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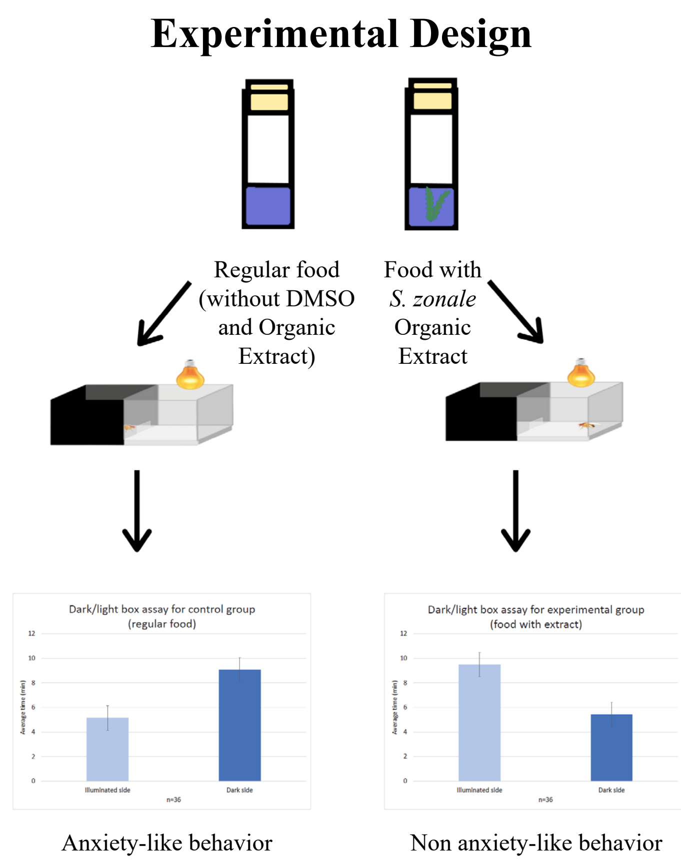
Long-term exposure to benzodiazepines for the treatment of anxiety can cause dependence and tolerance, which may lead to addiction. Alternative anxiety treatments that use bioactive substances found in organic extracts obtained from algae have been considered. This research explores the possible anxiolytic effects of brown algae crude extracts of *Stypopodium zonale* in *Drosophila melanogaster* using a dark/light box test. We hypothesize that *D. melanogaster*, upon consumption of food containing the crude extract derived from *Stypopodium zonale*, will exhibit less anxiety-related behaviors. To test our hypothesis, an organic extract of *S. zonale* brown algae was isolated and dissolved in 15% dimethyl sulfoxide (DMSO). The crude extract was administered in the food of wild-type, developing and adult *D. melanogaster* subjected to a dark/light box test. The control group consisted of *D. melanogaster* exposed to regular food. The amount of time the flies spent on both sides of the box was recorded. Results show that *Drosophila* exposed to the extract showed less anxiety-related behaviors when compared to the control group. In conclusion, the crude organic *S. zonale* extract seemed to exert anxiolytic effects in an anxiety model of *D. melanogaster.* This project is a step in the future potential use of specific or combined metabolites of brown algae extracts as a pharmacological alternative for the treatment of anxiety disorders.



Introduction

Anxiety is a normal reaction to stress and can be beneficial in some circumstances. It helps us anticipate and assume potential danger in ambiguous situations, keeping us alert. However, anxiety disorders differ from normal feelings of nervousness or anxiety involving fear. Notably, anxiety disorders are the most common mental disorders, affecting almost 30 percent of adults at some point in their lives (Griffin et al., 2013). Benzodiazepines remain as the standard treatment of care for individuals suffering from anxiety disorders, acting as indirect agonists of gamma-aminobutyric acid (GABA), the primary inhibitory neurotransmitter of the central nervous system (Zimmermann et al., 2019). However, benzodiazepines are psychotropic drugs that have adverse effects, which limit their use due to the potential of developing psychological and physiological dependence (Pérez, 2002). The risk of drug dependence increases with an increase in dose, duration of treatment, and half-life of the benzodiazepine. In addition, common adverse effects of benzodiazepines may include excessive sedation, lack of concentration, cognitive disorders, alteration of motor coordination, ataxia, slow reflexes, rebound insomnia, retrograde amnesia, and withdrawal syndrome (Ranstam et al., 1997).

Alternative anxiety treatments that use bioactive substances present in organic extracts obtained from algae have been considered. Ninety percent of the planet's biomass is found in the oceans, including marine species and algae. It has been estimated that there are about one million species of algae (Guiry, 2012). Despite this, algae are still underutilized marine biological resources. These aquatic organisms present an excellent potential for the study of bioactive compounds. Compounds with antiviral, antioxidant, and anti-inflammatory activity have been isolated in algae (Pangestuti et al., 2011), as well as several compounds with neuroprotective properties, such as dieckol (a phenolic compound in brown algae), fucoxanthin (an antioxidant in *Padina tetrastromatic*), florotannins, alginates, fucoidan, sargaquinonic acid, and carotenoids (Ohba et al., 2007). Studies show that a fraction rich in florotanins obtained from the *Ecklonia cava* brown algae has the potential to reduce anxiety in mice (Cho et al., 2012). Based on these findings, organic extracts obtained from brown algae could possess metabolites with the potential to decrease anxiety-related behaviors. Interestingly, there is a species of brown algae found in the coasts of Puerto Rico: *Stypopodium zonale*.

We hypothesize that *D. melanogaster*, upon consumption of food containing the crude extract derived from brown algae, will exhibit less anxiety-related behaviors in a dark/light box test (See Figure 1). The dark/light box test consists of an acrylic box that has a transparent half, illuminated with a light bulb, and a dark half painted with black enamel. The test is based on the innate aversion of rodents to brightly illuminated areas (transparent area illuminated with a light bulb) and on the spontaneous exploratory behavior of rodents in response to mild stressors, that is, novel environment and light (Crawley et al., 1980; Neckmeyer et al., 2015). There is a conflict between the tendency to explore and the initial tendency to avoid the unfamiliar (neophobia). These two conflicting emotions lead to observable anxiety-like symptoms. In a dark/light box test, the administration of drugs increases the time spent in the transparent area compartment, suggesting anxiolytic activity. In a previous investigation, we reported that the organic extract of *Stypopodium zonale* decreased centrophobia in *Drosophila melanogaster* (Mejías et al., 2016). *D. melanogaster* has emerged recently as a versatile invertebrate model for the study of neuropsychiatric disorders, with many neurological, physiological, and neurochemical similarities with the vertebrate brain, including neurotransmitter systems, such as GABA, serotonin, and dopamine, among others. Besides, *D. melanogaster* has an excellent ability for learning, memory formation, and the development of tolerance, dependence, and drug addiction (O'Kane, 2011).

Materials and Methods

Collection and Storage of Stypopodium zonales

S. zonale was collected at Flamenco Beach, on the island of Culebra, and at Pozuelo Beach in the town of Guayama. The specimens were placed in plastic bags. The bags were sealed, covered with iced water, and taken to the laboratory to be washed, dried, and stored at -20°C.

Food Preparation

The control group was fed regular food, consisting of 5 g of Formula 4-24, obtained from Carolina Biological Supply Company. Food was placed in a cylindrical tube and mixed with 10 mL of water. After this, yeast was added to increase food yield, and 3-5 drops of vinegar were added to prevent fungal growth. For the experimental group, regular food was mixed with the extract. In a small beaker, 9,733 uL of water and 267 uL of the crude extract were mixed and then added to the food in a cylindrical tube. The final extract concentration was 1mg per gram of food. As in the control group, yeast was added to increase food yield, and 3-5 drops of vinegar were added to prevent fungal growth.

Larvae and Adult Count

The number of larvae between the control group (regular food without *S. zonale* crude extract in DMSO) and the experimental group (food with crude extract) was compared to assess possible effects in developing *D. melanogaster*. A random tube was selected for each group and was placed at -20ºC for several minutes to sacrifice adult *D. melanogaster* in order to facilitate larvae counting. The number of larvae was compared between groups. This control was performed in duplicate, i.e., two *D. melanogaster* culture tubes for the control and the experimental groups.

Ring Assay

The objective of the ring assay analysis was to verify that the motor ability of *D. melanogaster* was not affected either by the crude extract or by DMSO. *Drosophila melanogaster* shows negative geotaxis, which is the tendency to fly against gravity. *Drosophila* (n = 20) were placed in a tube, which was gently knocked against a

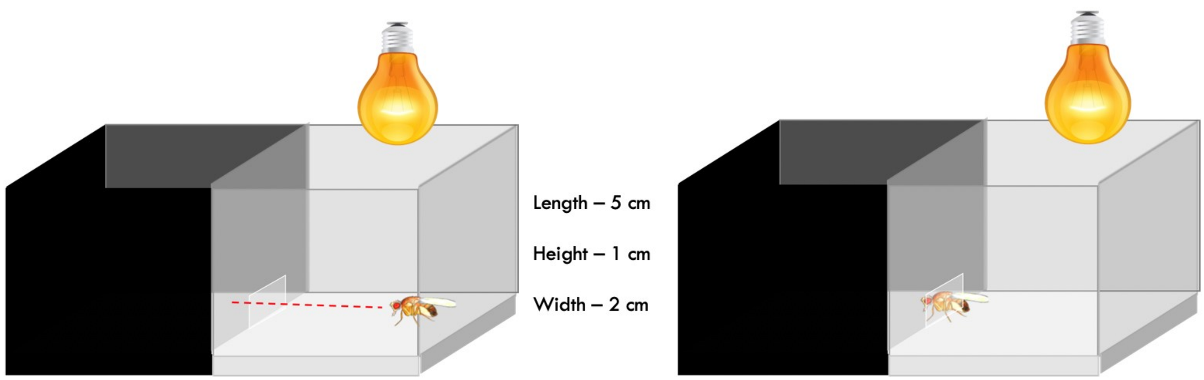


Figure 1: Dark/light box test model.

bench several times to force them to the bottom of the tube. At a three-second count, the tube was photographed. The *Drosophila* were allowed to fly in the tube for a full minute. This procedure was repeated four times. The height reached by each *Drosophila* from the bottom of the tube was measured in centimeters.

The Dark/Light Box Test

The dark/light box consisted of a 2 cm wide, 5 cm long, and 1.5 cm high box, made with transparent acrylic. One half of the box was painted with black enamel to create a dark side, while the other remained transparent. The dark/light box was placed inside a bigger wooden box, painted black on the inside, with a 16W bulb emitting white light atop the clear part of the box. The lights outside the work area were turned off for 30 minutes. A male *Drosophila* was placed on the illuminated side of the box and its behavior recorded in the timeframe previously established. The first 15 minutes were allocated for habituation, while the other 15 minutes were recorded on video for behavioral analysis. The time spent by each *Drosophila* on each side of the box was also recorded for the control group (regular food) and the experimental group (food with 1 mg extract/g of *Stypopodium zonale*).

Results

Larvae and Adult Count

For larval counting, both the height of the culture tube (9.20 cm) and the height of the food (1.70 cm) were measured. The number of larvae was counted from the 1-cm mark up to the 7-cm mark. Two *Drosophila* culture tubes were used for each group (regular food and food with the organic extract). For the control group, the average was 68 larvae and 38 adults, while for the experimental group (1 mg extract/of food), the average was 147 larvae and 55 adults. The results for adult *Drosophila* are shown in Figure 2.

Ring Assay

For this test, two runs were performed with two different tubes per group, using only male *Drosophila*. The regular food assay was made with n=30 per tube, two tubes in total (n = 60 total), while the extract assay was made with eight (n=8) and twelve (n=12) *Drosophila,* for a total of 20 adult *Drosophila*. The average distance traveled in both tubes was 2.5 cm for the control group, and 2.4 cm for the experimental group. Figure 3 compares both the control and experimental group.

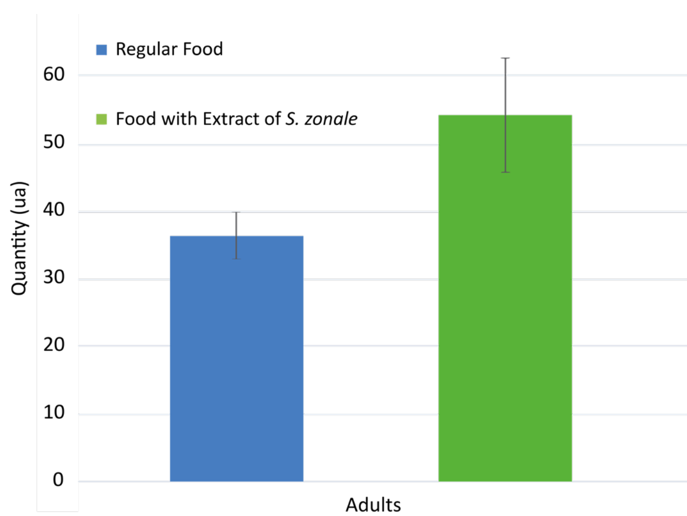


Figure 2: Results for adult *Drosophila* count. The student's t-test did not show a significant difference between the results obtained for the control group vs. the experimental group diluted in DMSO with p=0.25 ($p \leq 0.05$).

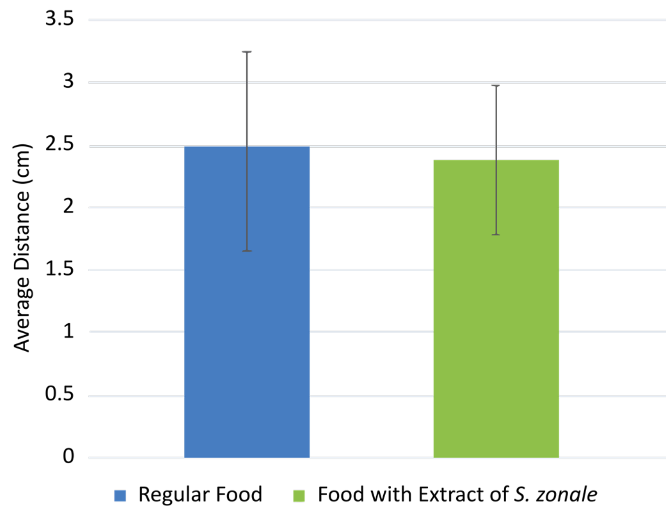


Figure 3: Average vertical distance (in cm) of *Drosophila* in the ring assay. *Drosophila* in the control group moved an average of 2.47 cm, while *Drosophilas* in the experimental group moved an average of 2.40 cm. These values demonstrate that the crude organic extract of *S. zonale* did not affect fly behavior and thus locomotor capabilities in *Drosophila melanogaster*. The student's t-test did not show a significant difference between the results obtained with regular food vs. the results of food with extract diluted in DMSO with p = 0.98 ($p \leq 0.05$).

Dark/Light Box Test

For the dark/light box test, there were 36 assays per group. Only male *Drosophila* were used for this assay. The results were analyzed using a student's t-test to determine if the results had significant differences ($p \leq 0.05$). For the t-test, the mean for each side was compared between the control group and experimental group (Figures 4C and 4D). The significant differences were p = 0.000018 between the illuminated side for each group, and p = 0.002 between the dark side for each group.

Discussion

Controls to identify potential effects of crude *Stypopodium zonale* extract or DMSO on motor activity (ring assay), reproduction, and development (larvae and adult count) show that neither the extract nor DMSO altered motor activity, reproduction or development in *Drosophila melanogaster*. Figure 2 shows that the number of adult flies in both groups was similar, and that the difference was not statistically significant. The number of larvae was also similar between the groups (data not shown), with no statistical difference. The average time of development at 25°C for both groups, from oviposition to adult, was eight days. Locomotor activity measured as negative geotaxis was similar in *Drosophila* fed with regular food, and the ones fed the *S. zonale* crude extract, as shown in Figure 3. The ring assay findings are crucial since the differences

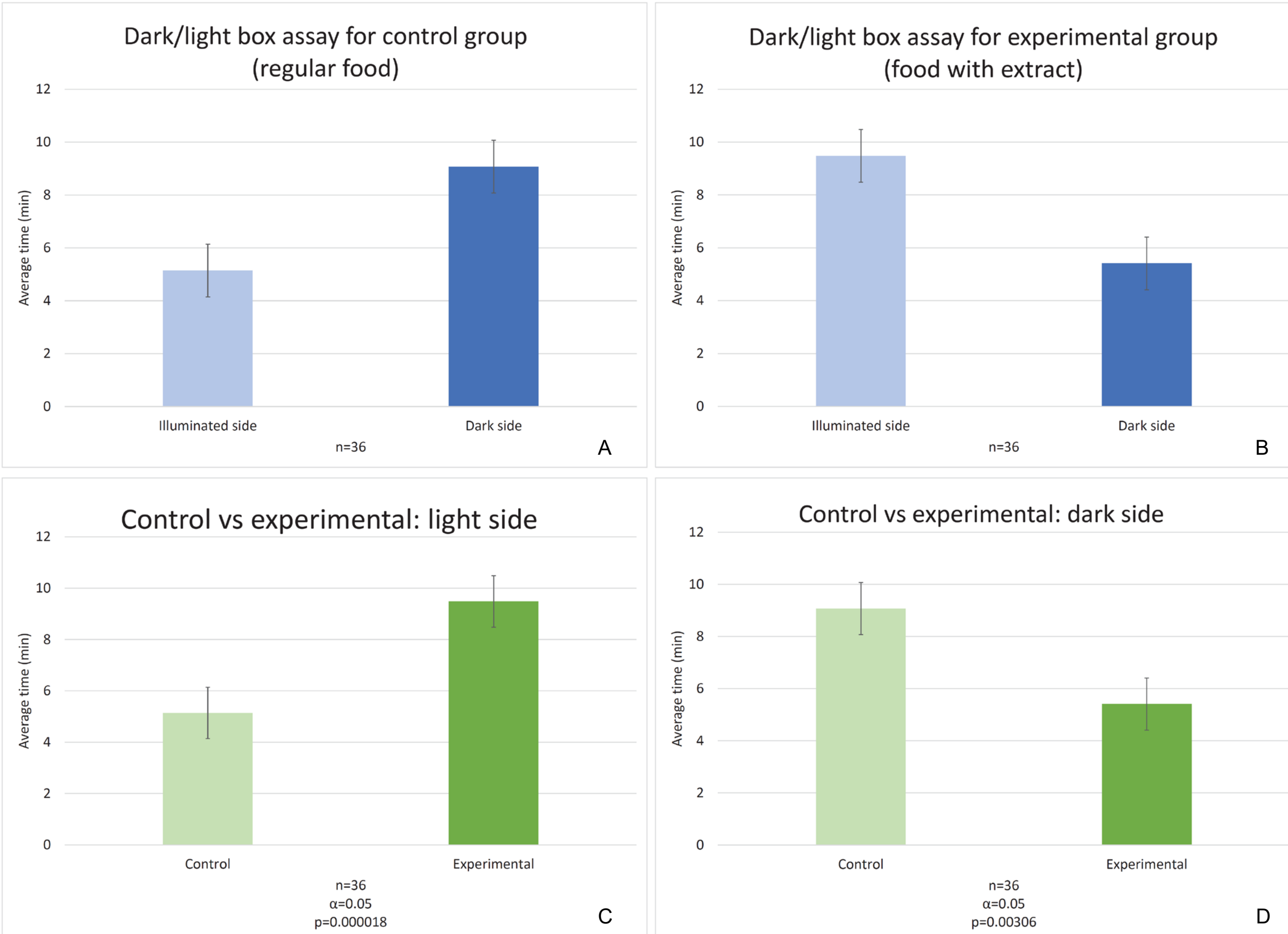


Figure 4: Results of the dark/light box assays. A) *Drosophila* exposed to Regular Food, B) *Drosophila* exposed to food with extract in DMSO, C) Comparison of the time in the illuminated side, regular food vs. food with extract. D) Comparison of the time in the dark side, regular food vs. food with extract. The student's t-test shows a significant difference between the results obtained with regular food versus food with extract in DMSO with $p = 0.003$ ($p < 0.05$).

in behavior observed in the dark/light box test between the control and experimental groups could not be attributed to any type of locomotor impairment caused by the crude organic extract or DMSO.

The behavior of *Drosophila* in the dark/light box test supports the hypothesis regarding the potential anxiolytic effects of *S. zonale* crude extract. As predicted, *Drosophila* fed with regular food spent more time on the dark side of the box when compared to the time spent on the illuminated side (Figure 4A). However, *Drosophila* fed with extract-containing food spent more time on the illuminated side, when compared to the time spent on the dark side (Figure 4B). This difference is statistically significant, as can be observed in Figures 4C and 4D.

Conclusion

Administering *Stypopodium zonale* crude organic extract to *Drosophila melanogaster* reduced anxiety and fear-related behavior according to the results of a dark/light box test. To identify the organic compound or compounds responsible for decreasing anxiety-related behaviors on *Drosophila*, our research team is currently focusing on two efforts. First, to study the chemical characterization of the crude extract (using column chromatography, nuclear magnetic resonance (NMR) and infrared spectroscopy (IR) to identify the most abundant metabolites), and second, to conduct behavioral assays based on dark/light box tests using different fractions of the organic extract and combinations of solvents. Additionally, we plan to perform the behavioral assays with female *Drosophila* only, to determine if differences in the hormonal milieu influence the response to the organic extract.

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