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Article

Effects of antioxidants on the climbing abilities of *Drosophila melanogaster* exposed to dental resin.

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SUMMARY

Reactive oxygen species (ROS) are highly reactive free radicals containing oxygen that seek to fill in empty spaces within their valence shells. ROS buildup can lead to DNA damage and diseases including cancer, immune diseases, and neurodegenerative diseases. In contrast, antioxidants, which are found in foods such as asparagus and avocado, prevent ROS damage by pairing the unpaired electrons in ROS before oxidation can occur. Based on this information, we decided to study the effects of the antioxidant-containing foods asparagus and avocado on the climbing abilities of fruit flies exposed to dental resin, a ROS-releasing compound. We carried out this study by dividing the experiment into four groups of fruit fly food media based on the presence of dental resin, asparagus, and avocado, with each group consisting of six vials with five male and five female flies each. We measured the climbing abilities of the flies every 2 days for 6 days by counting the number of flies that passed the climbing line (6 cm) in 30 seconds. The results demonstrated that the dental resin group achieved significantly lower climbing percentages compared to the control group. While the asparagus group achieved higher scores than the dental resin group, the difference was not significant. No conclusions were made from the avocado group due to the thick avocado consistency that stuck to the flies and prevented them from climbing up the vial. The results of this study encourage further investigations on natural remedies for DNA damage and ROS-related diseases.

INTRODUCTION

Free radicals are highly reactive particles that seek to fill open spaces within the valence shell through oxidation. Reactive oxygen species (ROS) are the most common free radicals that contain oxygen. ROS, such as superoxide anion, peroxide, hydroxyl radicals, and hydroxyl ions, play a role in cell signaling and gene expression (1). In fact, ROS are produced daily in living organisms as part of cellular metabolism. For example, superoxide anions form as part of cellular respiration when molecular oxygen is reduced by electrons released by nicotine adenine dinucleotide phosphate (NAD(P)H), hydrogen peroxide (H_2O_2) is produced by superoxide dismutases (enzymes that break down superoxide anions), and hydroxyl radicals are produced via the Haber-Weiss and Fenton Reaction (a reaction in which H_2O_2 combines with iron) (2). In moderate concentrations, ROS play a role in cell life, activation, proliferation, and organ function. However, high concentrations of ROS can harm DNA, lipids, cell membranes, and proteins (2,3). For example, ROS oxidize lipids, forming lipid radicals. As these lipid radicals combine with oxygen, they form peroxyl radicals that initiate a chain reaction that transforms fatty acids into lipid hydroperoxides. These lipid hydroperoxides are very unstable and easily decompose, harming cell membranes and structures (2). ROS can also cause protein oxidation, leading to changes in protein function and behavioral changes. Additionally, an increase in ROS production has been linked to hypoxia, a condition in which cells are deprived of oxygen, and hyperoxia, a condition in which cells are exposed to an abundance of oxygen (1).

In our daily lives, ROS and free radicals increase through exposure to fried foods, smoking, and polluted air (3). Specifically, an increase in ROS can occur through the actions of white blood cells, as free radicals are used to fight off invading pathogens (1). Eventually, this buildup of ROS will lead to a chronic condition known as oxidative stress. While short-term oxidative stress harms macromolecules and aerobic respiration, long term oxidative stress can lead to DNA damage through genetic mutations, oxidative damage, and DNA strand breaks. Such genetic changes can lead to variances in protein formation and function, eventually causing cancer, immune diseases, and neurodegenerative disorders (4). One method in which ROS aid in the formation of cancerous tumors is through mutating oncogenes that cause tumorigenesis. Most widely, damage associated with ROS has been seen in substitutions in guanine and cytosine pairs. Evidence showing the role ROS plays in carcinogenesis was seen in human tumor cells that contained multiple modified bases and had an increased amount of H_2O_2 within cells (5). Additionally, ROS reduce immunity and enhance the formation of viruses. For example, ROS prolongs hepatic cell life, enhancing hepatitis C virus (HCV) RNA formation. Evidence has been found where high levels of H_2O_2 and low levels of glutathione (an antioxidant) were found to be present in the plasma of HIV-infected individuals (5). Finally, through lipid and protein oxidation, ROS have been associated with neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (ALS). In fact, oxidation causes around 10^4 DNA mutations per day in humans (6). Furthermore, in susceptible areas of the brain, high levels of ROS cause apoptosis and necrosis that leads to neuron death and neurodegenerative disease (5).

Research demonstrates that antioxidants combat oxidative stress by neutralizing free radicals and ROS. Specifically, antioxidants act as "free radical scavengers" by quickly filling in the ROS valence shell spaces before any other particles can be oxidized (3,4). While the human body produces antioxidants naturally through a defense system containing superoxide dismutase, catalase, and glutathione peroxidase, antioxidant production can be insufficient for combating abundant free radicals (1). Additionally, antioxidant production by the body decreases with age (7). While Vitamin C, Vitamin E, and N-acetyl cysteine (NAC) are all beneficial antioxidants, glutathione, which is an antioxidant that is responsible for

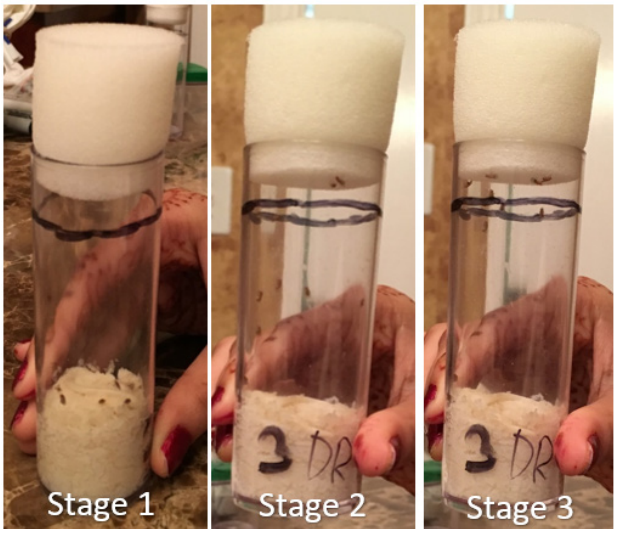

Figure 1. Demonstration of the Climbing Assay. Shown over three stages to demonstrate the upward movement of flies over the 30 seconds.

signal transduction, monitoring immune responses, and regulating cell proliferation, is the most common and beneficial antioxidant to the body (8,9). In particular, asparagus is the highest plant-based source of glutathione containing 28.3 mg of glutathione per gram. Avocado and spinach contain the next two highest contents of glutathione, containing 27.7 mg per gram and 11.4 mg per gram, respectively (10). Since ROS leads to DNA damage, asparagus and avocado should effectively reduce ROS presence along with associated DNA damage, as well.

The purpose of our study was to observe the effects of antioxidants on the climbing abilities of fruit flies exposed to dental resin, an ROS-releasing compound (11,12). We hypothesized that if fruit flies exposed to dental resin are fed antioxidant-containing foods, such as asparagus and avocado, then the flies will demonstrate better climbing abilities compared to fruit flies exposed only to dental resin. This research will hopefully provide people with an accessible method for limiting ROS damage and preventing chronic diseases.

RESULTS

To assess the effects of dental resin, asparagus, and avocado on fruit flies, we assessed the climbing abilities of flies exposed to four different media: regular fly medium, medium containing dental resin (a ROS-releasing component), medium containing dental resin and asparagus, and medium containing dental resin and avocado. For each group, we set up six vials, each with five male and five female flies and measured the percentage of flies that passed the climbing line (6 cm) in 30 seconds (**Figure 1**). We conducted three trials per vial every two days for six days (Day 2, 4, and 6).Before we analyzed the data, we removed outliers that did not fit into the dataset (**Table 1**). We collected data on all four test groups on three different days. We collected data from 3 different trials for each of the 24 test vials on Day 2, Day 4, and Day 6, respectively (**Table 2**). Using this raw data, we calculated the means, standard deviations, and 95% Confidence Intervals. On Day 2, the dental resin, asparagus, and avocado groups all received lower scores compared to the control group. Additionally, the dental resin group performed significantly worse than the control group (p = 0.007), showing the negative impact that dental resin was starting to have on the flies. Both asparagus and avocado received very low scores on Day 2, potentially due to the flies adjusting to their new environment (**Figure 2**). On Day 4, the control group performed significantly better than the dental resin group (p = 0.037), demonstrating that the dental resin was continuing to have a negative impact on the flies. While the scores in the asparagus group did increase between Day 2 and Day 4 from 52.8% to 85.6%, its scores were not significantly different from those of the dental resin group scores (p = 0.99) (**Figure 3**). On Day 6, the control group once again performed significantly better than the dental resin group ($p = 1.3 \times 10^{-5}$) showing that the dental resin's negative impact on the flies continued over the span of the experiment. Although the scores in the asparagus group increased from 85.6% to 88.2% between Day 4 and Day 6 and the dental resin group scores decreased from 85.6% to 82.0% in the same time period, the two groups were still not significantly different on Day 6 (p = 0.14). Similarly, the control and asparagus groups also achieved relatively close scores on Day 6 and were not significantly different from each other (p = 0.14). Therefore, both the control group and asparagus group can be considered equal to each other in performance and in impact on the flies (**Figure 4**).

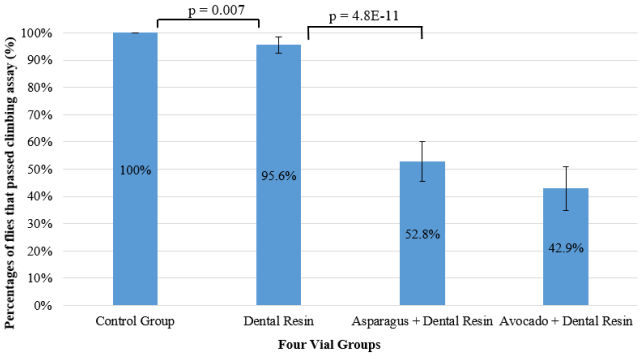

Figure 2. Average fruit fly climbing percentages (%) on Day 2. The error bars were set at the 95% Confidence Interval (as shown in Table 2) and p-values were calculated using two-sample t-tests of unequal variance.

Furthermore, through a line graph we analyzed the average climbing assay data over the span of the entire six days

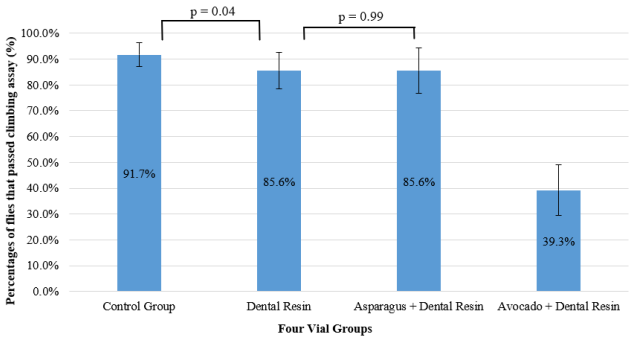

Figure 3. Average fruit fly climbing percentages (%) on Day 4. The error bars were set at the 95% Confidence Interval (as shown in Table 2) and p-values were calculated using two-sample t-tests of unequal variance.

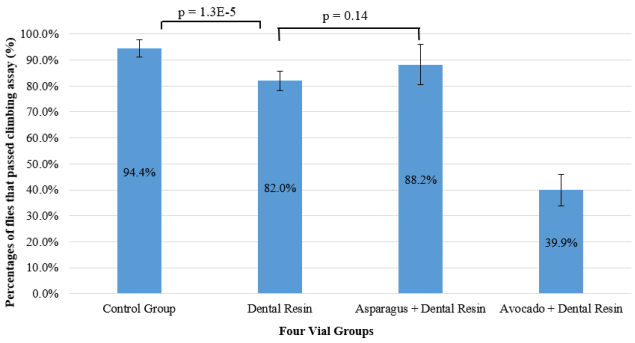

Figure 4. Average fruit fly climbing percentages (%) on Day 6. The error bars were set at the 95% Confidence Interval (as shown in Table 2) and *p*-values were calculated using two-sample *t*-tests of unequal variance.

for each of the four test groups. Throughout the course of six days, the control group's performance remained relatively stable. On the other hand, the dental resin group scores continuously decreased over Day 2, Day 4, and Day 6, demonstrating its negative impact on the flies. Contrary to the dental resin group, the scores in the asparagus group continuously increased over the six-day timespan demonstrating its growing beneficial impact on the flies. The scores in the avocado group remained very low over the six days. Therefore, it made it difficult to collect climbing assay data leading to very low scores in the avocado group (**Figure 5**).

DISCUSSION

In this study, we predicted that if fruit flies exposed to dental resin are fed antioxidants found in asparagus and avocado, then they will demonstrate better climbing abilities than fruit flies exposed only to dental resin. Our results showed that if Drosophila fruit flies exposed to dental resin are fed asparagus, then the climbing abilities of the flies are not significantly different from those of flies exposed only to dental resin. As shown in the Day 6 data, the dental resin group scores were significantly lower than the control group scores, indicating that dental resin has a negative impact on the flies. This could potentially suggest that dental resin released ROS, affecting the flies; however, a limitation of this study was our inability to determine whether ROS was truly released. Additionally, the statistical test demonstrated that the flies in the asparagus group did not perform significantly better than the flies in the dental resin group. This may be because the study was underpowered and the sample size was not high enough to see real differences. Being unable to confirm whether or not asparagus did release antioxidants is another limitation of this study. On the other hand, according to our data, if fruit flies exposed to dental resin are fed avocado, then the climbing abilities of the flies will worsen. This result is likely due to the sticky consistency of the avocado itself, which stuck to the feet of the flies and disrupted the fruit flies' climbing abilities, rather than the avocado's antioxidant properties.

When monitoring the changes in climbing assay performance over the span of the six days, we noted several observations. For example, the control group scores started off high on Day 2, decreased on Day 4, and climbed a little higher on Day 6. The reason for this is likely because the flies started

Table 1. Outliers Calculation Table.

	Q1	Q3	IQR	1.5*IQR	Lower Boundary (Q1-(1.5*IQR))	Upper Boundary (Q3+(1.5*IQR))	Outliers
Day 2 - Control Group	100%	100%	0%	0%	100%	100%	90%
Day 2 - Dental Resin	90%	100%	10%	15%	75%	115%	None
Day 2 - Asparagus	40%	60%	20%	30%	10%	90%	None
Day 2 - Avocado	33%	50%	17%	25%	8%	75%	None
Day 4 - Control Group	88%	100%	13%	19%	69%	119%	None
Day 4 - Dental Resin	80%	100%	20%	30%	50%	130%	40%
Day 4 - Asparagus	80%	100%	20%	30%	50%	130%	None
Day 4 - Avocado	22%	50%	28%	42%	-19%	92%	None
Day 6 - Control Group	90%	100%	10%	15%	75%	115%	70%
Day 6 - Dental Resin	70%	90%	20%	30%	40%	120%	10%
Day 6 - Asparagus	80%	100%	20%	30%	50%	130%	35%
Day 6 - Avocado	30%	44%	14%	22%	8%	66%	None

Table 1. Outliers Calculation Table. Method by which outliers were calculated for all four groups on Day 2, Day 4, and Day 6. Outliers were not included when statistics were calculated.

Table 2. Average Climbing Assay Results from Day 2, Day 4, and Day 6.

	Control Group	Dental Resin	Asparagus	Avocado
Day 2	100.0%±0.0 (100.0, 100.0)	95.6%±6.2 (92.5, 98.6)	52.8%±14.5 (45.6, 60.0)	42.9%±16.1 (34.9, 50.9)
Day 4	91.7%±9.2 (87.1, 96.3)	85.6%±13.1 (78.6, 92.6)	85.6%±17.6 (76.8, 94.3)	39.3%±19.7 (29.5, 49.0)
Day 6	94.4%±6.3 (91.0, 97.7)	82.0%±6.8 (78.3, 85.7)	88.2%±15.1 (80.5, 96.0)	39.9%±11.9 (34.0, 45.8)

Table 2. Average climbing assay percentages (%) from Day 2, Day 4, and Day 6. Climbing assay scores for the control group, dental resin, asparagus, and avocado groups over the span of six days. Values following the plus or minus represent the standard deviation and values in the parentheses represent the 95% Confidence Interval.

off very energized within their new environment, but slowly started to grow used to the environment and became less willing to climb during the climbing assay tests. Similarly, the dental resin group scores continuously decreased between Day 2, Day 4, and Day 6. One reason for this may be that the dental resin's impact on the flies could have gradually built up over the six days, causing more harm to the flies over time. Additionally, many of the flies died along the way, potentially due to the dental resin and ROS, decreasing the total count of flies in each vial and possibly impacting the climbing assay percentages. On the other hand, the percentages in the asparagus group continuously increased over the six days. This shows that the asparagus's impact on the flies improves over time and could have potentially continued to increase if the experiment had been conducted for a longer period of time. Finally, the scores in the avocado group all seemed to be around the same range throughout the six days, since the avocado hindered the flies' abilities to climb due to the sticky consistency.

In this experiment, we performed a variation of the Rapid Iterative Negative Geotaxis (RING) Protocol utilized by many scientists. Since past research shows that ROS causes DNA damage, we predicted that observing the flies' behavior through an assay would indicate whether or not damage had occurred. In order to observe differences in the flies' behavior, we had the option of conducting a larval crawling assay, the RING Protocol, or a Courtship and Mating Assay. Out of these three, we decided the RING Protocol was the most relevant for observing behavioral and physical differences of

fully grown flies. The standard RING protocol is conducted by measuring the height each individual fly has traveled after a three-second climbing period through the use of a camera capturing each fly's final position (13). Our climbing assay was based on this method; however, due to the flies moving very slowly, the assay was conducted for 30 seconds, and rather than measuring each individual fly's height, the total percentage of flies that crossed a 6 cm line was recorded. Additionally, unlike in the RING protocol where climbing assays are conducted in separate vials, in our experiment, due to the lack of time and resources, the climbing assay was conducted within the fly's living environment. These differences between our climbing assay and the RING Protocol may have caused variation in the results.

During experimentation, we observed few known errors that may have affected our results. One known error is that on Day 2 of testing the asparagus and avocado groups, we conducted the climbing assays in a separate climbing assay vial. However, we conducted all other climbing assays besides these two groups in the original vial (in which the food medium was located). While this may have caused slight variation in the results, variations were minimized by maintaining an approximate six cm height that the flies had to climb no matter the vial that tests were conducted in. Another known error is that the consistency of the avocado paste that was mixed into the avocado vials along with the Instant Food Medium was too thick and sticky. This caused variations in the results of the avocado group since many of the flies had paste stuck to their legs, making it difficult to climb up the sides of the vials. Due to the sticky paste, quite a few flies died, reducing the total number of flies in many of the avocado group vials to below ten. Two other known errors are that we accidentally retested one vial on Day 2 for the asparagus group, and we did not include yeast in the food media of the asparagus and avocado groups. Additionally, one limitation of this study was that we could not analyze direct release of ROS and the direct impact of asparagus on ROS, but rather we assessed the indirect effects via the climbing abilities of the fruit flies.

If this experiment were to be further tested, we would expand on certain components such as the length of observance (number of days), types of antioxidants, and stimulators of ROS. Additionally, we would assess reproductive ability and offspring growth in addition to climbing ability to determine the effect of antioxidants on the offspring of fruit flies. Also, with the help of lab access, we would measure direct increases and decreases in ROS released by cells and tissues. Some techniques that we could use to measure ROS include cytochrome c reduction (where superoxide is exposed to ferricytochrome c and optical density is measured using a spectrophotometer) and chemiluminescence methods (lucigenin is exposed to superoxide and the release of photons are measured using luminometer or scintillation counter) (14). This deeper experimentation would help confirm the role of antioxidants in reducing DNA damage and other potential uses, as well.

In conclusion, the asparagus group did not receive significantly higher scores than the dental resin group. On the other hand, no conclusions can be made about avocado's antioxidant properties as the consistency of the avocado hindered the flies' capability to climb. Our findings suggest that increasing dietary consumption of antioxidant-containing foods like asparagus could positively benefit humans in reducing ROS

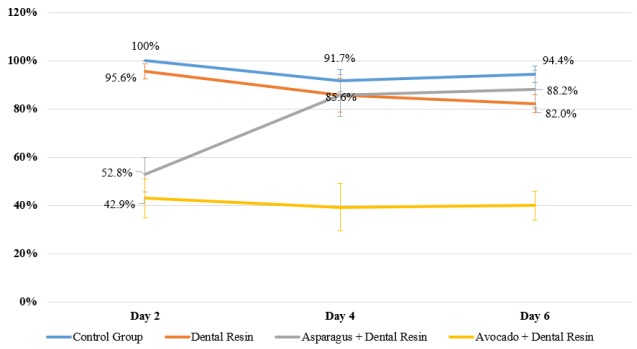

Figure 5. Average climbing assay percentages (%) over the span of six days. Demonstrates average scores of all four groups over the six day time frame. The error bars were set at the 95% Confidence Interval (as shown in Table 2).

levels within their bodies. However, due to the lack of significance in the asparagus results, we would have to conduct further research with larger sample sizes or increased observation time in order to support this prediction. In the long run, this could lead to a reduction in cancer, immune diseases, and neurodegenerative diseases.

METHODS

Preparing the Food Medium

24 Culture Vials and 24 Vial Plugs (Carolina Biological) were separated into groups of 6 (control group, dental resin, asparagus + dental resin, and avocado + dental resin).

Control Group and Dental Resin Vials

First, 14 g of the Formula 4-24 Instant Drosophila Medium (Carolina Biological) and 5-8 grains of yeast were added to each of the 24 vials. Then, two Esthet X HD 0.25 g Compules Tips were injected into each "DR", "AS", and "AV" vial using the Esthet X HD Compules Tips Gun. Next, 15 mL of water were added into each "CG" and "DR" vial.

Asparagus and Avocado Vials

While the vials were settling, three asparagus were cut up into small pieces (around 20 pieces each). These pieces were then placed in the mixer along with 240mL of water. After grinding for around 15 seconds, an asparagus paste with a liquid consistency had formed. Then, the asparagus paste was poured through a tea strainer. To collect the asparagus liquid, another bowl was placed underneath the tea strainer. This bowl was set aside and labeled "Asparagus Paste", and the mixer, tea strainer, and spoon were cleaned for their next use. Next, one avocado was cut into two halves and the large seed was removed. The soft inside of the avocado was scooped out and placed in the mixer. After adding 240 mL of water to the mixer, the paste was ground for around 15 seconds to form an avocado paste with a liquid consistency. Similar to the asparagus paste, the avocado paste was also poured through a tea strainer and collected in a separate bowl labeled "Avocado Paste." In each "AS" vial, 15 mL of the Asparagus paste was poured in. The vial was slightly mixed using the glass mixing stick. Once mixed, the dental resin was carefully lifted up using the mixing stick so that it could be seen at the top of the food medium. Finally, the sides of the vial were cleaned using a cotton swab so that no remnants remained. Similarly, this

was repeated for the "Avocado Paste" and "AV" vials.
Once the food media were complete, all vials in each group were numbered 1-6.

Live Transfer

Next, a live transfer was conducted in order to add Drosophila fruit flies to each of the fruit fly food media. First, a small funnel was placed in one on the test tubes and the plug of one Drosophila fruit fly vial (Carolina Biological) was slightly opened. In order to conduct the live transfer, the vial plug was quickly removed and the vial was turned upside-down and positioned at the top of the funnel. The vial was continuously tapped down until almost all of the flies were in the bottom test tube. Quickly afterward, the vial was turned back around, the funnel removed, and the cap of the test tube closed. This test tube containing flies was then placed in the refrigerator for 10 minutes (in order to temporarily sedate the flies). These steps were repeated for all Drosophila culture vials until all of the flies were inactive.

Sorting and Adding Flies

Next, two bowls each filled halfway with water were gathered. Five to seven ice cubes were placed in each bowl. The top half of one petri dish was placed in one bowl's water, and the bottom half of the petri dish on the other bowl's water. Then, two test tubes containing sedated flies were poured into one petri dish. Similarly, the flies in two other test tubes were poured into the other petri dish. It was made sure that the flies did not touch the water as they would have died. Using the paint brush, the male flies were carefully separated from female flies in both petri dishes. The male flies were identified by their dark abdomens and smaller bodies, while female flies were identified by their pointed abdomens and larger bodies. Then, using the paint brush, 5 male fruit flies and 5 female fruit flies were placed into each of the "CG" vials and "DR" vials. It was made sure that the flies did not fall into the food when they were asleep due to the risk of them dying. After placing the fruit flies in each vial, the vial plugs were put in place and the vials were positioned on their sides until the flies awakened. This entire procedure was repeated for the four other test tubes containing sedated flies using the "AS" and "AV" vials. In the end, there were a total of 240 fruit flies in the vials. All vials were placed in a dark room in order to prevent the dental resin from hardening.

Using the Climbing Assay

The climbing assay was used to gather data on the climbing abilities of the Drosophila fruit flies. First, a bold line was marked 9 cm up the vial (3 cm food medium, 6 cm climbing assay). Then, the vial was carefully tapped so that all the fruit flies moved downwards. As soon as most of the flies had reached the bottom, the stopwatch was quickly started. As the fruit flies moved upward (6 cm), the number of fruit flies that cross the marked line was counted. After thirty seconds, the watch was stopped, and the number of flies that crossed the line was recorded. This process was repeated three times for each of the twenty-four vials. Additionally, this was performed every two days for six days (Day 2, Day 4, Day 6).

Disposal

For disposal, all the vials were taken outside. Each vial was unplugged and the flies were released. The remaining food including dental resin from all vials was poured out into a plastic bag. Finally, the remnants of food were washed out with water.

Analysis

Before analyzing the data, outliers, very large or very small numbers that did not fit into the dataset, were removed. First, using the raw data, Quarter 1(Q1), Quarter 3(Q3), and the Inner Quartile Range (IQR - difference between Q3 and Q1) were calculated. Then 1.5 × IQR was subtracted from Q1 to calculate the lower boundary and 1.5 × IQR was added to Q3 to create the upper boundary. Any numbers that fell outside of these boundaries were considered outliers and were not considered when calculating means or stats (**Table 1**).

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