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The Effect of Amyloid Beta on Symptoms Caused by Traumatic Brain Injury in *Drosophila melanogaster*

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ABSTRACT

Traumatic brain injury (TBI) is the cause of one third of all injury-related deaths in the United States alone, and approximately 1.7 million people sustain a TBI each year. Depending on the severity of the, a TBI could lead to sustained damage to cognitive and locomotive abilities. Treatments for TBI have been previously investigated; however, prior research does not include the investigation of amyloid beta protein (Aβ), a protein found to accumulate rapidly after TBI, as having a role in recovery from TBI. This experiment focused on studying the effect of the presence of Aβ on cognitive and locomotor ability in *Drosophila melanogaster* after TBI. The cognitive and locomotor skills of D. melanogaster were tested through the means of a food-based choice assay and a climbing assay respectively. After data collection, Mann Whitney statistical tests were done between *D. melanogaster* groups to determine the significance of the results. From the statistical analysis of the results, the following was derived: the presence of Aβ is related to improved locomotion in *D. melanogaster* after TBI according to a significant difference in assay success rates between *D. melanogaster* with and without Aβ (p-value < 0.0001), however there was no demonstrated relation between Aβ and improved cognition (p-value = 0.0663). Therefore, the hypothesis regarding the possible neural repair properties of Aβ is partially supported. It can be concluded that while Aβ had no effect on cognition in *D. melanogaster* after TBI, its presence is directly related to the improvement of locomotion.

1 | Introduction

1.0 Traumatic Brain Injury

One third of all injury-related deaths in the United States are a result of traumatic brain injury, as depicted in Figure 1 (Centers for Disease Control and Prevention, 2022). Traumatic brain injury (TBI) is a form of injury that results in damage to the brain and is most often caused by impact on the head. Brain injury severity varies, as TBI cases can be identified as mild, moderate, or severe. Concussions are often considered as mild forms of TBI, so most patients with concussions fully recover. However, severe cases of TBI can lead to coma or death (National Library of Medicine, 2020). Both physical and psychological symptoms can develop as a result of TBI. Such symptoms may include a poor cognitive state, characterized by symptoms such as a depleted memory, poor decision-making, and a weakened ability to control emotions.

Figure 1. Current Prevelance of Traumatic Brain Injuries in the United States.

Specifically for severe cases of TBI, one significant symptom may be a poor physical state characterized by weak motor function, lessened limb strength, and damaged coordination. For mild TBI, recovery mainly includes rest. Severe TBI may require specific interventions, including surgeries to remove clotted blood within the brain, remove damaged tissue, or repair skull fractures to relieve pressure in the skull. Additionally, a TBI patient may need to take anticoagulants to prevent blood clots, anticonvulsants to prevent seizures, or muscle relaxants to reduce muscle spasms. The medications are dependent upon the type of symptoms the patient experiences. Finally, forms of therapy may also be required, which may include physical therapy to build physical strength, psychological therapy to help the patient learn coping skills, and cognitive therapy to improve memory, perception, and judgment (National Library of Medicine, 2020). According to a study done by Brett et. al., TBI has also been named a cause for the rapid accumulation of amyloid beta protein (Aβ) in the brain (Brett et. al., 2022).

Amyloid Beta Protein

Aβ is produced by the amyloid precursor protein (APP), which is a large membrane protein that is in the neural system and specializes in neural growth and repair. Aβ is often villainized for its notorious role in the development of Alzheimer's Disease (AD). The upregulation of Aβ, which leads to aggregations, or plaques, that are mostly insoluble within the neural system, plays a crucial role in the development of AD (Ashrafian et. al., 2021). While Aβ has been researched as a cause of AD, its function as a protein has yet to be thoroughly investigated. Knowing that Aβ is a derivative of protein known for its role in neural growth and that, according to Smith et. al., it is produced rapidly in the brain after severe head injury, it is possible that the true purpose of Aβ is to help the brain recover after injury instead of being a negative consequence.

The Drosophila melanogaster Model

Research on the status of TBI and Aβ in the neural system has been done utilizing *Drosophila melanogaster*. More commonly referred to as fruit flies, *D. melanogaster* models are often used in scientific experimentation and research as they share many genes and neurons with human brains. In fact, nearly 75% of genes found in humans are also found in fruit flies, which makes flies a great model for studying human traits (Pfizer, 2022). Flies are inexpensive to maintain and can produce great amounts of progeny in a short amount of time for experimentation. Although the brain of *D. melanogaster* does contain fewer neurons compared to that of a human brain, it is complex enough to perform a range of behaviors, including learning and navigation (Scheffer et. al., 2020). Thus, fruit flies are a fitting model for representing how humans may react to certain experiments that may impact cognitive and physical skills.

D. melanogaster can also be engineered to express certain proteins that humans contain, which allows for Aβ to be produced within a laboratory setting. A process known as the GAL4/UAS system is one of the methods used to create fruit flies with certain inducible proteins and genes. The GAL4/UAS system has demonstrated over the past two decades that it is an essential tool in managing gene expression in animal models, which include *D. melanogaster* (di Pietro et. al., 2021). GAL4 is a transcriptional activator that binds to UAS enhancer sequences. The GAL4 activator then recruits transcription machinery in order to induce downstream gene expression. A lot of UAS enhancer sequences are composed of heat shock proteins, meaning that the crosses of the GAL4 and the UAS parent flies produce progeny that are able to express the chosen gene through heat shock. A cross between flies with the GAL4 transcriptional activator gene for Aβ and flies with heat shock protein UAS enhancer sequences would create progeny that express Aβ from heat shock. This GAL4/UAS model has been used previously in a study by Prüßing et. al., which concluded that this GAL4/UAS model is a reliable and valuable *in vivo* model of the effects of Aβ (Prüßing et. al., 2014).

In addition to Aβ, D. melanogaster has also been investigated for its reliability when modeling injury. One study conducted by Putnam et. al. focused on analyzing TBI in *D. melanogaster*. Fruit flies have been deemed a good model for these experiments since TBI impacts cognitive and physical function, which fruit flies model in a similar manner to humans. The study conducted by Putnam et. al. focused on the development of methodology for inducing TBI in flies to record their symptoms after injury (Putnam et. al., 2019). The Putnam study mainly focused on an overview of apparatuses for TBI induction in *D. melanogaster*. One method for inducing TBI involved using a centrifuge to spin fruit flies around at fast speeds, resulting in injury. This method utilized minimal material and could be carried out in approximately two minutes, making it an efficient model for inducing TBI.

The Role of Amyloid Beta Protein

A current problem in the field of present research involves the lack of knowledge surrounding the role of Aβ. As AD is a devastating disease that draws a lot of attention in hopes of finding treatments and causes, most knowledge surrounding Aβ has only to do with such. It is necessary to understand the function of Aβ so that the levels of it in the brain are not suppressed without proper knowledge of their roles within the neural system, for it is possible that tasks meant to be fulfilled by Aβ go unfulfilled because of artificial suppression. By gaining knowledge of the Aβ protein through research rather than focusing solely on its connection to AD, more can be learned about what its intended functions are and how those functions can be carried out without the development of AD. Additionally, new research involving Aβ, a product of APP, could lead to discoveries in terms of Aβ's neuroprotective differences or similarities with APP.

Is the role of amyloid beta protein to repair damage and improve symptoms, including those related to cognition and locomotion, after traumatic brain injury*?* To conduct this research, the relationship between TBI and Aβ was analyzed. *D. melanogaster* has been used prior to model both Aβ and TBI, and it was utilized for this research. The hypothesis formed for this research was that if there is an increased amount of Aβ in the neural system of *D. melanogaster*, then both cognitive and locomotive symptoms caused by TBI will be less severe because of Aβ's hypothesized neural repair properties, which stem from the rapid accumulation of Aβ after brain injury, suggesting that Aβ after TBI could be combating damage to the brain, and the fact that Aβ is a derivative of APP, a known neuroprotector (Dar & Glazner, 2020).

The independent variables for this research are the presence of Aβ and TBI in *D. melanogaster.* There will be three control groups of flies in the experiment: flies that receive only Aβ, referred to as the genetic control, flies that receive only TBI, referred to as the untreated control, and normal flies that do not receive Aβ or TBI, referred to as the negative control. The experimental group will be flies that are given TBI and receive Aβ. The dependent variables are both the physical and cognitive state of *Drosophila*, which will be measured through a climbing assay and a food-based choice assay. For the climbing assay, flies will be placed in a vial and they will be tested on how they respond to tapping on the glass. Normal flies should respond by climbing against the force of gravity, as that is their natural response to agitating stimuli. If the flies do not do so, then their response will be labeled as a sign of poor locomotive skills. For the food-based choice assay, flies will be supplied with a sucrose solution and an arabinose solution to test their decision-making skills in terms of nutrition. Both solutions will be introduced to the flies prior to the test. The sucrose solution is metabolizable, and therefore the ideal choice. If the flies choose to ingest the arabinose solution, their choice will be labeled as a sign of poor decision-making skills (Yu et. al., 2021).

TBI has been researched in terms of the causes and new forms treatments for systems. However, such research never included the possibility of Aβ serving as a form of therapy for TBI, which would be the main novel aspect of this new research. If Aβ shares the same properties as APP, then it is possible that it could contribute towards treatment for TBI and other forms of injury.

2 | Methods

2.0 Materials

The fly stock expressing heat shock inducible GAL4 (stock #1799) and fly stock producing Aβ under the control of UAS (stock #33769) were both obtained from the Bloomington *Drosophila* Stock Center. The red and blue food dye was purchased from Amazon.com. All other materials for the maintenance and disposal of fly stocks, food preparation, tapping, cold sorting, CO_2 sorting, and assay procedures were obtained from the science labs at the Academy of Science (AOS).

2.1 Maintenance and Disposal Procedures

All flies were housed in plastic vials, which were covered with foam flugs. These vials were stored at a temperature of 22°C and monitored regularly to ensure that the vials did not become overpopulated. For fly stocks being expanded, flies were transferred to different vials every 4 days using the tapping method (refer to Section 2.3). If a stock was being maintained, flies were transferred to different vials every 3 weeks. If a vial of flies had to be disposed of, the vial was placed in a -20°C freezer for a minimum of an hour, and then the vial was discarded in the trash.

2.2 *Drosophila* Food Preparation Procedure

Using a scoopula, a weight boat, an electronic weighing scale, 6.75 grams of yeast, 3.9 grams of soy flour, 28.5 grams of yellow cornmeal, and 2.25 grams of agar were weighed out and emptied into a 1000 mL beaker. Next, 30 mL of light corn syrup and 390 mL of water were respectively measured out using a 100 mL graduated cylinder and poured into the same 1000 mL beaker. The fluid and dry ingredients were combined in the beaker through thoroughly mixing the contents using a glass stirring rod. Once the mixture was smooth, the beaker containing the mixture was moved to the microwave, where it was heated for 30 second increments until the contents were bubbling; in between these increments, the mixture was stirred using the glass stirring rod to ensure that all contents remained combined.

Once the mixture was boiling, the beaker was removed from the microwave, covered with a cheesecloth, and had a weighted object on top of the cloth to prevent any form of contamination. A thermometer was used to measure the temperature of the mixture; once the temperature was between 60°C and 70°C, 1.88 mL (1880 microliters) of 10% propionic acid was added to the beaker using a micropipette and stirred in with the glass stirring rod (the acid inhibits fungal growth in the food). After having completely stirred the propionic acid into the food mixture, the food was poured into individual vials; in each vial, the food had an approximate height of 1 inch. The cheese cloth was then placed over all the vials, and the weighted object was placed on top to secure the cloth. The food was left to cool until it reached room temperature at around 22°C, where it solidified. Once this point had been reached, the vials were covered with flugs.

2.3 Tapping *Drosophila* Procedure

The tapping procedure consists of transferring *Drosophila* from one vial to another and was always performed on a tabletop. To begin this procedure, a new vial for the flies to be transferred into was held in the nondominant hand, and the old vial was held in the dominant. Once this had been set up, the empty vial was positioned over the vial with the *Drosophila*. Next, the vial with *Drosophila* was tapped on the table and its flug was pulled out soon after, followed by the empty vial being quickly placed over the uncovered vial. The assembly was then turned over and tapped against the table to transfer the flies from the old vial into the new vial. To successfully disassemble this apparatus without any flies escaping, the old vial was quickly removed from the assembly and a flug was placed on the new vial. The old vial was kept upside down so that any flies that were not successfully transferred into the new vial would not be

inclined to fly out of the old vial, seeing as flies naturally demonstrate a negative geotaxis. This procedure was repeated every four days when a stock was being expanded but was only repeated every three weeks if a stock was being maintained.

2.4 Cold Sorting Procedure

Flies were sorted by sex using the cold sorting method. To prepare for sorting, flies were tapped into an empty vial (refer to Section 2.3) and then anesthetized by being placed in a bucket of ice for approximately 2 minutes. The cold plate in use was set to a temperature of 2°C and a piece of weight paper was placed on top of the plate. Flies were then taken from the bucket of ice and sorted on the cold plate using a magnifying glass and a sorting feather. After sorting, female flies were placed in an empty vial on its side, and then transferred to a vial with food once they had woken up. Male flies were disposed of (refer to Section 2.1).

2.5 CO_2 Sorting Procedure

Virgins were identified from groups of *Drosophila* through the CO_2 sorting procedure. First, the CO_2 tank was turned on, allowing CO_2 to flow to the sorting pad and the CO_2 needle gun. The flies meant to be sorted were first anesthetized using the CO_2 gun, and then the flies were moved to the sorting pad. Using a sorting feather and a microscope, the flies were sorted. Virgin flies were collected and transferred to a vial. Flies that were not virgins were disposed of (Section 2.1).

2.6 GAL4/UAS Scheme

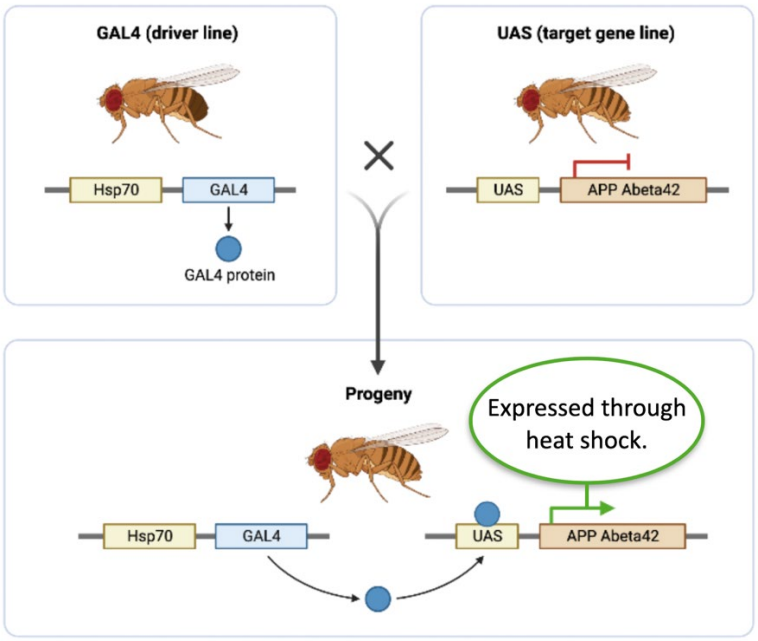

Figure 2. GAL4/UAS Cross Scheme. A cross was made between virgin female flies that produced Aβ under the control of the UAS and males that expressed heat shock inducible GAL4. From this cross, progeny with heat shock inducible Aβ were created.

The parent flies from this cross were housed in the same vial and were transferred to a new vial every 3 to 4 days, and the old vials were stored. On the 14th day since the cross was started, the adult fly progeny from the cross were collected for experimentation from the old vials. The progeny from this cross was used for testing for the genetic control and experimental group. On the 16th day since the cross was started, all flies yet to be collected for experimentation were disposed of (refer to Section 2.1).

2.7 Inducing TBI

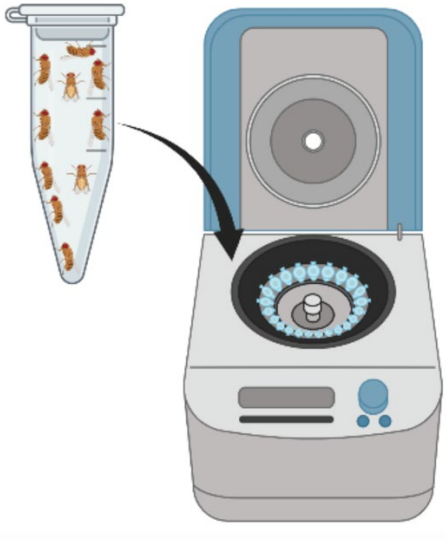

Figure 3. Apparatus for TBI Induction. Flies were first anesthetized by placing them in a bucket of ice for 1 to 2 minutes and were then transferred to 2mL microcentrifuge tubes. 10 flies were placed in a microcentrifuge tube at a time. The microcentrifuge tubes were then placed in the centrifuge. For 2 minutes, the tubes spun around in the centrifuge at a speed of 2000 rpm.

After flies received brain trauma, they were placed in vials that were placed on their side. Flies were allowed a 48-hour recovery period before any tests were performed. This procedure was carried out for flies that were a part of the untreated control and the experimental group.

2.8 Heat Shocking *Drosophila*

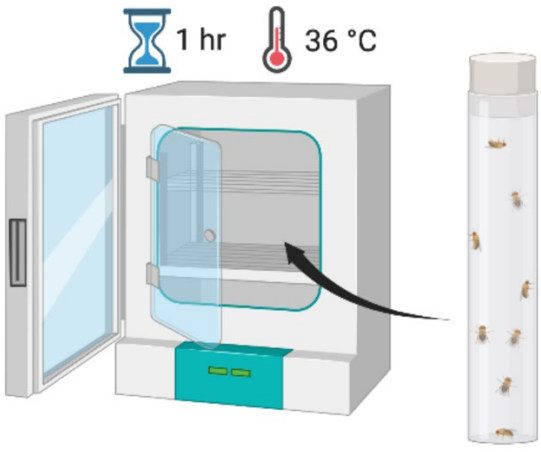

Figure 4. Apparatus for Heat Shock. 4 days after progeny from the GAL4/UAS cross (refer to Section 2.6) had been collected, the progeny was exposed to heat shock to induce Aβ. Flies were first transferred into empty vials using the tapping procedure (refer to Section 2.3) and then placed in an incubator. The incubator was set to a temperature of 36℃ and flies were left in the incubator for approximately 1 hour.

2.9 Climbing Assay, Food Based Choice Assay, & Statistical Analyses

A climbing assay and a food-based choice assay were conducted for every group of *Drosophila* in this experiment to assess cognitive and locomotive ability. The pass rates for each trial of the climbing assay and the food-based choice assay were calculated using Equation 1 and Equation 2, respectfully.

Figure 5. Apparatus for Climbing Assay. The climbing apparatus consisted of two vials taped together, with an 8-centimeter height mark on the side. 10 flies were exposed to agitation in the form of tapping on the side of the vial and were expected to respond to said agitation in the form of climbing up the apparatus. 10 seconds were allotted to determine if the flies responded normally (by climbing past 8 centimeters) or abnormally (by failing to climb past 8 centimeters).

Equation 1: Pass Rate Percentage for Climbing Assay

$$\frac{\#\ of\ flies\ that\ passed\ the\ 8\ cm\ mark}{\#\ of\ flies\ tested} \cdot 100\%$$

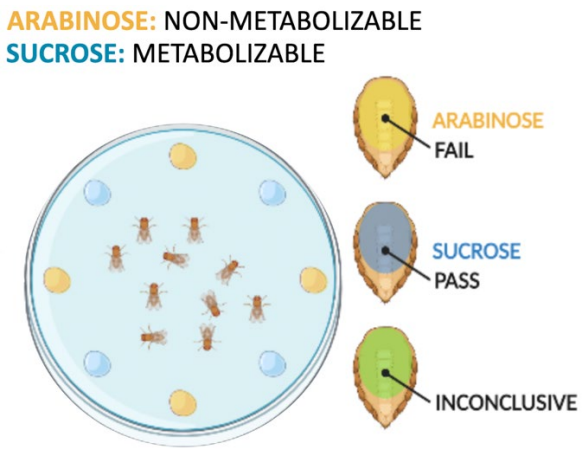

Figure 6. Apparatus for Food-Based Choice Assay. Flies were starved for 24 hours before this test. For the test, 10 flies were placed in a 10-centimeter petri dish with 4 drops (20 microliters per drop) of each solution along the perimeter. The arabinose was colored yellow, and the sucrose was colored blue. Flies were left in the apparatus for 2 hours and were then immediately anesthetized with CO_2 and observed under a microscope. The solution ingested by a fly was determined by the color of its abdomen. If an abdomen color was indistinguishable or green, the result was labeled as "inconclusive".

Equation 2: Pass Rate Percentage for Food-Based Choice Assay

$$\frac{\#\ of\ flies\ that\ ingested\ the\ sucrose}{\#\ of\ flies\ tested} \cdot 100\%$$

Mann-Whitney U tests were used to determine statistical significance between *Drosophila* group results from the climbing and food-based choice assays.

3 | Data & Results

Table 1. Pass Rates of the Climbing Assay for Each *Drosophila* Group.

Trial	Pass Rate (%)			
	Negative Control	Genetic Control	Untreated Control	Experimental Group
1	86	82	30	66
2	88	80	38	64
3	70	90	34	66
4	76	66	32	72
5	84	72	38	68
6	82	78	22	60
7	86	78	40	66
8	82	78	32	62
9	80	88	40	82
10	84	74	30	66
Mean (%)	**81.8**	**78.6**	**33.6**	**67.2**

Table 2. Pass Rates of the Food-Based Choice Assay for Each *Drosophila* Group.

Trial	Pass Rate (%)			
	Negative Control	Genetic Control	Untreated Control	Experimental Group
1	70	70	40	60
2	70	50	60	50
3	70	60	30	40
4	60	60	30	60
5	70	70	0	40
Mean (%)	**68.0**	**62.0**	**32.0**	**50.0**

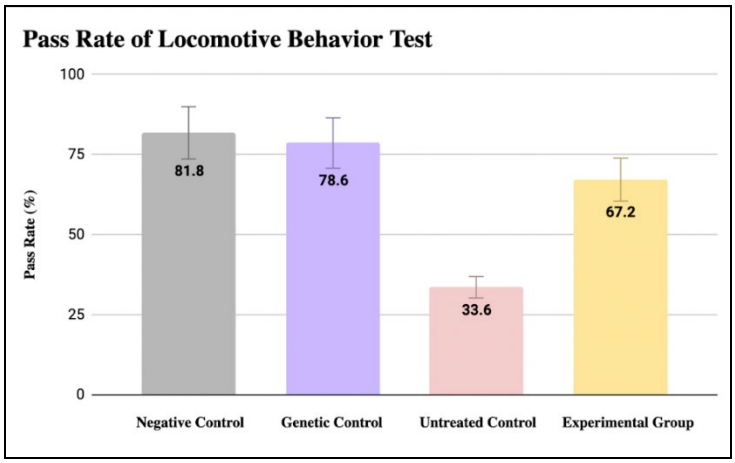

Figure 7. Graphed Mean Pass Rates of the Climbing Assay. This graph includes the mean pass rate from the climbing assay (measured by the y-axis) of each *Drosophila* group from this experiment (labeled the x-axis). As depicted in the graph, the negative control group had the highest mean pass rate, followed by the genetic control group and the experimental group respectively. The untreated control group had the lowest mean pass rate.

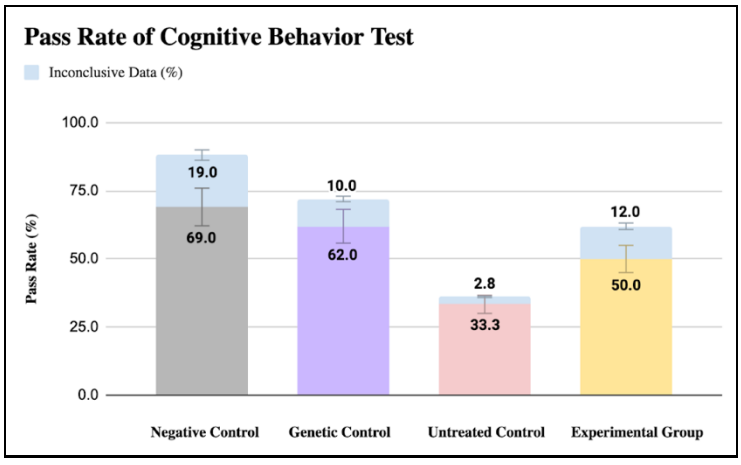

Figure 8. Graphed Mean Pass Rates of the Food-Based Choice Assay. This graph includes the mean pass rate from the food-based choice assay (measured by the y-axis) of each *Drosophila* group from this experiment (labeled the x-axis). As depicted in the graph, the negative control group had the highest mean pass rate, followed by the genetic

control group and the experimental group respectively. The untreated control group had the lowest mean pass rate. This graph also contains the representations of inconclusive results for each *Drosophila* group.

Table 3. Two groups broken down with age ranges and the difference.

Test	***p*-values**[1]
Negative v. Untreated	**<0.0001**
Negative v. Genetic	0.0652
Untreated v. Experimental	**<0.0001**

[1]All *p*-values in boldface are significant, as they are less than the significance level of 0.05.

Table 4. Two groups broken down with age ranges and the difference.

Test	***p*-values**[2]
Negative v. Untreated	**0.0063**
Negative v. Genetic	0.1160
Untreated v. Experimental	0.0663

[2]All *p*-values in boldface are significant, as they are less than the significance level of 0.05.

4 | Discussion

The Mann-Whitney U tests done between the negative control data and untreated control data were done to determine whether the procedure for inducing TBI was successful or not. For both the climbing assay and the food-based choice assay, the Mann-Whitney U test done between the negative control and untreated control yielded significant p-values (refer to Table 3 and Table 4, specifically the "Negative v. Untreated" test section). The significant difference between the medians of the negative control and the untreated control for both the climbing and food-based choice assay supports that the flies that received TBI experienced greatly impaired cognitive and motor performance as a result. Thus, the procedure for inducing TBI was effective.

The Mann-Whitney U tests for the climbing assay and the food-based choice assay also yielded similar results for the tests between the negative control data and the genetic control data. Tests between the negative control data and the genetic controls data were run to test for a significant difference in cognitive and locomotive performance between normal flies and flies with Aβ. The Mann-Whitney tests for climbing assay and the food-based choice assay yielded insignificant p-values of 0.0652 and 0.1160 respectively (refer to Table 3 and Table 4, specifically the "Negative v. Genetic" test section), indicating that the presence of Aβ did not have a significant effect on an uninjured *Drosophila* brain.

For the climbing assay and the food-based choice assay, results from the Mann-Whitney U tests determining the statistical significance of the difference between the medians of the untreated control and the experimental group were different. The Mann Whitney test between the untreated control and the experimental group for the climbing assay yielded a significant p-value of <0.0001, whereas the Mann Whitney test between the same *Drosophila* groups for the food-based choice assay yielded an insignificant p-value of 0.0663 (refer to Table 3 and Table 4, specifically the "Untreated v. Experimental" test section). These statistical results suggest that the presence of Aβ in the *Drosophila* neural system is related to improved locomotion after traumatic brain injury but does not have a significant effect on cognition.

5 | Conclusion

From the results of the experiment, the original hypothesis can be partially accepted: Aβ is related to improved locomotion in *Drosophila* after TBI according to the significant p-values produced by the Mann-Whitney U test between

the untreated control and experimental group for the climbing assay (refer to Discussion), but there was no demonstrated relation between Aβ and improved cognition.

6 | Limitations

Future research could identify the cause for this difference in the relationship between Aβ and locomotor ability after TBI and the relationship between Aβ and cognitive ability after TBI. One independent variable that may be a key part of this future investigation could be the amount of amyloid beta in the *Drosophila* neural system. This experiment only focused on the sole presence of Aβ in the neural system but did not investigate different accumulated amounts of Aβ in *Drosophila* brains in order to determine the effect the accumulated amount has on cognitive and motor ability. Thus, this could be a direction for the future.

7 | Acknowledgments

I would like to thank my mentor, Dr. Jessica Eliason, for all her guidance during the duration of my project. I would also like to thank the Academies of Loudoun, particularly the Academy of Science, for providing me with all the necessary equipment and materials for my experiment.

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