions during the light/dark preference testing, across the three testing days.