Effects of caffeine on the mitochondrial pathology of the *SdhB*^{EY12081} mutant in *Drosophila melanogaster*

Brianna Pluchino

Amanda Iacometta

Table of Contents

Abstract

Caffeine is a central nervous system (CNS) stimulant that enhances oxidative fat metabolism. This is facilitated by several mechanisms including the regulation of the cyclic adenosine monophosphate protein (cAMP) and protein kinase A (PKA), blocking adenosine receptors, and stimulating ATPase through the Na+/K+ pump. The *SdhB*^{EY12081} strain has a mutation in the gene encoding for succinate dehydrogenase (subunit B of Complex II) of the electron transport chain (ETC) in flies, decreasing motor performance and longevity, and has been used as a genetic model for mitochondrial disorders in humans. In this experiment we tested the hypothesis that caffeine supplementation will alleviate the pathology of *SdhB*^{EY12081} mutants. Our results show that 0.75mg/ml of caffeine does marginally improve climbing ability in the *SdhB*^{EY12081} flies (p-value = 0.050), although not wild-type flies (p-value = 0.328). A caffeine titration was also carried out to determine which concentration was the best at improving climbing without having a toxic effect on the strain. After four days of 0.25mg/ml of caffeine treatment, climbing was improved (p-value = 0.000) without affecting the mortality rate (p-value = 0.128). In a demography study, longevity of the mutated flies was tested using the 0.25mg/ml caffeine concentration. Caffeine-treated food did not improve the overall life span of the *SdhB*^{EY12081} flies when compared to the control food. In fact, caffeine treatment decreased the longevity of the *SdhB*^{EY12081} (p-value = 0.000). Overall, my research showed that caffeine may ameliorate some of the deleterious effects that the *SdhB*^{EY12081} mutation has in the mitochondria involving high-energy activities; however, it has a detrimental effect on the organismal longevity.

Introduction

In eukaryotic organisms, the mitochondria are primarily responsible for the production of the cell's energy currency, adenosine triphosphate (ATP), through various metabolic processes (Olsen et al., 2009). Eukaryotic cells contain two forms of genetic material: nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) (El-Hattab and Scaglia 2016). Both forms of genetic material can encode for mitochondrial located proteins, which play a crucial role in mitochondrial structure and the biological process of producing ATP. ATP is generated by a mechanism located in the inner membrane of the mitochondria known as the electron transport chain (ETC). A series of four complex proteins (I - IV) are responsible for transferring electrons via carrier molecules to create a proton gradient and ultimately produce ATP through the mechanism of oxidative phosphorylation (Larsson and Oldfors 2001).

Mutations occurring in any of the genes encoding for these complexes can result in a variety of different defects, including the leakage of electrons and accumulation of reactive oxygen species (ROS), compounds toxic to cells. ROS, like hydrogen peroxide (H_2O_2) and superoxide anion (O_2), may build up during oxidative phosphorylation, overwhelming the cellular antioxidant defense system, causing oxidative stress (Ray et al., 2012). The oxidative stress and ROS accumulation due to the inability to properly couple electrons has been directly linked to a series of pathologies including carcinomas, neurodegeneration and muscular dysfunction (El-Hattab and Scaglia 2016).

Dysfunctional mitochondria have the inability to meet the high-energy demands of multiple organs. Specifically, mitochondrial myopathies and other neuromuscular disorders are a result of the energy supply-and-demand imbalance in muscle and nerve cells. Kearns-Sayre

syndrome, Leigh syndrome, Mitochondrial DNA depletion syndrome, and Pearson's syndrome are some examples of mitochondrial myopathies affecting multiple biological systems and processes. Common symptoms of such diseases include muscle weakness, atypical cardiac rhythms, exercise intolerance, seizures, and ataxia (Pfeffer and Chinnery 2011).

This study examines the *SdhB*^{EY12081} strain in *Drosophila melanogaster* (commonly known as the fruit fly), which contains a mutation in the gene encoding for the iron-sulfur containing subunit B Complex II of the ETC, also known as succinate dehydrogenase (SDH). The genes from this complex are all encoded in the nucleus, unlike the other complexes, which have mitochondrial and nuclear encoded proteins (Cecchini 2003). SDH is an integral membrane protein composed of four subunits that converts succinate to fumarate during the Krebs cycle (Cecchini 2003). The free energy change resulting from the conversion of succinate to fumarate is too small to produce a proton gradient across the inner mitochondrial membrane (Cecchini 2003). However, because SDH is responsible for passing electrons to the other complexes of the ETC, it becomes necessary to produce ATP for energy use.

In *Drosophila melanogaster,* the gene coding for *SdhB* is crucial in preventing electron leaking and the accumulation of ROS (Walker et al., 2006). Therefore, *SdhB*^{EY12081} mutants have large accumulations of ROS, causing cellular oxidative stress (Walker et al., 2006) and inhibiting the proper production of ATP. In particular, the mutated *SdhB*^{EY12081} fly strain suffers from muscle weakness, decreased motor performance, and a shortened life span (Walker et al., 2006). Considering that both humans and fruit flies contain the ETC complexes that are subjected to certain mutations, specifically that of *SdhB*^{EY12081}, the mutated flies serve as model organisms for studying mitochondrial disorders.

Unfortunately there is no current cure for mitochondrial myopathies in humans. This is partially because mitochondria have only recently been linked to disease (1960s) and the symptomatology of the diseases is very wide, making the diagnosis of mitochondrial disorders challenging. Since mitochondrial diseases are still considered to be rare, funding is limited (Parikh et al., 2013). Fairly recently, research has suggested nutritional and exercise supplementations as a possible mitigation of symptoms. Coenzyme Q10, creatine and rapamycin supplementation as well as endurance and resistance exercise training have suggested symptomatic improvement for neurological and muscular degeneration (Hassani et al., 2010; Villa Cuesta et al., 2014). This study explores the potential effects caffeine supplementation may have on the high-energy output activities and the longevity of the $SdhB^{EY12081}$ strain.

Caffeine is a central nervous system (CNS) stimulant that has been shown to temporarily improve exercise tolerance in regards to prolonging fatigue in humans and facilitates ATP production (Davis and Green 2009). This drug affects organisms through the action of multiple mechanisms. First, caffeine blocks adenosine receptors and enhances epinephrine levels. This prevents a decline in neuronal activity while stimulating a calcium influx, which allows for the firing of motor units during muscle contraction (Davis and Green 2009). The drug also inhibits the oxidative enzyme phosphodiesterase, allowing adenosine monophosphate protein (cAMP) and protein kinase A (PKA) levels to rise, increasing overall fatty-acid oxidation for energy production (Magkos and Kavouras 2005). Research has suggested that caffeine may have the ability to facilitate the action of the sodium-potassium (Na+/K+) pump, creating an electrochemical gradient that allows ATPase to drive ATP production for cellular use (Renaud 2002). By preventing the accumulation of plasma K+, caffeine stimulates muscle contraction and

postpones fatigue (Maclntosh and Wright 1995), acting as an ergogenic aid in humans, as well as improving the strength of muscular contractions (Davis and Green 2009).

Previous studies have shown that increased caffeine consumption has been linked to a lower risk of neurodegenerative disorders such as Parkinson's disease and Alzheimer's (Ross et al., 2000). These diseases, like several other neurodegenerative and neuromuscular pathologies, are affected by dysfunctional mitochondria due to the improper coupling of electrons across the ETC complexes. This study uses the mutated *SdhB*^{EY12081} strain of *D. melanogaster* as a model organism to understand the mechanisms by which caffeine has a potential beneficial effect on certain human mitochondrial mutations. Motor performance and longevity were measured by climbing ability and a controlled demography respectively in order to observe the significance of the caffeine treatment.

Materials and Methods

Fly Strains

Drosophila melanogaster OreR wild type and the *SdhB*^{EY12081} mutant were used as the model organisms to observe caffeine's effect on energy output and longevity (Glittenberg et al., 2011). The *SdhB*^{EY12081} strain is a transposable element insertion developed by the Berkeley Drosophila Genome Project. A P-element transposon (white^{+} and yellow^{+}) was inserted into the 5' untranslated region of the *SdhB* nuclear gene on chromosome 2R and then crossed with *L*^{2}*/CyO* (Bellen et al., 2011).

Five male and five female flies were bred in 25 x 95mm vials containing 5.0mL of food. After two weeks, flies were sorted through the use of carbon dioxide gas. All flies were maintained in normal fly media: 11% sugar, 5.2% cornmeal, 0.79% agar in water, 2% autolyzed yeast and 0.2% tegosept/methyl *p*-hydroxybenzoate. The flies were incubated at 25 degrees Celsius and 70% humidity.

Caffeine Treatment

According to past studies, 0.3125mg of caffeine/mL water to 0.625mg of caffeine/mL water was shown to have the best results regarding wild-type survival rate (Nikitin et al., 2008). Based on this information, a titration of caffeine concentration ranging from 0.25mg/mL to 0.75mg/mL (0mg/mL, .25mg/mL, .5mg/mL and .75mg/mL) was performed and climbing and toxicity was observed. Flies treated with 0.25mg of caffeine/mL of water had the greatest survival rate among the other concentrations, and this concentration was therefore used as the treatment for the longevity assay.

Climbing Assay

51.56mg (0.0516g) caffeine was added to 25mL of experimental food. Five 25 x 95mm vials of each experimental group, control and caffeine, were prepared and ten wild-type females or ten homozygous *SdhB*^{EY12081} females were placed in the corresponding vials for one, two, three and four days. Climbing and/ or survival assays were performed, depending on the experiment.

Flies climb up the vial against gravity through negative geotaxis. To test the climbing ability of the fly strains given their respective treatments, the flies were transferred to empty vials, which were lightly struck against the table to cause the flies to fall to the bottom. Each vial was marked with a 3cm line from the top, and after 40 seconds the number of flies that reached this line was recorded. Three consecutive trials were conducted with each of the vials. Statistical analysis was performed using an analysis of variance (ANOVA) on SPSS software.

Demography

A demography was used to determine the longevity of the *SdhB*^{EY12081} flies with and without the treatment of caffeine. A total of fifty vials of control food and fifty vials of caffeine-treated food were prepared for this assay. While the control food contained no treatment, the caffeine food contained 0.25mg caffeine/mL of water. There were six one-liter cages measuring 8cm in diameter at the base, 11.5cm diameter at the top, and 14.5cm in length used for the demography. Three cages contained control food and three contained caffeine-treated food. Female homozygous *SdhB*^{EY12801} flies were sorted so that thirty flies were placed in each demography cage. The cages were maintained at 25 degrees Celsius and 70% humidity until all flies were found dead. Each day the dead flies from every cage were counted, removed and

recorded. Every three to four days the food from each cage was changed. Statistical analysis was performed using ANOVA on SPSS software in addition to a Kaplan-Meier log rank comparison.

Results

Caffeine improves the climbing ability of the *SdhB*^{EY12081} strain but not the wild-type strain.

The climbing activity of wild type and *SdhB*^{EY12081} *D. melanogaster* flies was analyzed after they had been given control or caffeine food. For the wild-type flies the mean of both control and caffeine-treated flies showed similar climbing trends (Figure 1). While the box plot displays that some of the wild-type flies decreased in climbing after being treated with caffeine, this effect was not statistically significant according to the values found in Table 1 (p-value for treatment = 0.328).

While wild-type flies are typically good climbers under normal conditions, *SdhB*^{EY1201} flies suffer from impaired motor performance (Walker et al., 2006). These results were replicated in my experiments as control fed *SdhB*^{EY12081} flies climbed poorly (Figure 1). In addition, the results from the climbing performance of *SdhB*^{EY12081} mutated strain demonstrate that flies given caffeine-treated food climb better as compared to flies given control food (Figure 1). The control flies were seen to perform very poorly, having a range never exceeding .20 actively climbing (Figure 1). The proportion of caffeine-treated flies had a much larger climbing range, spanning from .00 to about .80, and a mean closer to .20 (Figure 1). Statistical evidence (p-value = 0.050) confirms that caffeine does marginally improve the climbing of the mutated *SdhB*^{EY12081} flies.

Since the caffeine treatment improved the climbing ability of the *SdhB*^{EY12081} strain but not the wild type, the mutated *SdhB*^{EY12081} flies were selected for further study.

0.25mg/mL caffeine improves climbing ability of the *SdhB*^{EY12081} strain without affecting mortality rate.

Considering that 0.75mg caffeine/mL of water yielded climbing results with borderline significance, various caffeine titrations were carried out to determine the proper amount of caffeine that would improve climbing ability of the mutated strain without a negative impact on survival rate. The titration included 0mg caffeine/mL of water, 0.25mg/mL, 0.50mg/mL and 0.75mg/mL. 0.25mg/mL, 0.5mg/mL and 0.75 mg/mL all elicited improvement in climbing ability as compared to the vehicle food (Figure 2). Although all concentrations expressed similar trends, 0.25 mg/mL was the lowest concentration seen to improve climbing ability (p-value =0.000) (Table 3).

Survival analysis of the $SdhB^{EY12081}$ strain under varying concentrations of caffeine showed that mutated flies were not affected after being treated with 0.25mg/mL of caffeine (p-value = 0.128) (Table 4). However, when treated with the higher concentration of 0.75mg/mL, the survival of the mutated flies decreased as compared to the control flies (p-value= 0.000) (Table 3). $SdhB^{EY12081}$ flies typically have a shortened life span as compared to wild-type *Drosophila melanogaster* (Walker et al., 2006). Thus, while a moderate amount of caffeine may aid in motor functioning, it does not seem to influence the life span of the mutated strain.

0.25mg/mL of caffeine treatment does not improve the life span of the $SdhB^{EY12081}$ strain

Because the concentration 0.25mg/mL improved climbing ability without having a toxic effect, a demography was conducted to determine the longevity of the mutated strain after being given control or caffeine-treated food. A total of 180 $SdhB^{EY12081}$ flies were used; 90 were given control food and 90 were given caffeine-treated food. After counting the number of dead flies each day, the data collected was used to analyze the effects of caffeine on life span.

The data suggests that the cumulative survival rate of the caffeine treated $SdhB^{EY12081}$ is significantly lower than that of the control group (p = 0.000) (Table 5), demonstrating not only

that caffeine does not improve longevity in the mutant strain, but also that caffeine treatment is detrimental (Figure 4).

Discussion

Caffeine is a CNS stimulant that is continuously studied due to its potential effects on human performance in terms of exercise. This drug has several mechanisms of action, all

enabling for prolonged motor function and often extended exercise tolerance. Some of these processes include the translocation of calcium into muscle cells, preventing accumulation of K+, and increasing the secondary messenger, cAMP, through the inhibition of the enzyme PDE (Lindinger et al., 1993; Mustard 2014). The cAMP/PKA process specifically occurs in both humans and *D. melanogaster*, increasing the fatty acid oxidation in muscle cells for free energy (Wu 2002). While multiple studies have evaluated caffeine's influence on human exercise performance in regards to energy availability for active muscles, little is known about the impact caffeine has on high energy output when it comes to different mitochondrial disorders. This experiment therefore aimed to explore the effects caffeine has on mitochondrial myopathies, using *D. melanogaster* as the model organism.

Considering the $SdhB^{EY12081}$ strain of *D. melanogaster* contains a mutation encoding for subunit B of succinate dehydrogenase, which allows for accumulation of ROS, total energy output is compromised when compared to wild-type flies (Walker et al., 2006). Climbing ability was used to determine caffeine's effect on motor functioning in flies. The results indicate that the wild-type flies had a much better climbing ability than did the mutated fly strain when given vehicle control food (Figure 1). However, while 0.75mg/mL caffeine treatment did not improve the climbing ability of wild-type flies, this treatment had a significant effect on the $SdhB^{EY12081}$ flies (Table 2). Because caffeine has the ability to extend exercise tolerance by prolonging fatigue, the treatment seems to also overcome some of the deleterious effects the mutation has on the motor performance of fruit flies. This was expected due to caffeine's influence on human activity (Davis and Green 2009).

One must question caffeine's toxic effect when it comes to administering this drug as a potential treatment for mitochondrial disorders. Although moderate caffeine ingestion has been

shown to pose no harm, in high quantities this drug proves fatal (Kerrigan and Lindsey 2005). A titration of caffeine treatment was carried out to determine the proper concentration needed to improve climbing while maintaining viability. Results demonstrate that the 0.25mg/mL concentration was most effective at benefiting climbing ability without affecting life span, while the 0.75mg/mL concentration was toxic to the mutant strain (Table 4). This proves that moderate consumption of caffeine may be advantageous to high-energy output of fruit flies, while increased caffeine ingestion is fatal.

Compared to the wild-type strain, the *SdhB*^{EY12081} mutant has been seen to have a 66% decrease in overall mean survival (Walker et al., 2006). The accumulation of ROS seems to be one of the main factors affecting survival rate. When electrons are not properly coupled down the ETC, not only are free radicals built up in the mitochondria, but also ATP production is compromised due to oxidative stress (Turrens 2003). Therefore, caffeine was hypothesized to be a treatment that overcomes this negative consequence. The demography results indicate that caffeine-treated food did not improve the longevity of the mutant strain when compared to the control food (Figure 4). The mean number of survival days (control- 19.235 and caffeine- 9.689) demonstrate this decline (Figure 5). Given that the *SdhB*^{EY12081} strain already has a compromised life span, the caffeine treatment proves to be further deleterious with respect to longevity, disproving the initial hypothesis.

This study seems to suggest that while caffeine does improve the climbing ability of the *sdhB*^{EY12081} strain of *D. melanogaster*, the drug does not have the positive effect of prolonging life span. Even though our research suggests that caffeine has a positive influence on motor functioning and a negative influence on longevity, experimental error can be accounted for. Because flies have the ability to escape their respective vials and cages, many more repetitions

are needed to provide for significant data. In addition, the various concentrations of caffeine throughout the experiment may not have been measured accurately while creating each stock solution. More extensive titrations with varying quantities of the drug may produce more finite data when establishing a proper caffeine treatment for the flies. It may be helpful to asses caffeine's impact on other subunits specifically in SDH, or one of the other complexes in the ETC. ROS production can also be measured after flies are treated with caffeine to determine whether this accumulation is limited by the supplementation. Because all the complexes play a vital role in the production of energy, expanding research in this direction may validate the effects caffeine may have. While our research provides some insight on caffeine's influence on motor function and longevity, further experimentation is needed to expand on these results.

Due to the vital importance of mitochondria in regards to cellular metabolism, a defect in any aspect of this organelle can result in several different pathologies. These include common disorders like Alzheimer's and Parkinson's disease and more extensive mitochondrial myopathies such as Leigh's syndrome and Kearns-Sayre syndrome (Smits et al., 2010). Because of the unfortunate lack of pharmaceutical treatment for such disorders, many suffer from the coinciding health conditions that result. Even though caffeine had a detrimental effect on the longevity of the flies, the treatment did improve some of the pathology associated with the SDH deficiency. Given this information, the data serves as evidence that caffeine may be used as a potential intermittent treatment for these various neuromuscular disorders and mitochondrial myopathies. While caffeine treatment over an extended period is not favored, this supplement may prove beneficial when given on an occasional basis. With further research, one can hope that many unfavorable symptoms of dysfunctional mitochondria may be alleviated with the use caffeine.

Acknowledgements

Firstly, I want to thank Dr. Eugenia Villa-Cuesta for her continuous guidance, support, insight and mentoring throughout this research project. I would also like to acknowledge the Adelphi University Biology Department staff for the reagents to complete the experimental

assays. I would like to thank Amanda Iacometta, my research partner, for her undivided collaboration efforts and assistance in experimental procedures. I also thank the Adelphi University Research Conference for providing me the opportunity to present this work, the Deans of the Honors College for supporting the entirety of this project, and for my readers who dedicated their time and efforts for the improvement of my research.

References

Bellen, H. J., Levis, R. W., He, Y., Carlson, J. W., Evans-Holm, M., Bae, E., . . . Spradling, A. C. (2011). The Drosophila Gene Disruption Project: Progress Using Transposons With Distinctive Site Specificities. *Genetics, 188*(3), 731-743.

Cecchini, G. (2003). Function and Structure of Complex II of the Respiratory Chain. *Annual Review of Biochemistry, 72*(1), 77-109.

Davis, J., & Green, J. M. (2009). Caffeine and Anaerobic Performance. *Sports Medicine, 39*(10), 813-832.

El-Hattab, A. W., & Scaglia, F. (2016). Mitochondrial cytopathies. *Cell Calcium, 60*(3), 199-206.

Hassani, A., Horvath, R., & Chinnery, P. F. (2010). Mitochondrial myopathies: developments in treatment. *Current Opinion in Neurology, 23*(5), 459-465.

Kerrigan, S., & Lindsey, T. (2005). Fatal caffeine overdose: Two case reports. *Forensic Science International, 153*(1), 67-69.

Larsson, N., & Olfors, A. (2001). Mitochondrial myopathies. *Acta physiologica Scandinavica, 171*(3), 385-93.

Lindinger, M. I., Graham, T. E., & Spriet, L. L. (1993). Caffeine Attenuates The Exercise Induced Increase In Plasma [K+] In Humans. *Medicine & Science in Sports & Exercise, 74*(3), 1149-55

Magkos, F., & Kavouras, S. (2005). Caffeine use in sports, pharmacokinetics in man, and cellular mechanisms of action. *Critical Reviews in Food Science and Nutrition, 45*(7-8), 535-62.

Mustard , J. A. (2014). The buzz on caffeine in invertebrates: effects on behavior and molecular mechanisms. *Cellular and Molecular Life Science , 71*(8), 1375-82.

Olsen , L. F., Anderson, A. Z., Brasen, J. C., & Poulsen, A. K. (2009). Regulation of Glycolytic Oscillations by Mitochondrial and Plasma Membrane H -ATPases. *Biophysical Journal 96*(9), 3850-61.

Parikh, S., Saneto, R., Falk, M. J., Anselm, I., Cohen, B. H., & Haas, R. (2009). A Modern Approach to the Treatment of Mitochondrial Disease. *Curr Treat Options Neurology, 11*(6), 414-430.

Pfeffer, G., & Chinnery, P. F. (2011). Diagnosis and treatment of mitochondrial myopathies. *Annals of Medicine, 45*(1), 4-16.

Ray, P. D., Huang, B., & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling, 24*(5), 981-990.

Renaud, J. (2002). Modulation of force development by Na , K , Na K pump and KATP channel during muscular activity. *Canadian journal of applied physiology, 27*(3), 296-315.

Ross, G. W., Abbott, R., Petrovitch, H., Morens, D., Grandinetti, A., & Tung, K. (2000). Association of Coffee and Caffeine Intake With the Risk of Parkinson Disease. *Jama, 283*(20), 2674-9.

Smits, P., Smeitink, J., & Heuvel, L. V. (2010). Mitochondrial Translation and Beyond: Processes Implicated in Combined Oxidative Phosphorylation Deficiencies. *Journal of Biomedicine and Biotechnology, 2010*, 1-24.

Turrens, J. F. (2003). Mitochondrial formation of reactive oxygen species. *The Journal of Physiology, 552*(2), 335-344.

Villa-Cuesta, E., Holmbeck, M. A., & Rand, D. M. (2014). Rapamycin increases mitochondrial efficiency by mtDNA-dependent reprogramming of mitochondrial metabolism in Drosophila. *Journal of Cell Science, 127*(10), 2282-2290.

Walker DW, Hajek P, Muffat J, Knoepfle D, Cornelison S, Attardi G, Benzer S. 2006. Hypersensitivity to oxygen and shortened life span in Drosophila mitochondrial complex II mutant. Proceedings of the National Academy of Sciences of the United

States of America 103 (44): 16382-16387

Wu, M. N., Ho, K., Crocker, A., Yue, Z., Koh, K., & Sehgal, A. (2009). The Effects of Caffeine on Sleep in Drosophila Require PKA Activity, but not the Adenosine Receptor. *J Neurosci*, *29*(35), 11029-11037.

Tables and Figures

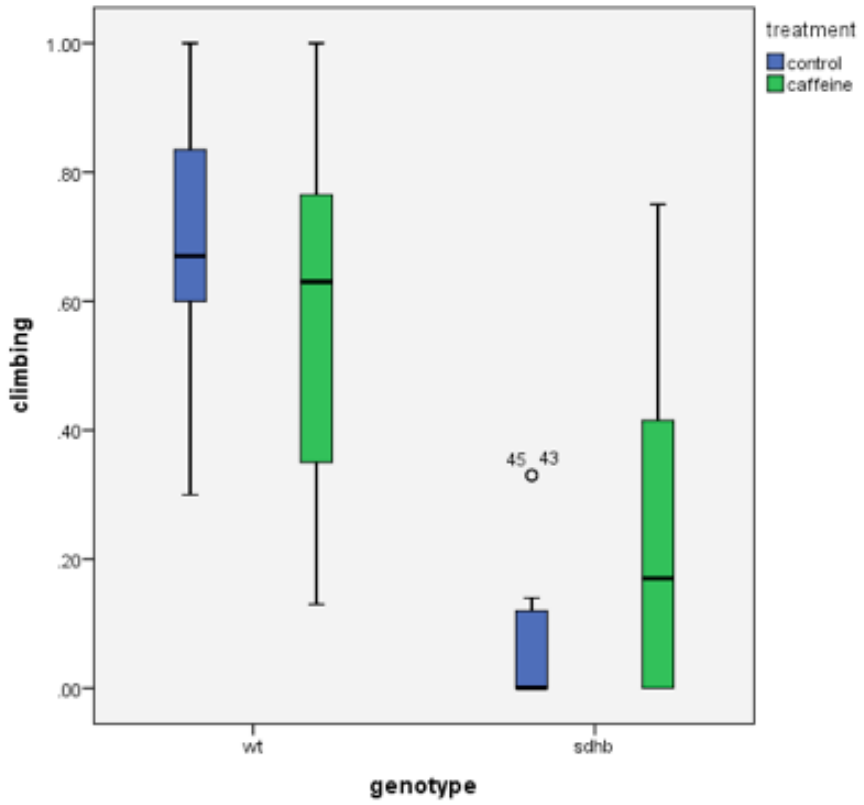

Figure 1: Climbing ability of wild-type and *SdhB*^{EY12081} flies after treated with the vehicle control and caffeine food for two days. Climbing ability was measured by observing the negative geotaxis movement of the flies. Those able to reach the top of the vial (about 3cm from the cotton stopper) were considered to have maximal climbing ability. The x-axis demonstrates the genotype and the y-axis includes the proportion of flies actively climbing in relation to those dead or poorly climbing.

Table 1: The significance of the climbing ability of wild-type *Drosophila melanogaster* (with and without caffeine) was determined by ANOVA Univariate Statistical Analysis using SPSS software ($p = 0.328$).

Tests of Between-Subjects Effects[a]

Dependent Variable: climbing

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.061[b]	1	.061	.991	.328
Intercept	12.250	1	12.250	199.794	.000
treatment	.061	1	.061	.991	.328
Error	1.717	28	.061		
Total	14.027	30			
Corrected Total	1.777	29			

a. genotype = wt

b. R Squared = .034 (Adjusted R Squared = .000)

Table 2: The significance of the climbing ability of *SdhB*^{EY12081} *Drosophila melanogaster* after being treated with and without 0.75mg/mL caffeine was determined by ANOVA using SPSS software ($p = 0.050$).

Tests of Between-Subjects Effects[a]

Dependent Variable: climbing

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	.179[b]	1	.179	4.192	.050
Intercept	.894	1	.894	20.898	.000
treatment	.179	1	.179	4.192	.050
Error	1.198	28	.043		
Total	2.272	30			
Corrected Total	1.378	29			

a. genotype = sdhb

b. R Squared = .130 (Adjusted R Squared = .099)

Climbing Ability

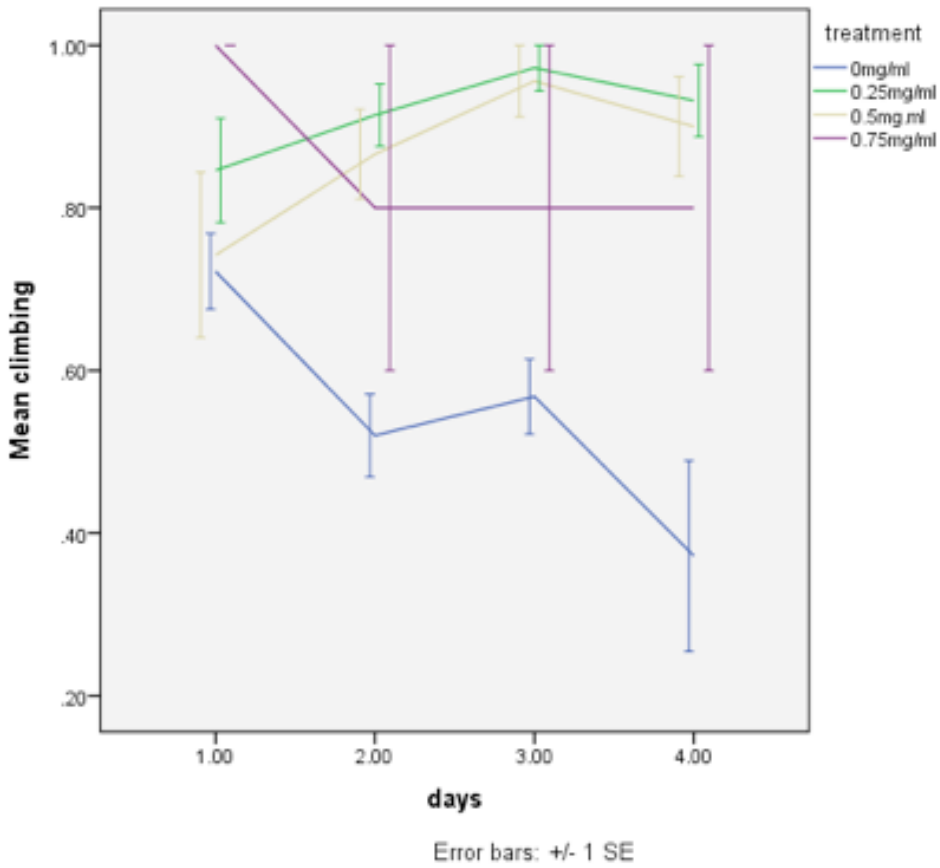

Figure 2: Linear graph displaying the proportion of live *SdhB*^{EY12081} flies that were actively climbing. The flies were observed over a four-day period after being treated with various caffeine concentrations (0mg/mL, 0.25mg/mL, 0.50mg/mL, 0.75mg/mL). Climbing ability was measured by a 40-second negative geotaxis assay.

Table 3: The significance of the varying concentrations of caffeine on the climbing ability of the *SdhB* flies. Each concentration was compared to the control food (0mg/mL) to determine the

effect caffeine has on climbing. The data was analyzed by ANOVA on SPSS software using Tukey HSD for a multiple comparison.

Multiple Comparisons

Dependent Variable: climbing

	(I) treatment	(J) treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval
						Lower Bound
Tukey HSD	0mg/ml	0.25mg/ml	-.3705*	.07247	.000	-.5617
		0.5mg.ml	-.3205*	.07247	.000	-.5117
		0.75mg/ml	-.3045*	.07247	.000	-.4957
	0.25mg/ml	0mg/ml	.3705*	.07247	.000	.1793
		0.5mg.ml	.0500	.07247	.901	-.1412
		0.75mg/ml	.0660	.07247	.799	-.1252
	0.5mg.ml	0mg/ml	.3205*	.07247	.000	.1293
		0.25mg/ml	-.0500	.07247	.901	-.2412
		0.75mg/ml	.0160	.07247	.996	-.1752
	0.75mg/ml	0mg/ml	.3045*	.07247	.000	.1133
		0.25mg/ml	-.0660	.07247	.799	-.2572
		0.5mg.ml	-.0160	.07247	.996	-.2072

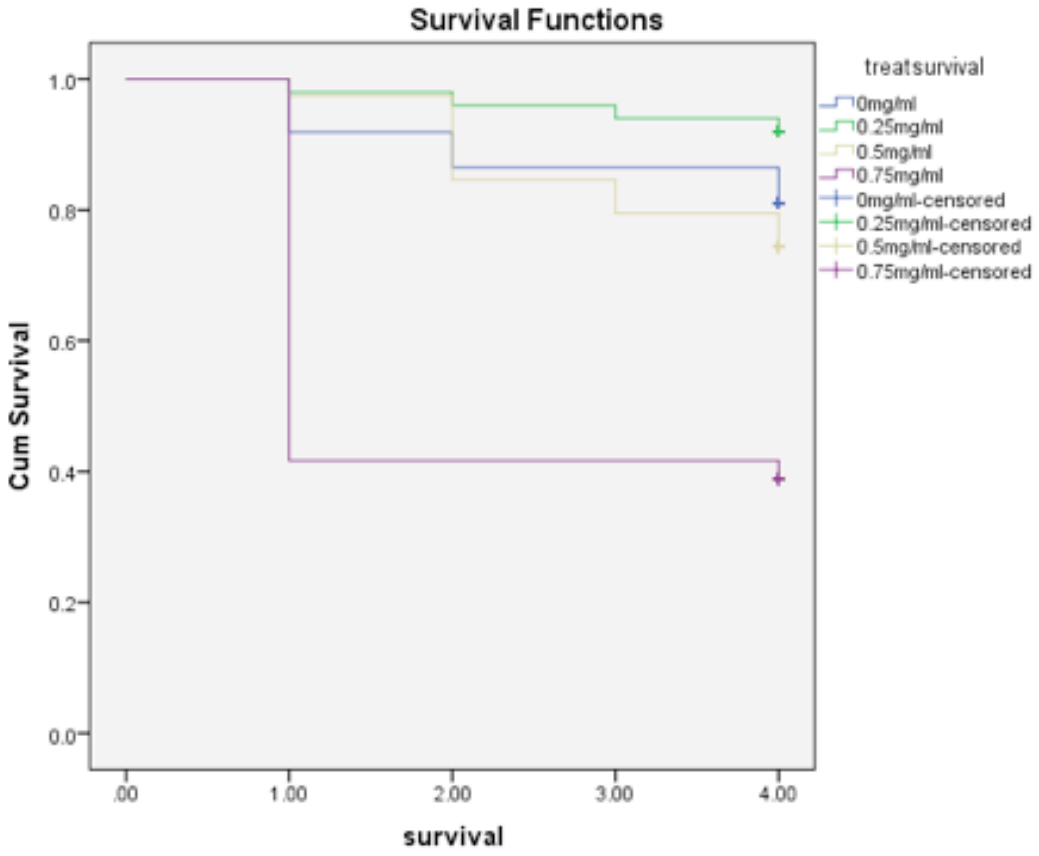

Figure 3: A survival plot was used to depict the survival rate of *SdhB* flies after being treated with the caffeine concentrations. Each day for four days, the number of dead flies was counted.

Table 4: The significance of varying caffeine concentrations on the viability of S*dhB*^{EY12081} flies was determined by a Log-Rank pairwise comparison using Kaplan-Meier software. This comparison was used to observe the effects multiple caffeine titrations have on the survival rate of flies.

Pairwise Comparisons

		0mg/ml		0.25mg/ml		0.5mg/ml	0.5mg/ml	0.75mg/ml	
	treatsurvival	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
Log Rank (Mantel-Cox)	0mg/ml			2.321	.128	.403	.526	14.422	.000
	0.25mg/ml	2.321	.128			5.091	.024	29.466	.000
	0.5mg/ml	.403	.526	5.091	.024			11.981	.001
	0.75mg/ml	14.422	.000	29.466	.000	11.981	.001		

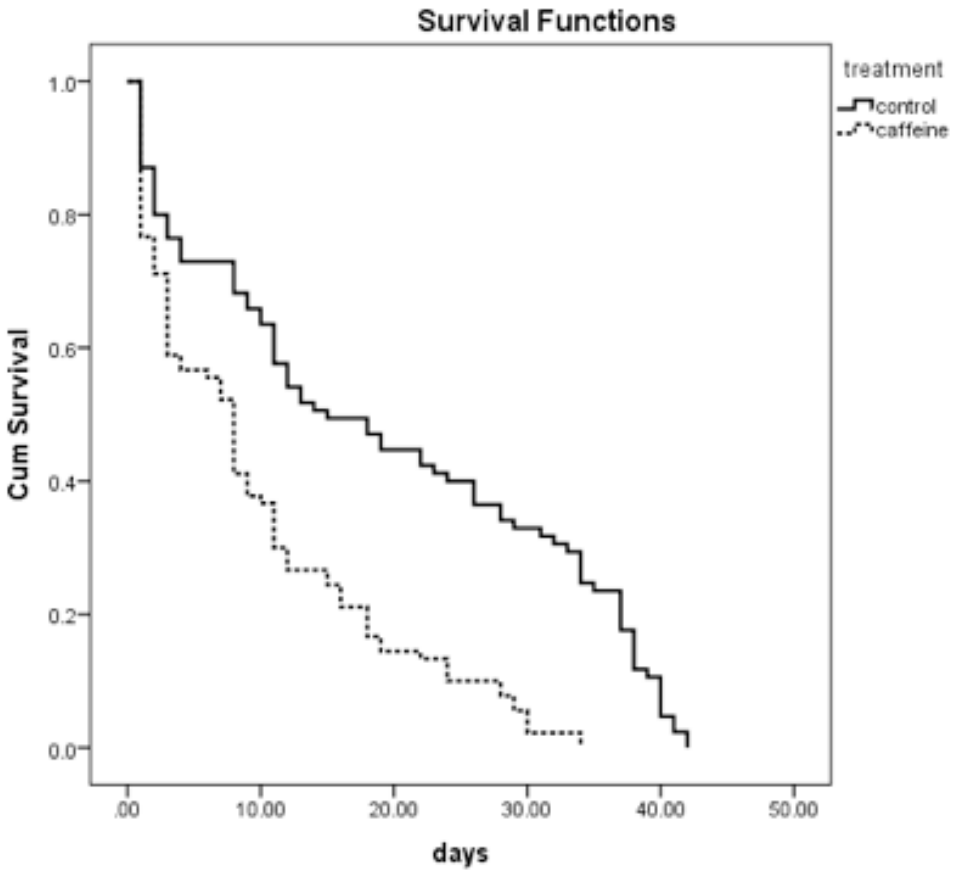

Figure 4: A survival line graph depicts the linear relationship between cumulative survival rate and the length (in days) comparing the control and caffeine-treated experimental groups. The demography lasted until all the flies in each cage were found dead.

Table 5: The significance of caffeine on longevity of *SdhB*^{EY12081} strain (p = 0.000) was determined using the Kaplan-Meier log rank test and the Mantel-Cox pairwise comparison. The analysis was used to compare the life span of control and caffeine-treated mutant strain.

Overall Comparisons

	Chi-Square	df	Sig.A
Log Rank (Mantel-Cox)	**30.585**	**1**	**.000**

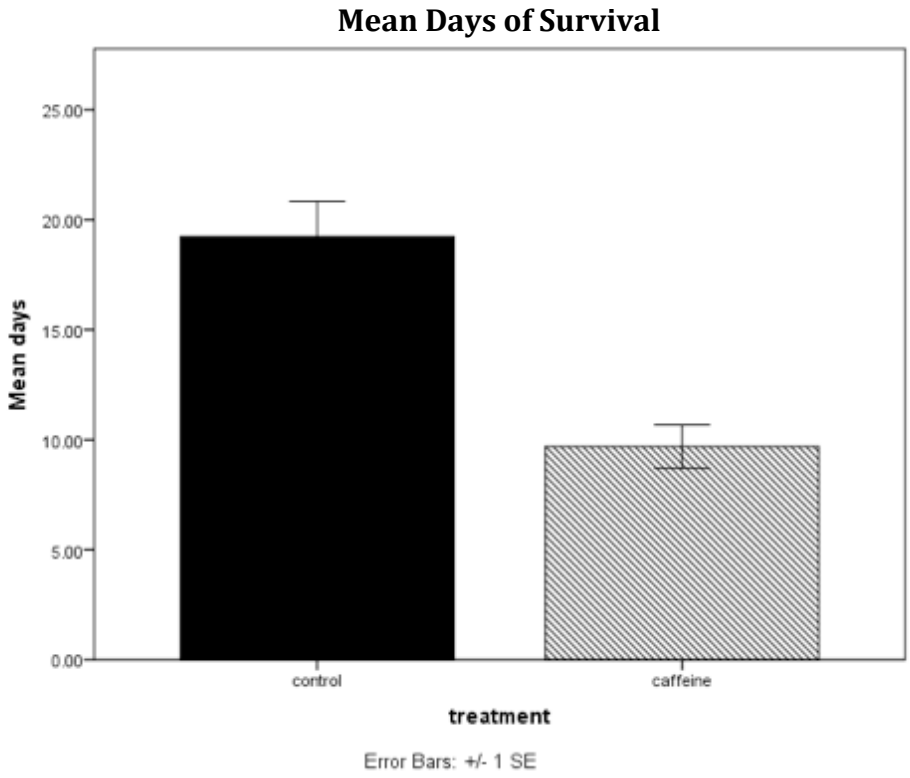

Figure 5: This bar graph illustrates the mean number of days in which the *SdhB* mutants died. The longevity of the flies treated with caffeine was compared to that of the control group. The data was interpreted using a Student T-test on SPSS software.