Soothing the Savage Fish: The Effects of Anxiolytic Plant Extracts on *Betta splendens*

by Sharon Eastburn
High School: South Jersey Sudbury School
Medford, NJ
Home Address: 16 Venture Lane
Levittown, PA 19054

ACKNOWLEDGEMENTS

Very special thanks to the professors who gave their advice and ethical clearance on this project, including Dr. Stephanie da Silva from Columbus State University (Columbus, GA), Dr. Kennon Lattal from West Virginia University (Morgantown, WV), Dr. Mirari Elcoro from Framingham State University (Framingham, MA), and Dr. Andres Bendesky from Columbia University (New York, NY). Thanks as well to Gabrielle Goddard of Princeton High School in Princeton, NJ, who volunteered to review all videos and participated in data entry. All of the experimental procedures and data analysis were conducted by Sharon Eastburn, and Mark Eastburn (mentor) provided advice on edits to the research paper and article citations.

Research involving non-human vertebrates was conducted under the supervision of an experienced teacher and followed state and federal regulatory guidance applicable to the humane and ethical conduct of such research.

ABSTRACT

Betta splendens is a highly aggressive species of fish with strong behavioral responses, which makes it useful in testing the effects of anxiolytic or "anxiety breaking" substances to determine their effectiveness in living organisms. This experiment measured the aggressive responses of 14 male *Betta splendens* (also known as "bettas," or Siamese fighting fish) when allowed to see their images in a mirror, because males of this species are known to react aggressively towards their own kind. The effects of four different plant extracts on betta behavior were investigated, each of which is believed to have anxiolytic effects. By counting the number of flares (gill openings) and strikes at a mirror over ten-minute periods, and comparing frequencies between fish that were treated and untreated, it was concluded that reserpine (from Indian snakeroot--*Rauvolfia serpentina*), cannabidiol (a legal extract from industrial hemp--*Cannabis sativa*), and tea made from holy basil (*Ocimum tenuiflorum*) may have anxiolytic properties after results were compared with one-way ANOVA and post-hoc Tukey honestly significant difference test (HSD). Cilantro (coriander or Chinese parsley--*Coriandrum sativum*) did not show an effect at tested concentrations. Due to the related evolutionary origins of fish and humans, this non-invasive method of testing has been shown to yield reliable results without long-term effects on test subjects, and may be used to explore the effectiveness of many different plant extracts and medications for calming anxious or aggressive behavior.

TABLE OF CONTENTS Page

INTRODUCTION

Vertebrate brains are the most complex of all animals (Shepherd, 1994), and backboned organisms share brain structures such as the medulla oblongata (Rovainen et al., 1985), a region that is responsible for automatic functions, including heart rate and breathing (Mackie, 2008). Vertebrates and some of their close relatives also have basal ganglia (Grillner et al., 1998), which is involved in thought processes and emotional responses (Weyhenmeyer and Gallman, 2007). Another portion of the brainstem, called the locus coeruleus, appears to be activated when a test subject is under stress (Steimer 2002). Each of these features was most likely present in the last common ancestor of fish and land-living vertebrates, which lived during the Devonian period (Ruta et al., 2007) between 358.9 million years and 419.2 million years ago (Gradstein et al., 2004). As a result of this common ancestry, several experiments have tested the effects of various chemicals on fish, both for the influence of environmental contaminants on aquatic habitats, and for the possibility of finding new medications that might treat disorders in humans.

The study of ethology, or natural behavior, has been investigated by scientists as a means of understanding interactions among fish, along with interactions between fish and their environment (Baerends, 1971). Among these studied behaviors is scototaxis, or the preference of dark to light regions of a habitat, and this response has been considered a measure of anxiety in goldfish (*Carassius auratus*), zebrafish (*Danio rerio*), and guppies (*Poecilia reticulata*) when tested in captive situations (Maximino et al., 2010). Two other species of fish that are commonly used in ethological experiments are the Siamese fighting fish (*Betta splendens*) and the three-spined stickleback (*Gasterosteus aculeatus*). Three-spined sticklebacks are considered potential models for neuroscience research into anxiety and aggression, both in their response to attacking images in mirrors, and their tendency to dive to the bottom of a container when exposed to stress (Norton and Gutierrez, 2019). Siamese fighting fish (*Betta splendens*), also known as bettas, have been studied for their complex combat displays in male-male encounters and courtship behavior in male-female encounters (Bronsten, 1984). These highly visible responses have allowed scientists to investigate the effects of exposure to anxiety medications intended for humans (Dzieweczynski and Hebert, 2012) and environmental contaminants such as arsenic (Tudor et al., 2019) and mercury (II) chloride (Mansur et al., 2012) in order to understand their influence. Added benefits are that bettas are available in most pet stores throughout the United States, and are relatively inexpensive.

Anxiety can be considered an emotional reaction to, or anticipation of, a threatening stimulus, and it can be induced by placing animals in situations where stressors are applied (Neumann et al., 2010). Anxiety can be correlated with aggression, because ethologists who study natural behavior consider anxiety to be a trigger for both defensive actions (which may include aggression) or the impulse to escape from danger; this is the “fight or flight” response that many animals share (Steimer, 2002). Aggression in males of all species is also considered necessary to meet basic needs such as nutrition, territory, and mating partners (Neumann et al., 2010). To put this in perspective, human behaviors in stressful situations are very similar to those

of many other animals, although humans may show a greater degree of diversity in their responses to threats (Steimer, 2002). Some of this diversity may actually help explain an individual human's risk of developing stress-induced diseases (Steimer, 2002), and therefore understanding the spectrum between anxiety and aggression in other species can have a direct link to human health. Tests for anxiety in animals such as rats may use a "black-white" box (Neumann et al., 2010), and can induce behaviors similar to scototaxis in fish. If a rat placed in one of these boxes spends the majority of its time in the black section, they are considered to be experiencing elevated levels of anxiety (Neumann et al., 2010), and if a rat spends equal times in both the white and black sections, they are considered to be experiencing lower levels of anxiety (Neumann et al., 2010). The fact that rats, which have brains that are very similar to humans (Palmer, 2020), exhibit anxious behaviors from stress that are nearly identical to fish, indicate that a treatment which might be given to fish to relieve symptoms of anxiety might also apply to rats and humans.

In rats, aggression among males can be measured through the resident-intruder test, which is where male rats are housed with a female for a period of several days or weeks, allowing the male rat to develop a sense of his "territory" (Neumann et al., 2010). After an appropriate amount of time has passed, the female is removed and a smaller, male rat is introduced. Interactions between these two males are recorded, and measurements such as the time between introduction to initial attack, anxious behavior, and the duration of combat are recorded (Neumann et al., 2010). Interestingly, aggression in both LAB (low anxiety behavior) and HAB (high anxiety behavior) rats were found to be higher than those rats that exhibited normal levels of anxiety behavior (NAB) (Neumann et al., 2010). These results indicate a linkage between aggression and anxiety, which means that treatments that alter levels of aggression may also have an impact on anxious behavior.

One of the major neural systems that responds to stressful situations in mammals is called the HPA axis, and it refers to interactions between the pituitary gland (at the base of the brain), the adrenal glands (located near the kidneys), and the hypothalamus (in the brain) (Neumann et al., 2010). The locus coeruleus may also be involved in this responsive pathway (Steimer, 2002), and these structures share similarities with a feature in the forebrain of fish known as the pallium (Rodriguez et al., 2006). This pathway in mammals is well-studied; the HPA axis begins a stress response with the release of corticotropin-releasing hormone in the hypothalamus, which causes a release of ACTH (adrenocorticotropic hormone) from the pituitary gland, which then leads to the secretion of glucocorticoids from the adrenal glands (Neumann et al., 2010). These glucocorticoids then trigger physiological and behavioral adaptations throughout the body (Neumann et al., 2010). Experiments that monitor the levels of glucocorticoids in the blood show that both high and low levels of glucocorticoids have been associated with high aggression (Neumann et al., 2010), which provides a physiological basis for understanding why both high-anxiety and low-anxiety rats (and possibly other animals) can show aggressive tendencies.

Various plant extracts and herbal supplements have been marketed for their anti-anxiety or stress-reducing benefits, although very few have been thoroughly investigated, and many studies are in need of replication before any conclusions can be made (Hoenders et al., 2018). Among the most studied is reserpine, having been used both as an antipsychotic and to lower blood pressure (Baumeister et al., 2003). Since the 1960s, this compound extracted from the Indian snakeroot plant (*Rauvolfia serpentina*) has also been considered a possible cause for depression in certain patients, because it decreases the brain's concentration of monoamine neurotransmitters such as dopamine, epinephrine, and serotonin (Mulnari, 2012), while other researchers have debated this claim (Baumeister et al., 2003). Of these three neurotransmitters, serotonin appears to have the strongest link to anxiety, because several classes of medications, including monoamine oxidase inhibitors (MAOIs) are used to treat anxiety symptoms by maintaining higher levels of serotonin in the brain through slowing its natural breakdown by enzymes (Buigues and Vallejo, 1987). As reserpine appears to have the opposite effect, depleting levels of serotonin and other monoamines, this is the main reason that it has been linked to depression (Mulnari, 2012), although certain preparations made from the Indian snakeroot plant appear to have antidepressive effects (Baumeister et al., 2003) and the earliest studies suggested that it could be used as an anxiety treatment (Davies and Shepherd, 1955).

One of the newest substances to be considered in anxiety treatments is cannabidiol (CBD), which is derived from the industrial hemp plant (*Cannabis sativa*) and was legalized through the Agricultural Improvement Act of 2018 (U.S. Food and Drug Administration [FDA], 2019). Though this legalization is very recent, traditional use of *Cannabis sativa* extends back many thousands of years (Schrot and Hubbard, 2016). Industrial hemp has very low concentrations of the psychoactive compound tetrahydrocannabinol (THC), another derivative of *Cannabis sativa* that, along with CBD, is considered for medications that affect the basal ganglia (a group of structures found within the cerebral hemispheres of the brain), such as Huntington's disease (Fernández-Ruiz et al., 2013). At present, plants with high levels of THC content are still considered illegal under federal law, although some states have challenged this prohibition in recent years through medical marijuana and recreational marijuana legislation (Clarke and Merlin, 2016). Cannabinoids such as THC and CBD are believed to interact with cannabinoid receptors, which are proteins in cell membranes of the basal ganglia that appear to have connections to pain, memory, and mood (Aizpurua-Olaizola et al. 2017). There appear to be two main types of cannabinoid receptors, which are designated CB_1 and CB_2; CB_1 receptors are found in parts of the brain that are known to interact with cannabinoid-like brain chemicals called endocannabinoids, while CB_2 receptors are found in cells that are part of the immune system (Mackie, 2008). Interestingly, the medulla oblongata does not appear to have any cannabinoid receptors (Mackie, 2008), which suggests that compounds like THC and CBD will not have an impact on the most basic bodily functions like heartbeat and breathing. Nevertheless, these compounds have been investigated as treatments for anxiety (Shannon et al., 2019), and one medication derived from CBD has been approved by the U.S. Food and Drug Administration to

treat epilepsy (Caceres, 2019), but many researchers believe that more research is needed before any conclusions can be drawn about the therapeutic potential for CBD and other cannabinoids (Kluger et al., 2015).

With regard to the legality of CBD products, industrial hemp (from which CBD is produced) is legal to grow under federal law (U.S. Food and Drug Administration, 2019), under Pennsylvania law ("CBD in Pennsylvania", n.d.), and is legally available for use in pets through online retailers, although the formulations that are used for pets may differ from those that are intended for people ("Are CBD oils for pets legal?," 2020). Though many CBD products are sold in age-restricted dispensaries and vape shops (where products containing THC or tobacco might also be offered), there are no legal age restrictions for purchasing CBD products online, whether they are intended for animals or people ("Is CBD safe for all ages?", n.d.). Aside from these considerations, the main ingredients of CBD products intended for pets and people are identical, and so it appears that one might be able to draw conclusions about the effectiveness of CBD products on anxiety and aggression whether they are intended for pets or for human beings.

Three other plants with potential interest for studies of anxiety and aggression are kava (*Piper methysticum*), tulsi (*Ocimum tenuiflorum*) and coriander (*Coriandrum sativum*). These three plant extracts have not been as extensively studied as the previous two compounds, although all have been marketed for their supposed anxiolytic properties. Kava has a variety of names across the South Pacific region where it has traditionally been consumed as a drink (Lynch, 2002), and extracts from the roots of this plant appear to contain several different compounds known as "kavalactones" (Tzeng and Lee, 2015). Tests on kava extracts with human patients suggests that they provide benefits for elderly patients suffering from anxiety (Kuchta et al., 2017), although other studies indicate that it might be more effective in younger patients and women (Witte et al., 2005). In high doses, kava extracts might also be toxic, especially to the liver, which has led them to be banned in Canada and the European Union (Clouatre, 2004). Nearly all studies of kava so far urge the need for further investigation.

Like kava, tulsi or "holy basil" has been grown and used as a medicinal plant for thousands of years, and is considered by many to have a wide range of health effects, including as an anxiolytic (Cohen, 2014). Also like kava, tulsi extracts contain a variety of compounds (Sundaram et al., 2012), although none of these appear to be directly related to anxiety or aggression at the present time. Coriander, which is also known as cilantro, has been used as an ingredient in cooking (Zohary and Hopf, 2012), and may be useful in treating anxiety at high concentrations (Mahendra and Bisht, 2011), although with all of these additional compounds, further investigation is required before any conclusions can be made.

MATERIALS AND METHODS

To begin this experiment, 14 *Betta splendens* were purchased from local pet stores, and included both the blue (n = 6) and red (n = 8) varieties. Research has shown that females prefer red males (Tudor et al., 2019), which may underlie differences in overall physiology, although this has not been conclusively determined. Bettas were kept individually in 19 L aquaria, and provided with filtration and a heater that maintained water temperatures at 27.0ºC (±1.0 ºC).

For testing, bettas were introduced into a smaller, 2880 mL container that was covered on the bottom and sides. This testing chamber was divided into three equal sections (Fig. 1) in order to track fish preferences for one area of the test tank when compared to another. Once fish were introduced into this container, they were allowed to acclimate for ten minutes (Tudor et al., 2019) before any plant extracts were introduced, and before a mirror was lowered into the container.

Plant extracts were all obtained through online sources, and arrived in dropper bottles after the extraction had already been performed. Care was taken to ensure that all compounds were water-soluble, to allow proper uptake by fish through normal gill exchange with the surrounding water. This includes the plant extract that contained cannabidiol (CBD), which is often available in an oil-based (water insoluble) form, although some online pet medication vendors do offer CBD in a water-soluble formula. The exact nature of the modifications to make CBD water soluble were not disclosed, as this is considered a proprietary secret from the supplying company.

After the ten minutes for acclimation had passed, fish were treated with a plant extract or left untreated as a control, the water was gently stirred with a plastic spoon, and a mirror that spanned the test tank's entire width was lowered for the fish to see. In each test tank, the mirror was placed in Section 1 (see Fig. 1) in order to ensure consistency. Lighting and temperature were kept constant, and fish that received treatments were chosen to ensure that each fish was represented approximately the same number of times throughout this experiment.

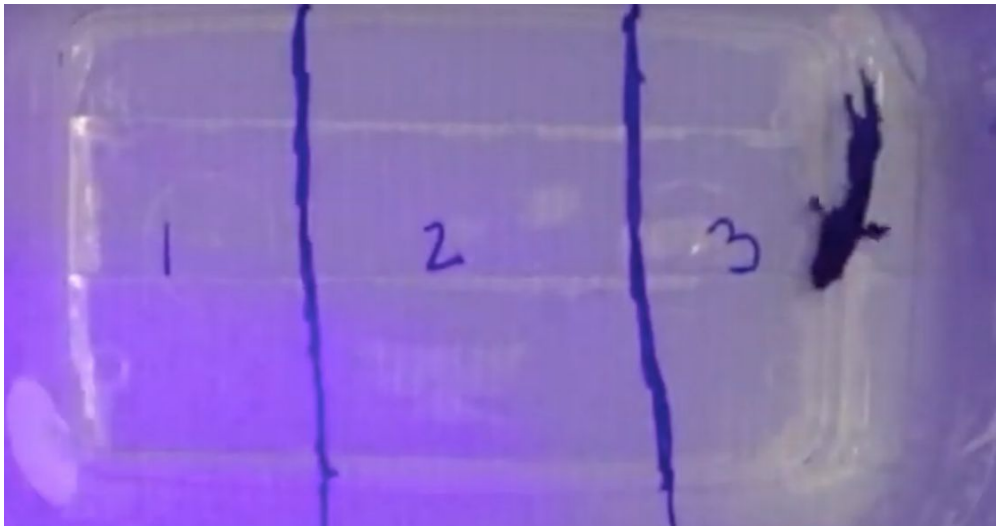

Figure 1. The testing tank for all betta experiments, which was divided into three equal sections. The mirror to test for aggressive responses was always placed on the leftmost side of Section 1.

As soon as the mirror was introduced into each test tank, a video was recorded for twenty minutes on an iPad 2 or iPad Mini. These videos were then divided into two segments, in order to record fish response to both the mirror and the plant extract treatment over ten-minute intervals when compared to the controls. This was, in part, to determine the approximate time that it would take an extract to take effect, as well as to determine consistency in the number of aggressive acts that male bettas would make towards an image of themselves in a mirror (Fig. 2). Ten videos were made for each treatment, as were ten videos of controls.

Figure 2. Gill flare (opening of the operculum, or gill cover) from a male *Betta splendens*. Flares and mirror strikes were both counted as aggressive acts.

Each ten-minute segment was given a number, posted on YouTube, and viewed by two different observers without any information attached regarding the treatment, in order to avoid potential viewer bias (Norton and Gutierrez, 2019). Counts were made of two different acts of aggression--the "flare," when male bettas open the operculum (gill covering) in order to appear larger, and the "strike," when the fish makes contact with the mirror to try and bite its perceived rival. These two acts were tabulated for each video by each observer, an average was taken, and values for each treatment were compared through a one-way ANOVA (analysis of variance) and a post-hoc Tukey HSD (honestly significant difference) test. Data from fifty seven videos were analyzed and compared, although some of these videos were eliminated from the final tally in order to ensure consistency of numbers for each treatment. The website used for all statistical analysis is https://astatsa.com/OneWay_Anova_with_TukeyHSD/.

Though several different concentrations of each plant extract were originally tested, the data reported in this experiment were the lowest doses given, because those were the most extensively used. Low concentrations were preferable to high doses due to concerns for each

fish's health. Concentrations were as follows: 0.2 µg/mL for reserpine, 0.1 µg/mL for CBD, 20.0 µg/mL for tulsi, and 3.0 µg/mL for coriander. These doses were similar to prior experiments with three-spined sticklebacks at 25 µg/mL for the anxiolytic compounds fluoxetine and buspirone (Norton and Gutierrez, 2019), 10 µg/mL to 100 µg/mL for arsenic (Tudor et al., 2019), and 40 µg/mL for mercury (II) chloride (Mansur et al., 2012). For an initial set of tests, fish exposed to kava root extract showed signs of distress at dosages of 34 µg/mL, therefore it was decided to discontinue testing of this extract. No fish died as a result of any treatment, or showed any long-term negative health effects.

RESULTS

When fish movements were observed in the test tank during the ten minutes of acclimation before the mirror was inserted, fish occupied each section almost exactly 33% of the time. When the mirror was placed in Section One, male *Betta splendens* remained exclusively in that section, except for two fish that appeared to completely lose interest in their reflection under the influence of cannabidiol (CBD). Of the five extracts tested, kava root was discontinued after two trials, because one fish's gill contractions became alarmingly rapid upon exposure, while another swam in a very agitated fashion and appeared ready to leap from the test tank. Both of these fish were removed, placed back in their home aquaria, and made full recoveries.

For the remainder of this experiment, male *Betta splendens* in the untreated videos (n =10) demonstrated aggression an average of 116.77 times over a ten-minute period (standard deviation = 43.39), while the results for reserpine (n =10, $\bar{x}$ = 68.67, standard deviation = 29.97, p = 0.0074), tulsi (n =10, $\bar{x}$ = 39.67, standard deviation = 11.85, p = 0.0054) and CBD (n =10, $\bar{x}$ = 6.55, standard deviation = 8.03, p = 0.0010) all indicate notably lower levels of aggression when compared to the untreated controls. The only extract that did not show this trend was coriander (n =10, $\bar{x}$ = 77.00, standard deviation = 34.56, p = 0.1017). These results are all shown graphically in Figure 3.

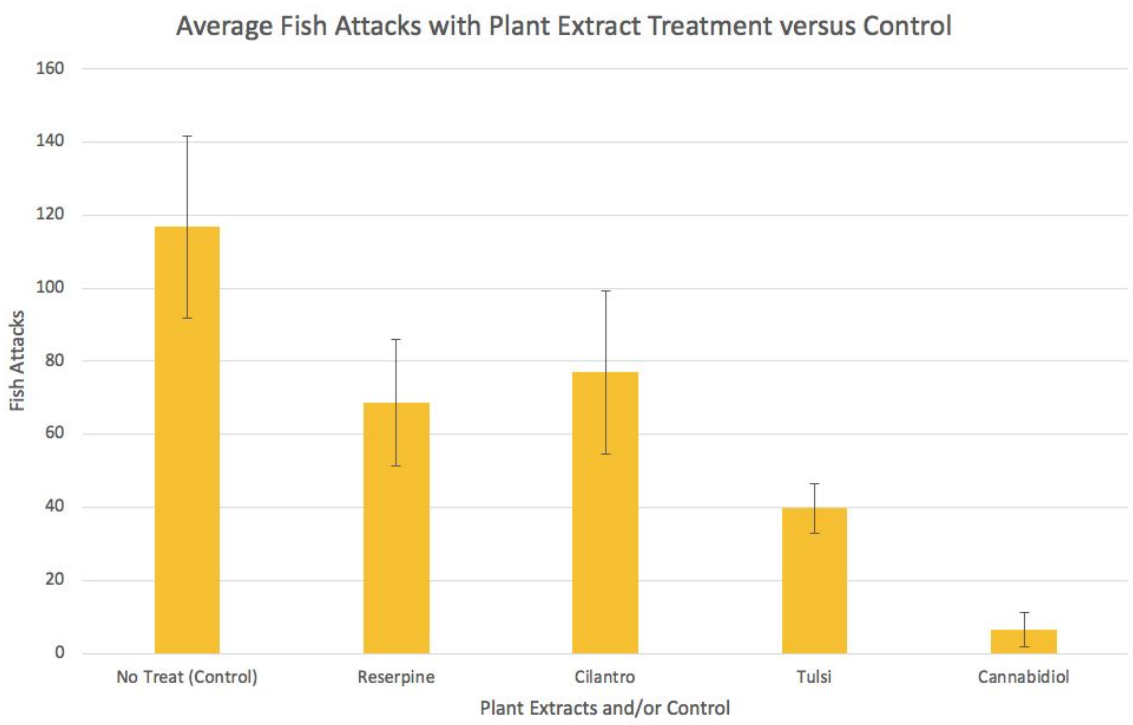

Figure 3. The effects of plant extracts on the aggressive behavior of *Betta splendens* when compared with a control. Reserpine, tulsi, and cannabidiol (CBD) all appear to produce statistically-significant calming effects, while the anxiolytic effects of coriander (cilantro) have not been confirmed. Due to possibly toxic effects on bettas, kava extract was discontinued, and not reported.

DISCUSSION

Though efforts were made to be as objective as possible over the course of this experiment, by separating observations from treatment data and averaging the numbers of aggressive acts from different observers, some fish were so highly aggressive that it was difficult to determine the exact numbers of strikes and flares that occurred in rapid succession when the fish became especially agitated. Though this may have resulted in a slight undercount in the total numbers of aggressive acts, results of this experiment largely confirm that plant extracts with suspected anxiolytic properties do actually have a significant effect on the aggressive behavior of male *Betta splendens*, which also supports the hypothesis that aggressive behavior and anxiety are linked. This hypothesis was previously suggested when male bettas showed lower levels of aggression towards "dummy" males after exposure to the antidepressant fluoxetine (Dzieweczynski and Herbert, 2012).

Interestingly, just as fluoxetine was demonstrated to have an effect on fish behavior even at extremely low doses (Dzieweczynski and Herbert, 2012), cannabidiol was also shown to

reduce fish aggression most effectively, and at the lowest dose of any plant extract. Of all of the trials with CBD, even down to a concentration of 0.1 μg/mL, the fish appeared to settle into an extremely relaxed state, settling on the bottom or top of the test tank and showing no interest in the mirror. Those attacks that were measured during the CBD tests usually occurred during the first fifteen minutes after exposure, and then decreased to zero, which means that CBD sometimes took longer than ten minutes to have its maximum effect. Though these fish appeared to have been sedated, they did respond quickly when a net was inserted into the water, and were fully capable of swimming away from the approaching "danger" when transferring them back to their home tank. This outcome may be due to the fact that the medulla oblongata, which is in charge of autonomic functions such as breathing (in mammals) and heart rate, does not appear to have any cannabinoid receptors (Mackie, 2008) and would therefore not be influenced by this compound, while structures that process more complex behaviors might be impacted. These periods of relaxation in *Betta splendens* as a result of CBD exposure lasted for several hours, although respiration and heartbeat did not appear to be affected, because these fish are still healthy at the time of writing and show no signs of lasting effects. It should be noted that these fish persisted in this extremely relaxed state upon returning to their home tanks for several hours, and did not make the usual exploratory swims upon reintroduction to their habitat, so the sedative effects of CBD could not be completely discounted. However, by the next day, these fish returned to normal behavior, including high levels of aggression when exposed to a mirror.

Initial tests with nonaggressive fish (goldfish and guppies) and similar concentrations of CBD did not demonstrate any observable decrease in swimming behavior, nor did these concentrations have a clear effect on the walking behaviors of crayfish (*Procambarus clarkii*), although these tests will have to be repeated and explored in greater detail in order to determine if there was any sedative effect of CBD on other species. It will also be interesting to pursue additional research about the effects of CBD on crayfish aggression, because *Procambarus clarkii* is considered another model organism for neuroscience research (de Abreu et al., 2019).

Though measuring the exact volume of water pumped over a betta's gills over the course of ten minutes would be difficult to determine accurately, it would not be unreasonable to assume that a fish might pump 10 mL of water over its gills over that time period, and assuming that 10% of the CBD that is dissolved into the water diffuses into the fish's bloodstream, that would mean that a betta might receive a dose of 0.1 μg during these tests. If the average male *Betta splendens* weighs 1.9 grams ("What is the weight of a betta fish?," 2011), and the average male human weighs 194.7 pounds ("What is the average weight of a human being?," n.d.), or 88,300 grams, then the dosage that a human would need for a similar anxiolytic effect is 88,300 / 1.9 x 0.1 μg, which would be 4,650 μg, or 4.65 mg. In the units of mg/kg that are usually considered for toxicity studies, this would translate to 4.65 mg/ 88.3 kg, or 0.0527 mg/kg, which is far below the "low dose" safe level for CBD at 246 mg/kg that has been calculated for mice (Ewing et al., 2019). The first human CBD medication for epilepsy is given at a dose of 2.5 mg/kg ("Epidiolex: side effects, dosage & uses," 2018), which is relatively close to the dosage

calculated from fish data, and avoids harmful side effects such as hepatotoxicity that are possible with high doses of CBD (Ewing et al., 2019). One reason that the effective doses are about five times higher in humans than fish might be due to brain size, because humans have larger brains and many more CB_1 receptors that could be targeted. Both dosages are much lower than what might be considered a potentially hazardous level, and so CBD may be an effective, and relatively safe, anxiolytic compound that needs further investigation.

Tulsi (*Ocimum sanctum*) appeared to be the second most effective plant extract out of those tested for lowering aggression in *Betta splendens*, and this suggests that at least one of the many claims made for this plant--along with lowering blood glucose levels and antimicrobial activity (Cohen, 2014)--may have scientific merit. This particular extract was taken from tea that also included dried rose petals (*Rosa* sp.), which may have medicinal properties (Boskabady et al., 2011); however, none of the compounds in rose petal extract are known to have definite anxiolytic properties, although inhaling rose oil may have calming effects on rats (de Almeida et al., 2004).

Results observed with reserpine appear to confirm its anxiolytic and possibly antidepressant effect, although the mechanism by which it might have this effect is still not understood if it reduces the levels of monoamine neurotransmitters such as serotonin in the brain (Mulnari, 2012). Due to the fact that it did lower fish aggression, it is not unreasonable to conclude that reserpine does indeed have a depressive effect on fish, although it was beyond the scope of this experiment to determine and compare the levels of neurotransmitters within the brains of the *Betta splendens* test subjects, because these animals could not be harmed. Further research might investigate scototaxis rather than aggression in *Betta splendens* or another fish species, in order to draw a better conclusion about the anxiolytic effects of reserpine.

Though coriander extracts were not shown to have a significant effect on betta aggression over the course of this experiment, previous research suggested that in mice, only doses of 100 mg/kg to 200 mg/kg would have an anxiolytic effect that was similar to the anti-anxiety medication diazepam, while a lower dose of 50 mg/kg did not have any effect (Mahendra and Bisht, 2011). Calculations for the dosage given to male *Betta splendens*, making the same assumption that was made with CBD that 10% of the extract would enter the fish's blood through its gills, would be as follows: 330 mg whole leaf extract in 1.0 mL of liquid was dissolved in 2880 mL of water in the test tank, and would produce a concentration of 0.115 mg/mL. Estimating a flow rate through the betta's gills of 10 mL over a 10 minute period, it would mean that 0.115 mg would be ingested if 10% were diffused into the fish's blood. Assuming again that an average male *Betta splendens* weighs 1.9 g, or 0.0019 kg, the dosage in mg/kg for this extract would be 60.5 mg/kg, which might be too low to have an anxiolytic effect. Further research should increase the concentration of this extract, as long as it can be determined that there will be no negative side effects. These harmful consequences could not be ruled out for kava (*Piper methysticum*), and this extract was not tested when initial trials suggested that it might be irritating or bothersome to fish. Research into the toxicity of kava has indicated that this

might be due to certain kava extracts being contaminated with compounds that cause liver damage (Clouatre, 2004), but on the other hand, one promising study claims that kavalactones might interact with CB_1 receptors that are part of the endocannabinoid system (Ligresti et al., 2012), raising the question of whether an uncontaminated kava root extract might have a similar effect to CBD.

Though this experiment did not include female betta fish, it has been shown in a previous study that female *Betta splendens* tend to prefer males that are red in color, except when exposed to high levels of arsenic (100 μg/L), after which females showed no color preference and also exhibited signs of elevated anxiety (Tudor et al., 2019). Effects of plant extracts and other compounds on female preference is quite an interesting angle to consider for future experiments, and so might the influence of compounds that could lower the anxiety caused by environmental contaminants.

These responses to arsenic exposure do indicate that certain known toxins can have behavioral effects on *Betta splendens*, and arsenic has long been known to be a dangerous poison (Vahidnia et al., 2007). This outcome could lead to an alternative hypothesis about the effect of plant extracts on betta behavior: that these males are being poisoned, making them less inclined to fight. While no observational evidence supports this hypothesis (aside from a possible negative response with kava extract), this would mean that these plant compounds might affect behavior in a manner that is similar to arsenic and mercury (II) chloride, which is known to accumulate in body tissues and the brain (Bernhoft, 2012), lowering incidence of many fish activities, including feeding, fighting, and swimming (Mansur et al., 2012). When mercury (II) ions enter the brain, they require more than a month for half of these ions to be excreted (Bernhoft, 2012), and this suggests that both mercury (II) and arsenic could have long-lasting effects (Vahidnia et al., 2007). Such a response would be different from the pharmacological reaction that one might expect from medications, and due to the fact that the fish in this experiment recovered to normal behavior in less than twelve hours, it is more likely that the plant extracts had a drug-like effect on these fish, rather than longer-term poisoning, because the fish would need more time to recover if there had been damage to the brain or body tissues. It should also be noted that none of the previous studies on these compounds suggested any long-term toxic effects, aside from kava, which was discontinued.

One final argument that might be made to refute the findings of this experiment is that betta fish would respond with lowered aggression to any contaminant that might be dissolved in otherwise pure water. While it is true that other compounds with no known pharmacological effect should be included in future experiments, it should be noted that the betta's natural environment includes rice paddies, drainage ditches, and slow-moving rivers (Rainboth, 1996), which surely contain a wide variety of dissolved substances and in no way could be considered "pristine" or "pure." In fact, it is recommended by many people who keep and breed betta fish that they should place leaves of the Indian almond leaf (*Terminalia catappa*) in their aquaria so that beneficial plant compounds such as tannins may dissolve in the water, helping bettas to feel

more “at home” (“Bettawan: How to Use Indian Almond Leaves (IAL) in the Aquarium,” n.d.). Further testing might include “teas” made from this plant and other leaves, or possibly using decaffeinated green or black tea to investigate how fish will respond to it.

CONCLUSION

The research presented in this paper provides evidence that certain plant extracts have anxiolytic (anxiety-breaking) properties, including cannabidiol (CBD), reserpine, and holy basil (tulsi), while coriander will need to be tested at higher concentrations in order to determine if it has an actual anxiolytic effect. This investigation continues to build on prior research of the linkage between anxiety and aggressive behavior, and further supports the use of *Betta splendens* as a useful model organism for behavioral research.

REFERENCES

Aizpurua-Olaizola, O., Elezgarai, I., Rico-Barrio, I., Zarandona, I., Etxebarria, N., & Usobiaga, A. (2017). Targeting the endocannabinoid system: future therapeutic strategies. *Drug Discovery Today*, *22*(1), 105–110. https://doi.org/10.1016/j.drudis.2016.08.005

Are CBD oils for pets legal? (2018, August 20). Augusta Free Press. Retrieved January 22, 2020, from https://augustafreepress.com/are-cbd-oils-for-pets-legal/

Baerends, G. P. (1971). The Ethological Analysis Of Fish Behavior. *Fish Physiology Environmental Relations and Behavior*, 279–370. doi: 10.1016/s1546-5098(08)60150-8

Baumeister, A. A., Hawkins, M. F., & Uzelac, S. M. (2003). The myth of reserpine-induced depression: Role in the historical development of the monoamine hypothesis. *Journal of the History of the Neurosciences*, *12*(2), 207–220. doi: 10.1076/jhin.12.2.207.15535

Bernhoft, R. A. (2012). Mercury toxicity and treatment: A review of the literature. *Journal of Environmental and Public Health*, *2012*, 1–10. doi: 10.1155/2012/460508

Bettawan: Using Indian Almond Leaves (IAL) in the Aquarium. (n.d.). BettaWan. Retrieved February 28, 2020, from https://www.bettawan.com/ial_method.html

Boskabady, M. H., Shafei, M. N., Saberi, Z., & Amini, S. (2011). Pharmacological effects of Rosa damascena. *Iranian Journal of Basic Medical Sciences*, *14*(4), 295–307.

Bronstein, P. M. (1984). Agonistic and reproductive interactions in Betta splendens. *Journal of Comparative Psychology*, *98*(4), 421–431. doi: 10.1037/0735-7036.98.4.421

Buigues, J., & Vallejo, J. (1987). Therapeutic response to phenelzine in patients with panic disorder and agoraphobia with panic attacks. *The Journal of Clinical Psychiatry*, *48*(2), 55–59.

Caceres, V. (2019, September 23). *Topical CBD Products May Offer Some Relief for Certain Types of Pain*. US News & World Report. Retrieved January 23, 2020, from https://health.usnews.com/wellness/articles/do-topical-cbd-products-work

CBD in Pennsylvania. (n.d.). Guide to CBD. Retrieved January 22, 2020, from https://www.guidetocbd.org/states/pa/

Clarke, R. C., & Merlin, M. D. (2016). *Cannabis: Evolution and Ethnobotany*. University of California Press.

Clouatre, D. L. (2004). Kava kava: examining new reports of toxicity. *Toxicology Letters*, *150*(1), 85–96. doi: 10.1016/j.toxlet.2003.07.005

Cohen M. M. (2014). Tulsi - Ocimum sanctum: A herb for all reasons. *Journal of Ayurveda and Integrative Medicine*, *5*(4), 251–259. https://doi.org/10.4103/0975-9476.146554

Davies, D., & Shepherd, M. (1955). Reserpine In The Treatment Of Anxious And Depressed Patients. *The Lancet*, *266*(6881), 117–120. doi: 10.1016/s0140-6736(55)92118-8

de Almeida, R. N., Motta, S. C., Faturi, C. B., Catallani, B., Leite, J. R. (2004). Anxiolytic-like effects of rose oil inhalation on the elevated plus-maze test in rats. *Pharmacology Biochemistry and Behavior*, *77*(2), 361-364. doi: 10.1016/j.pbb.2003.11.004.

de Abreu, MS, Maximino, C, Banha, F, et al. (2019). Emotional behavior in aquatic organisms? Lessons from crayfish and zebrafish. *Journal of Neuroscience Research*, 1– 16. doi: 10.1002/jnr.24550

Dzieweczynski, T. L., & Hebert, O. L. (2012). Fluoxetine alters behavioral consistency of aggression and courtship in male Siamese fighting fish, Betta splendens. *Physiology & Behavior*, *107*(1), 92–97. doi: 10.1016/j.physbeh.2012.06.007

Epidiolex: side effects, dosage & uses. (2018, November 12). Drugs.com. Retrieved February 21, 2020, from https://www.drugs.com/epidiolex.html

Ewing, L. E., Skinner, C. M., Quick, C. M., Kennon-McGill, S., McGill, M. R., Walker, L. A., ElSohly, M. A., Gurley, B. J., & Koturbash, I. (2019). Hepatotoxicity of a cannabidiol-rich cannabis extract in the mouse model. *Molecules*, *24*(9), 1694. doi: 10.3390/molecules24091694

Fernández-Ruiz, J., Sagredo, O., Pazos, M. R., García, C., Pertwee, R., Mechoulam, R., & Martínez-Orgado, J. (2013). Cannabidiol for neurodegenerative disorders: important new clinical applications for this phytocannabinoid?. *British Journal of Clinical pharmacology*, *75*(2), 323–333. doi: 10.1111/j.1365-2125.2012.04341.x

Gradstein, Felix M.; Ogg, James G.; Smith, Alan G. (2004). *A Geologic Time Scale.* Cambridge University Press. ISBN 978-0521786737.

Grillner, S., Ekeberg, Ö., Manira, A. E., Lansner, A., Parker, D., Tegnér, J., & Wallén, P. (1998). Intrinsic function of a neuronal network — a vertebrate central pattern generator. *Brain Research Reviews*, *26*(2-3), 184–197. doi: 10.1016/s0165-0173(98)00002-2

Hoenders, H. R., Bartels-Velthuis, A. A., Vollbehr, N. K., Bruggeman, R., Knegtering, H., & Jong, J. T. D. (2018). Natural medicines for psychotic disorders. *The Journal of Nervous and Mental Disease*, *206*(2), 81–101. doi: 10.1097/nmd.0000000000000782

Is CBD Safe for All Ages? (n.d.). SolCBD. Retrieved February 2, 2020, from https://www.solcbd.com/blogs/news/is-cbd-safe-for-all-ages

Kluger, B., Triolo, P., Jones, W., & Jankovic, J. (2015). The therapeutic potential of cannabinoids for movement disorders. *Movement Disorders: Official Journal of the Movement Disorder Society*, *30*(3), 313–327. doi: 10.1002/mds.26142

Kuchta, K., Nicola, P. D., & Schmidt, M. (2017). Randomized, dose-controlled double-blind trial: Efficacy of an ethanolic kava (Piper methysticum rhizome) extract for the treatment of anxiety in elderly patients. *Traditional & Kampo Medicine*, *5*(1), 3–10. doi: 10.1002/tkm2.1079

Ligresti, A., Vilano, R., Allará, M., Ujváry, I., DiMarzo, V., (2012). Kavalactones and the endocannabinoid system: The plant-derived yangonin is a novel CB1 receptor ligand. *Pharmacological Research*, *66*(2), 163-169. doi: 10.1016/j.phrs.2012.04.003.

Lynch, J. (2002). Potent Roots and the Origin of kava. *Oceanic Linguistics 41*(2), 493-513. doi:10.1353/ol.2002.0010.

Mansur, B. D. M., Cavalcante, C. N. S., Santos, B. R. D., & Gouveia, A. (2012). Effects of mercury chloride ($HgCl_2$) on Betta splendens aggressive display. *The Spanish Journal of Psychology*, *15*(1), 442–450. doi: 10.5209/rev_sjop.2012.v15.n1.37349

Mackie, K. (2008). Cannabinoid receptors: Where they are and what they do. *Journal of Neuroendocrinology*, *20*(s1), 10–14. doi: 10.1111/j.1365-2826.2008.01671.x

Mahendra, P., & Bisht, S. (2011). Anti-anxiety activity of Coriandrum sativum assessed using different experimental anxiety models. *Indian Journal of Pharmacology*, *43*(5), 574–577. doi: 10.4103/0253-7613.84975

Maximino, C., de Brito, T. M., ClaGellis De Mattos Dias, C. A., Gouveia, A., & Morato, S. (2010). Scototaxis as anxiety-like behavior in fish. *Nature Protocols*, *5*(2), 209–216. doi: 10.1038/nprot.2009.225

Mulinari, S. (2012). Monoamine Theories of Depression: Historical Impact on Biomedical Research. *Journal of the History of the Neurosciences*, *21*(4), 366–392. doi: 10.1080/0964704x.2011.623917

Neumann, I. D., Veenema, A. H., & Beiderbeck, D. I. (2010). Aggression and anxiety: social context and neurobiological links. *Frontiers in Behavioral Neuroscience*, *4*(12). doi: 10.3389/fnbeh.2010.00012

Norton, W., & Gutiérrez, H. C. (2019). The three-spined stickleback as a model for behavioural neuroscience. *PloS one*, *14*(3), e0213320. https://doi.org/10.1371/journal.pone.0213320

Palmer, S. (2020, February 20). *Rats' brains are more like ours than scientists previously thought*. Pennsylvania State University. Retrieved February 22, 2020, from https://news.psu.edu/story/270321/2013/03/26/research/rats-brains-are-more-ours-scientists-previously-thought

Rainboth, W. J. (1996). Fishes of the Cambodian Mekong. *FAO Species Identification Field*

Guide for Fishery Purposes. Food and Agriculture Organization of the United Nations, pg. 265

Rodriguez, F., Broglio, C., Durcin, E., Gomez, A., & Salas, C. (2006). *Fish cognition and behavior*. (C. Brown, K. N. Laland, & J. Krause, Eds.). Blackwell Publishing.

Rovainen, C. M. (1985). Respiratory bursts at the midline of the rostral medulla of the lamprey. *Journal of Comparative Physiology A*, *157*(3), 303–309. doi: 10.1007/bf00618120

Ruta, M., Pisani, D., Lloyd, G. T., & Benton, M. J. (2007). A supertree of Temnospondyli: cladogenetic patterns in the most species-rich group of early tetrapods. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1629), 3087–3095. doi: 10.1098/rspb.2007.1250

Schrot, R. J., & Hubbard, J. R. (2016). Cannabinoids: Medical implications. *Annals of medicine*, *48*(3), 128–141. doi: 10.3109/07853890.2016.1145794

Shannon, S., Lewis, N., Lee, H., & Hughes, S. (2019). Cannabidiol in anxiety and sleep: A large case series. *The Permanente Journal, 23*, 18–041. doi: 10.7812/TPP/18-041

Shepherd, GM (1994). *Neurobiology*. Oxford University Press.

Steimer T. (2002). The biology of fear- and anxiety-related behaviors. *Dialogues in Clinical Neuroscience*, *4*(3), 231–249.

Sundaram, R. S., Ramanathan, M., Rajesh, R., Satheesh, B., & Saravanan, D. (2012). Lc-Ms quantification of rosmarinic acid and ursolic acid in the Ocimum sanctum Linn. leaf extract (holy basil, tulsi). *Journal of Liquid Chromatography & Related Technologies*, *35*(5), 634–650. doi: 10.1080/10826076.2011.606583

Tudor, M. S., Lopez-Anido, R. N., Yocius, C. A., Conlin, S. M., & Hamlin, H. J. (2019). Ecologically relevant arsenic exposure alters female mate preference and anxiety-like behavior in Betta splendens. *Heliyon*, *5*(10). doi: 10.1016/j.heliyon.2019.e02646

Tzeng, Y. M., & Lee, M. J. (2015). Neuroprotective properties of kavalactones. *Neural Regeneration Research*, *10*(6), 875–877. doi: 10.4103/1673-5374.158335

Vahidnia, A., van der Voet, G. B., & de Wolff, F. A. (2007). Arsenic neurotoxicity — A review. *Human & Experimental Toxicology*, *26*(10), 823–832. doi: 10.1177/0960327107084539

Wang, J., Qu, W., Bittenbender, H. C., & Li, Q. X. (2013). Kavalactone content and chemotype of kava beverages prepared from roots and rhizomes of Isa and Mahakea varieties and extraction efficiency of kavalactones using different solvents. *Journal of Food Science and Technology*, *52*(2), 1164–1169. doi: 10.1007/s13197-013-1047-2

Weyhenmeyer, James A.; Gallman, Eve. A. (2007). *Rapid Review of Neuroscience*. Mosby Elsevier.

What is the average weight of a human being? (n.d.). Reference.com. Retrieved February 20, 2020, from https://www.reference.com/science/average-weight-human-being-1186bf58b9307867

What is the weight of a betta fish? (2011, February 28). Answers.com. Retrieved February 22, 2020, from https://www.answers.com/Q/What_is_the_weight_of_a_betta_fish

U.S. Food and Drug Administration. (2019, November 25). *What you need to know (and what we're working to find out) about products containing Cannabis or Cannabis-derived compounds, including CBD.* Retrieved February 29, 2020, from https://www.fda.gov/consumers/consumer-updates/what-you-need-know-and-what-were-working-find-out-about-products-containing-cannabis-or-cannabis

Witte, S., Loew, D., & Gaus, W. (2005). Meta-analysis of the efficacy of the acetonic kava-kava extract WS®1490 in patients with non-psychotic anxiety disorders. *Phytotherapy Research*, *19*(3), 183–188. doi: 10.1002/ptr.1609

Zohary, D., & Hopf, M. (2012). *Domestication of Plants in the Old World: the Origin and Spread of Cultivated Plants in West Asia, Europe and the Nile Valley*. Oxford Univ. Press.