The Ketogenic Diet Ameliorates the Effects of Caffeine in Seizure Susceptible *Drosophila melanogaster*

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INTRODUCTION

Epilepsy is a neurological disease involving spontaneous and chronic seizures (Fisher et al., 2005). Seizures are a product of excessive and abnormal neuronal discharge and are defined by involuntary convulsions and muscle spasms (Stafstrom & Carmant, 2015). Such excessive neuronal discharge is caused by an imbalance in the concentrations of the neurotransmitters glutamate and gamma-aminobutyric acid (GABA) (Petroff, 2002). Glutamate stimulates the receiving neuron to fire, and thus an excess of glutamate will lead to seizures or seizure-like activity (SLA) (During, 1993). GABA, a metabolic product of glutamate, silences receiving neurons, and thus a scarcity of GABA can also lead to SLA (Petroff et al., 1996).

Glutamate and GABA levels are impacted by different factors. Drugs, such as caffeine, stimulate the release of glutamate (Solinas et al., 2002) while decreasing the binding efficacy of GABA (Roca, Schiller, & Farb, 1988). Therefore, it would be expected that caffeine increases seizure susceptibility. However, chronic caffeine exposure plays less of a role in seizure susceptibility than acute caffeine exposure does, indicating a possible protective effect associated with increased tolerance or physiological adaptation (Germé et al., 2015). Alternatively, some forms of dietary restriction such as the ketogenic diet (KD) inhibit seizures (Neal et al., 2008). The ketogenic diet is a low carbohydrate, low protein, high fat diet (Thiele, 2003). When subjected to KD, the body uses the surplus fat to produce high levels of acetyl coenzyme A (acetyl-CoA) (McNally & Hartman, 2012), which it then uses to produce ketone bodies such as β-hydroxybutyrate (βHB) (Melø, Nehlig, & Sonnewald, 2006; Hartman & Vining, 2007). Higher levels of acetyl-CoA create an increase in metabolization of acetyl-CoA into citrate and CoA via oxaloacetate (Wiegand & Remington, 1986). Oxaloacetate also plays an important role in the metabolism of glutamate into aspartate (Karmen, Wróblewski, & Ladue, 1955). By decreasing the amount of available oxaloacetate for glutamate transamination, more glutamate is available for metabolism into GABA, thus decreasing seizure susceptibility (Yudkoff et al., 2007). Another mechanism by which ketone bodies decrease SLA is the βHB-mediated opening of ATP-sensitive potassium (K_{ATP}) channels on neurons (Li, O'Leary, & Tanner, 2017). By opening these channels, potassium ions can exit the neuron and create a hyperpolarized, low-voltage membrane that is less susceptible to excess discharge due to the decrease of the membrane voltage and the subsequent increased difficulty of reaching action potential (Faivre & Findlay, 1990).

Other forms of dietary restriction have been shown to have a wide array of benefits in animals, ranging from mammals to insects (Weindruch & Walford, 1990; Turturro et al., 1999; Metaxakis & Partridge 2013). A common dietary restriction strategy with strong benefits in insects is a low-protein, high-carbohydrate diet (Chippindale et al., 2004). This diet will be referred to as DR henceforth. DR causes a decrease in target of rapamycin (TOR) signaling by reducing the consumption of amino acids, which stimulate the TOR pathway (Emran et al., 2014). Additionally, DR and the resulting decrease in TOR signaling mitigate the negative physiological effects of caffeine (Mashal, 2016). Little is known about the effects that these diets have on seizures.

Studies concerning DR (Chippindale et al., 2004), caffeine (Alshuaib & Mathew, 2006; Matsagas et al., 2009), and epilepsy (Parker et al., 2011; Saras et al., 2017) have each used *Drosophila melanogaster* as a model organism. Though that these factors influence one another (Figure 1), this is the first study to investigate what happens at the intersection of all three features. This study aims to elucidate the effects of caffeine and dietary restriction, when used concurrently, on seizure susceptibility in *Drosophila*. It was hypothesized that dietary restrictions will mitigate the effect of caffeine on seizure intensity because it moderates the negative effects of caffeine and seizure susceptibility individually. Additionally, it was hypothesized that DR would function similarly to KD as a seizure inhibitor, and that flies that chronically consumed caffeine would experience fewer seizures than flies that were acutely administered caffeine.

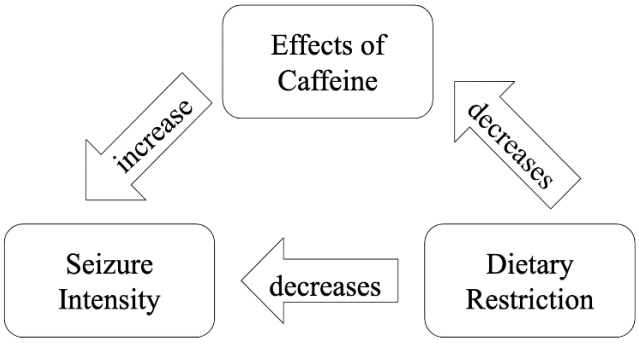


Figure 1: Effects of caffeine, dietary restriction, and seizure intensity on each other. Dietary restriction decreases both the effects of caffeine and intensity of seizures, while caffeine increases seizure intensity.

MATERIALS AND METHODS

In order to observe the interaction amongst diet, caffeine consumption, and seizure susceptibility, a nutritional profile consisting of a diet type and a caffeine frequency was assigned to each of three fly strains (wild type w^{1118}; seizure susceptible eas^{alaE13} and eas^{KO}) (Table 1). These flies were then induced to have seizures based on the protocols (Li, 2016) described below. There were 30 flies tested in each of the 27 experimental groups, meaning that 810 flies were tested for this study.

	Normal Diet	Dietary Restriction (DR)	Ketogenic Diet (KD)
No Caffeine (NC)	w^{1118}, eas^{alaE13}, eas^{KO}	w^{1118}, eas^{alaE13}, eas^{KO}	w^{1118}, eas^{alaE13}, eas^{KO}
Chronic Caffeine (CC)	w^{1118}, eas^{alaE13}, eas^{KO}	w^{1118}, eas^{alaE13}, eas^{KO}	w^{1118}, eas^{alaE13}, eas^{KO}
Acute Caffeine (AC)	w^{1118}, eas^{alaE13}, eas^{KO}	w^{1118}, eas^{alaE13}, eas^{KO}	w^{1118}, eas^{alaE13}, eas^{KO}

Table 1: Nutritional Profiles and Fly Strains. Each nutritional profile consists of one of three diets (normal diet, dietary restriction, or the ketogenic diet) and one of three caffeine frequencies (no caffeine, chronic caffeine, or acute caffeine). These nine profiles are then applied to three different fly strains, yielding 27 experimental groups for seizure observation.

Fly Stocks

eas Drosophila strains were used as seizure susceptible mutants. Hypomorphic allele eas^{alaE13} and its knockout counterpart eas^{KO} were kindly provided by J. Steinhauer, and described in (Ben-David, Miller, & Steinhauer, 2015). A wild type strain, w^{1118}, was used as a non-seizure susceptible control.

Flies of the *eas* strains have ethanolamine kinase loss-of-function mutations; ethanolamine kinase function is necessary to produce wild type levels of phosphatidylethanolamine (PE), a neuronal phospholipid (Pavlidis, Ramaswami, & Tanouye, 1994). Lower levels of PE compromise neuronal membranes and lead to seizure susceptibility (Pavlidis, Ramaswami, & Tanouye, 1994). The ethanolamine kinase mutation of the eas^{alaE13}

and eas^{KO} strains is not only manifested through decreased levels of PE, but also through an absence of the α lobes (*ala* stands for "α lobes absent") of the *Drosophila* mushroom body (Pascual, Chaminade, & Préat, 2005), a portion of the fly brain that controls memory and learning (Aso et al., 2014).

***Drosophila* Diets and Caffeine Administration**

All flies were raised on Caltech food (8.7% cornmeal, 7.5% sucrose, 1.5% live yeast, 0.45% agar, 1% propionic and phosphoric acid mix; by volume) (Lewis, 1960) throughout development (egg to early adult, 0-2 days post-eclosion). When flies reached 3-5 days in age, they were transferred onto food featuring one of nine nutritional conditions. DR media was made by reducing the amount of yeast in the Caltech recipe by two-thirds. KD media was made using a 2:1 ratio of coconut oil to carbohydrates as outlined in (Fogle et al., 2016). Paper towels were placed in solidified KD media in order to absorb excess moisture and thus prevent flies from sticking to the food. Flies that were placed on a normal diet continued to be fed the Caltech food that they were raised in.

Flies assigned to a chronic caffeine (CC) exposure group were fed KD, DR, or normal media with added caffeine at a concentration of 0.1mg/mL for three days. For three hours prior to seizure induction, all flies were placed on 5% agar, 2% sucrose media without caffeine or, if they were assigned to the acute caffeine (AC) exposure group, 0.3mg/mL caffeine (Wu et al., 2009).

Seizure Induction and Videography

24 hours prior to seizure induction, flies were anesthetized via a carbon dioxide pad and sexed under a microscope. Only female flies were selected for the study because they were readily obtainable for all strains; some strains yielded a considerably low frequency of males. Immediately prior to seizure induction, flies were placed individually into empty vials. They were then undisturbed for 20 to 30 minutes. Individual flies were vortexed for ten seconds at maximum speed (K550G. *Scientific Industries, Inc.* Bohemia, NY) before being transferred onto a petri dish with a diameter of 10 cm.

Seizures were filmed using an iPhone 6s mounted above the petri dish. Video was recorded at a frame rate of 30 fps and a size of 750 x 1334 pixels. Video was stopped at four minutes and 45 seconds if the fly had not yet recovered based on typical seizure output. Video footage was then used to determine the number of flies paralyzed and the number of flies

exhibiting SLA in each group, as well as the mean seizure duration. SLA was confirmed using the EthoVision XT software by treating the program response of "Subject not Found" as an indication of SLA as outlined in (Li, 2016).

Statistical Analyses

SPSS was used for all statistical analyses. A three-way ANOVA was performed to measure the variance of seizure durations across all samples. Seizure and paralysis frequencies were analyzed with a binary logistic regression model. Groups of interest were compared in pairs using two proportion z-tests. Average seizure durations were plotted against percentage of flies that exhibited SLA and percentage of flies paralyzed. Linear regression t-tests were performed. An α of 0.05 was used for all p-values.

RESULTS

This study aimed to investigate the effects of dietary restriction and caffeine consumption on seizure susceptibility. To promote a robust seizure phenotype, null and hypomorph mutations of the *eas* locus were used. This interaction was analyzed by two criteria: seizure duration and seizure susceptibility (proportion of flies in a group that experienced SLA). It was hypothesized that dietary restriction would ameliorate the effects of caffeine on seizure susceptibility in fruit flies.

A three-way ANOVA was performed on seizure duration data. There was no significant three-way interaction between fly strain, dietary restriction, and caffeine frequency when applied to seizure duration values; $F(2, 30) = 2.073$, $p = 0.129$. For this reason, post hoc testing was not warranted.

A logistic regression was performed to determine the effects of fly strain, dietary restriction, and caffeine frequency on the proportion of flies that experienced SLA (% SLA). The logistic regression model was statistically significant; $\chi^2(6) = 347.539$, $p < 0.0005$. The model explained 51.8% of the variance (Nagelkerke R^2) in % SLA and correctly classified 85.1% of cases. The same logistic regression was also performed to determine the effects of these variables on the proportion of flies that experienced paralysis, but the logistic regression model was not significant; $\chi^2(6) = 95.467$, $p = 0.589$. Due to the apparent significance of seizure susceptibility data, two proportion z-tests were performed on groups of interest. Since percentage paralyzed data was not significant, two proportion z-tests were not performed on paralysis data.

Baselines of seizure susceptibility in all three strains were confirmed prior to experimentation. When seizures were induced in w^{1118}, eas^{alaE13}, and eas^{KO} flies that were raised on normal media and were naïve to caffeine, it was established that eas^{alaE13} and eas^{KO} both exhibited increased susceptibility compared to w^{1118}; thus, it was appropriate to use *eas* flies as a seizure susceptible genotype (Figure 2). These findings affirm what previous studies such as (Pavlidis, Ramaswami, & Tanouye, 1994) have found.

Secondly, when seizures were induced in flies that were exposed to caffeine but were not under dietary restriction, it was established that caffeine increased seizure susceptibility (Figure 3). This affirms what previous studies had found regarding the link between caffeine exposure and seizure susceptibility (Alshuaib & Mathew, 2006; John, Kodama, & Siegel, 2014; Germé et al., 2015). Lastly, when seizures were induced in caffeine-naïve flies that either were or were not raised on KD media, it was confirmed that KD ameliorates seizure susceptibility (Figure 4). Results described in (Li, 2016) were successfully replicated, and the expected ketogenic effects were observable. Because the previously studied individual impacts of each of these factors were replicated, it is safe to interpret the significance of new findings in relation to these pre-established baseline relationships.

It was hypothesized that DR would ameliorate seizure susceptibility in a similar manner to that of KD because both diets can alter glutamate levels. Seizures were induced in non caffeinated flies of all strains in order to compare % SLA. DR did not impact seizure susceptibility and is not analogous to KD regarding anticonvulsant properties (Figure 5).

Because it was confirmed that KD is an effective inhibitor of SLA, it was also hypothesized that this inhibition ability would allow KD to counteract the effects of caffeine on SLA. Flies of all three strains were raised on either normal or KD media and exposed either chronically or acutely to caffeine. For both acute and chronic caffeine exposure, flies that were raised on KD had significantly lower seizure frequencies, suggesting that KD does, in fact, mediate the effects of caffeine (Figure 6).

Because chronic exposure to caffeine can lead to compensatory adaptive mechanisms (Roca, Schiller, & Farb, 1988; Rigoulot et al., 2003), it was hypothesized that, across groups, chronic caffeine exposure would be less conducive to increased seizure susceptibility than acute caffeine exposure. Flies of all strains and diets were compared across AC and CC exposure groups for seizure susceptibility values. Though no difference was found in KD flies, eas^{KO} flies

raised on either normal or DR media and eas^{alaE13} flies raised on DR media both showed significant decreases in seizure susceptibility when they were chronically, as opposed to acutely, exposed (Figure 7).

Additionally, both seizure and paralysis frequencies were plotted against average seizure durations for each group. Linear regression models were generated, and linear regression t-tests were performed for both graphs. There is a weak, yet significant positive correlation between seizure duration and both seizure and paralysis susceptibility (Figure 8).

Figure 2: Seizure susceptible genotypes exhibited appropriate seizure frequency phenotype. Though eas^{alaE13} and eas^{KO} were both confirmed as seizure susceptible strains, eas^{KO} had a much more pronounced phenotype.

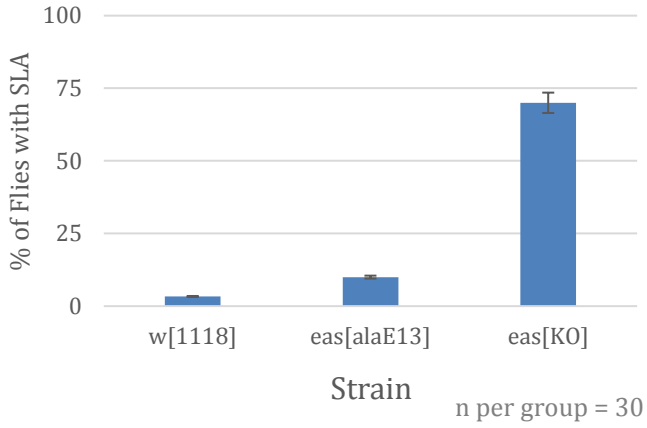


Figure 3: Caffeinated flies exhibited an appropriate increase in seizure susceptibility compared to non caffeinated counterparts. eas^{KO} flies were most affected by caffeination, with a p-value of 0.022 for the eas^{KO} AC group.

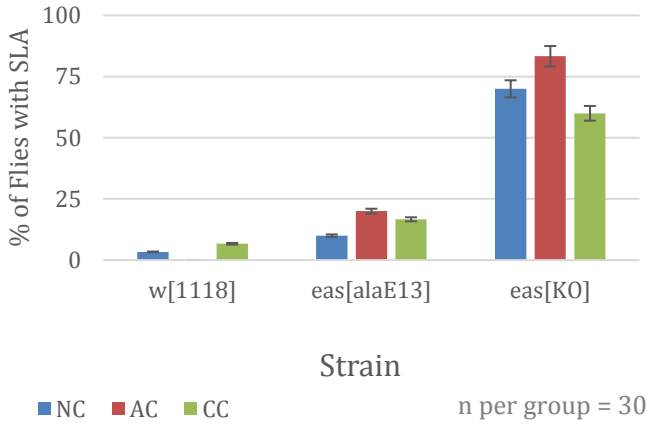


Figure 4: KD successfully ameliorated seizure susceptibility phenotypes. In non caffeinated *eas*^{alaE13} flies, KD completely eliminated SLA. KD also decreased SLA in *eas*^{KO} flies ($p < 0.05$).

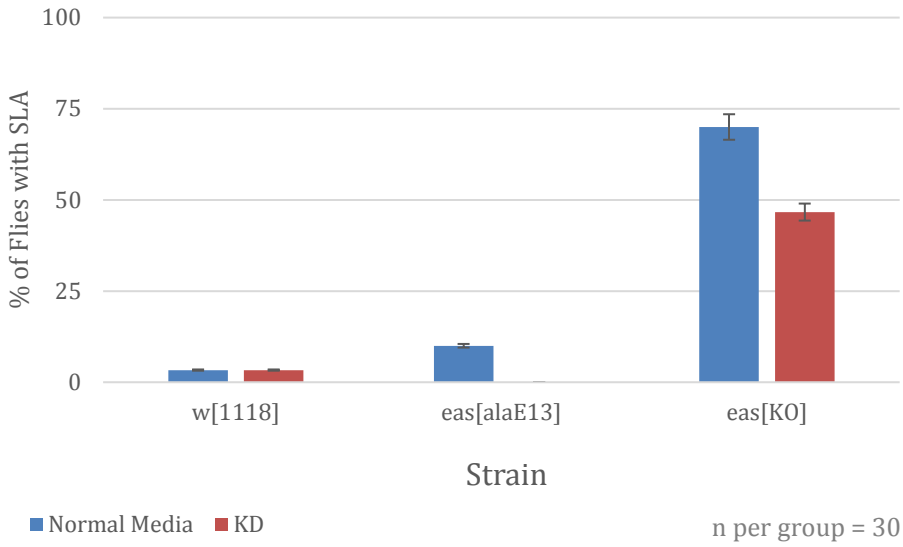


Figure 5: Overall, DR is not an effective mode of dietary restriction in relation to seizure phenotype amelioration. Though DR significantly decreased seizure susceptibility in *eas*^{alaE13} flies, for both *w*^{1118} and *eas*^{KO} flies, it increased seizure susceptibility. Due to this incongruence, and the fact that with every subsequent z-test performed, the probability of a Type I error increases, it was concluded that DR does not have anticonvulsant properties (*$p < 0.05$).

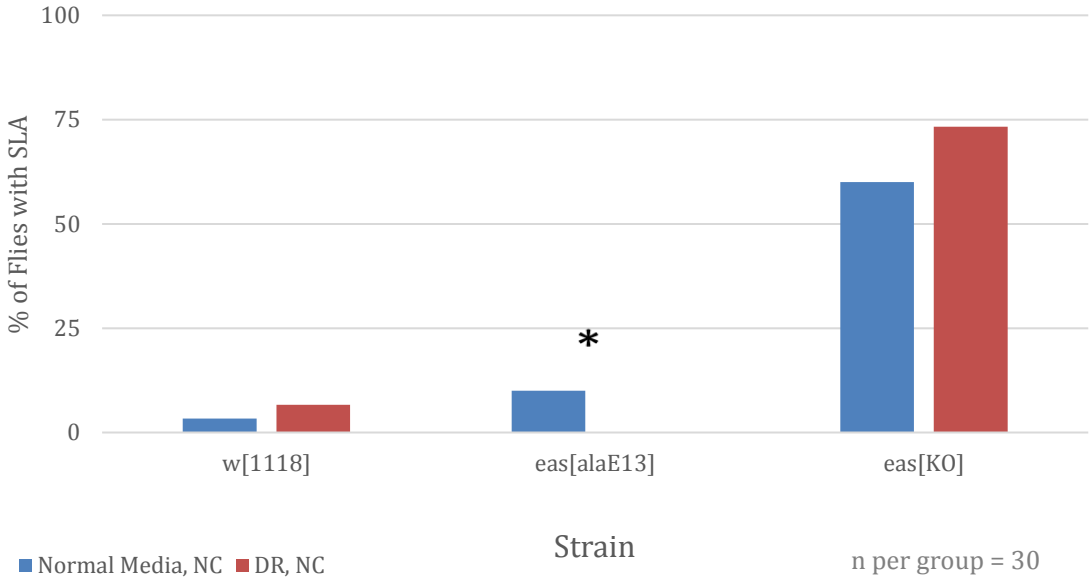
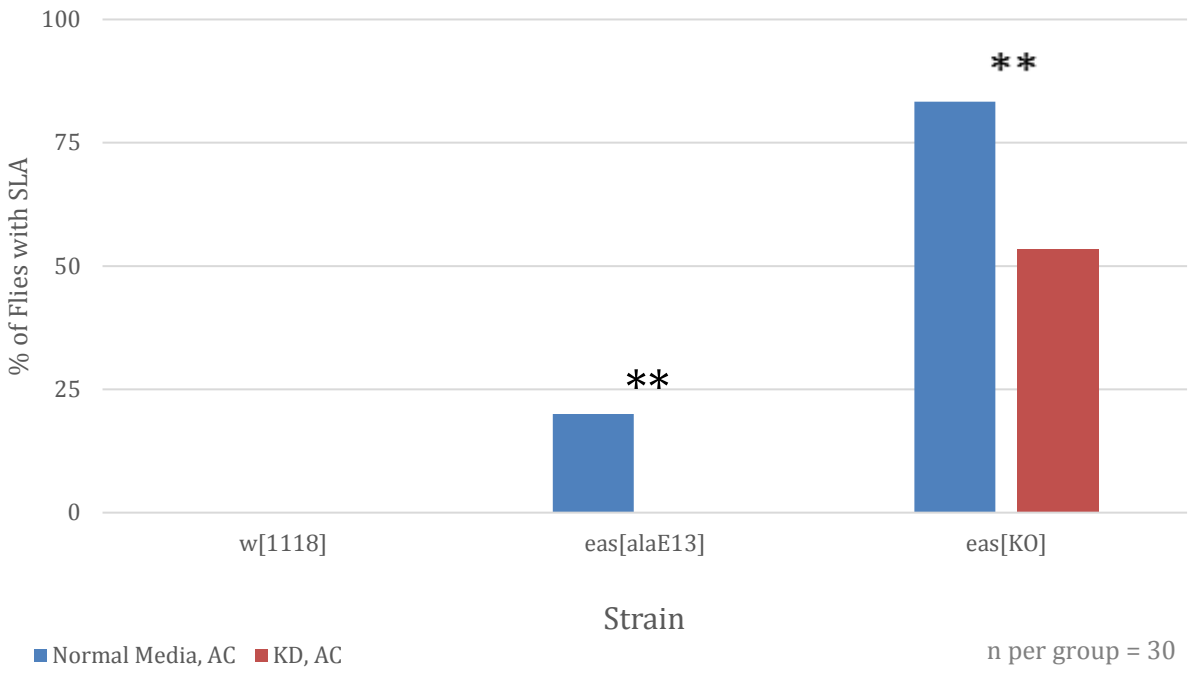


Figure 6: KD modulates the effects of caffeine exposure on seizure susceptibility. Under both **A.** acute and **B.** chronic caffeine exposure, flies that were concurrently exposed to caffeine and raised on KD exhibited markedly lower seizure frequencies (*p < 0.05, **p < 0.01).

A.



B.

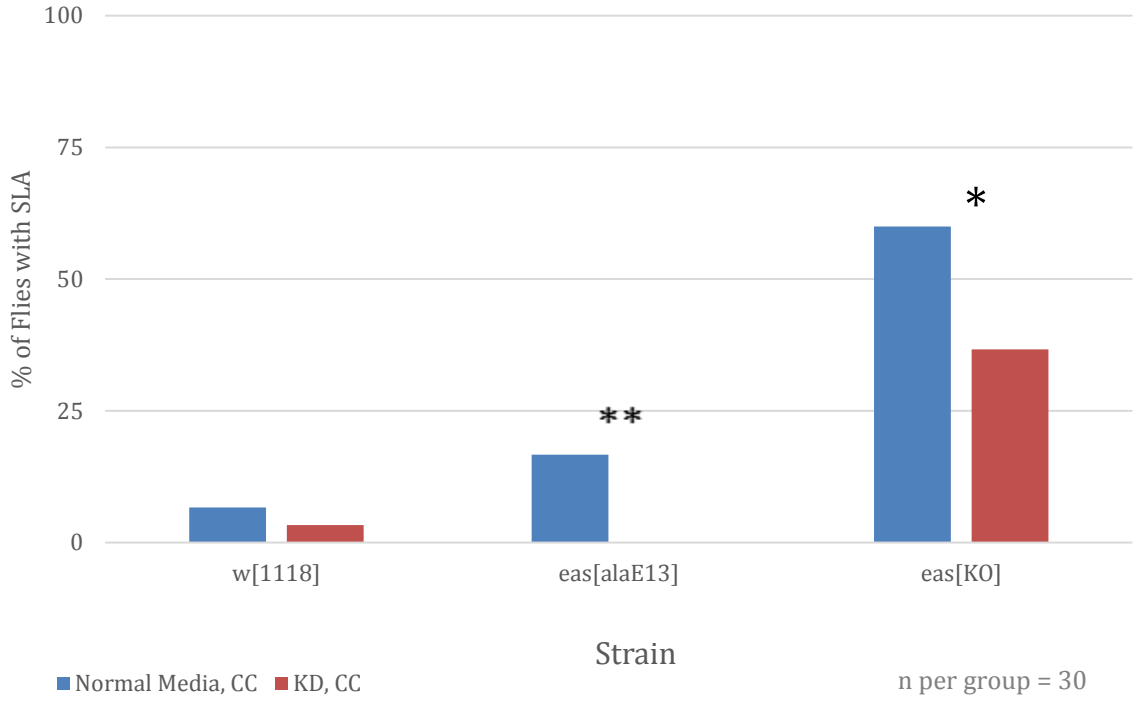
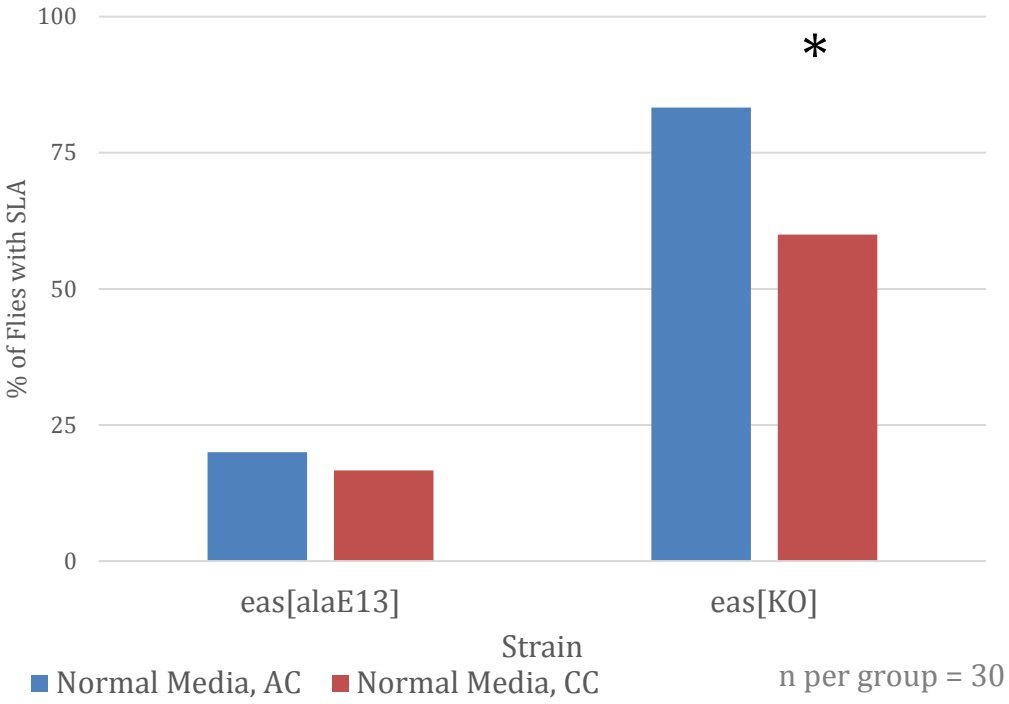


Figure 7: Chronic exposure to caffeine has protective effects under certain circumstances.

A. *eas*^{KO} flies on normal media has a significant decrease in seizure susceptibility when chronically (as opposed to acutely) exposed (*p < 0.05).



B. Both strains of *eas* flies on DR media had significant decreases in seizure susceptibility when chronically (as opposed to acutely) exposed (*p < 0.05, **p < 0.01).

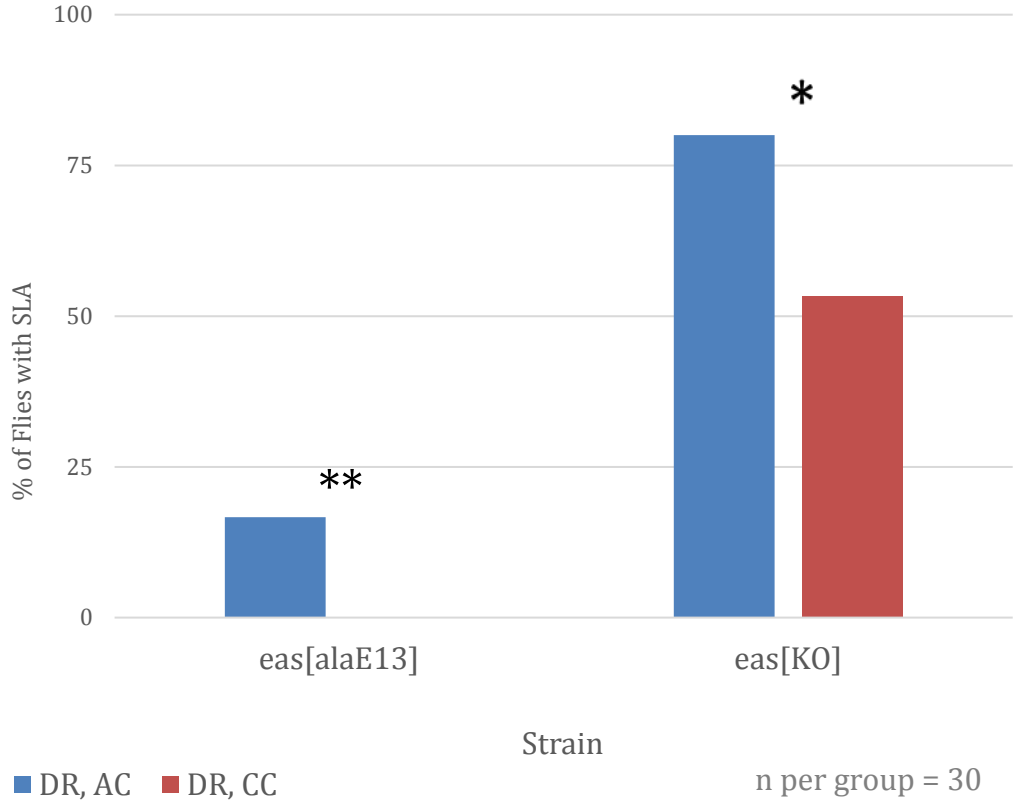
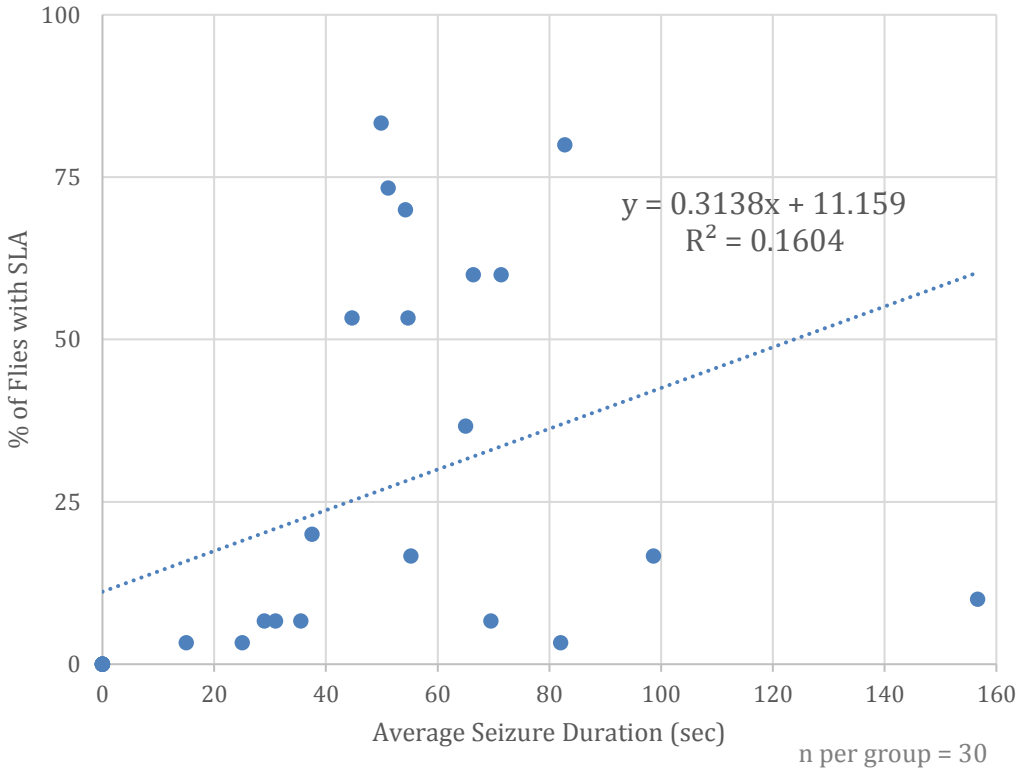
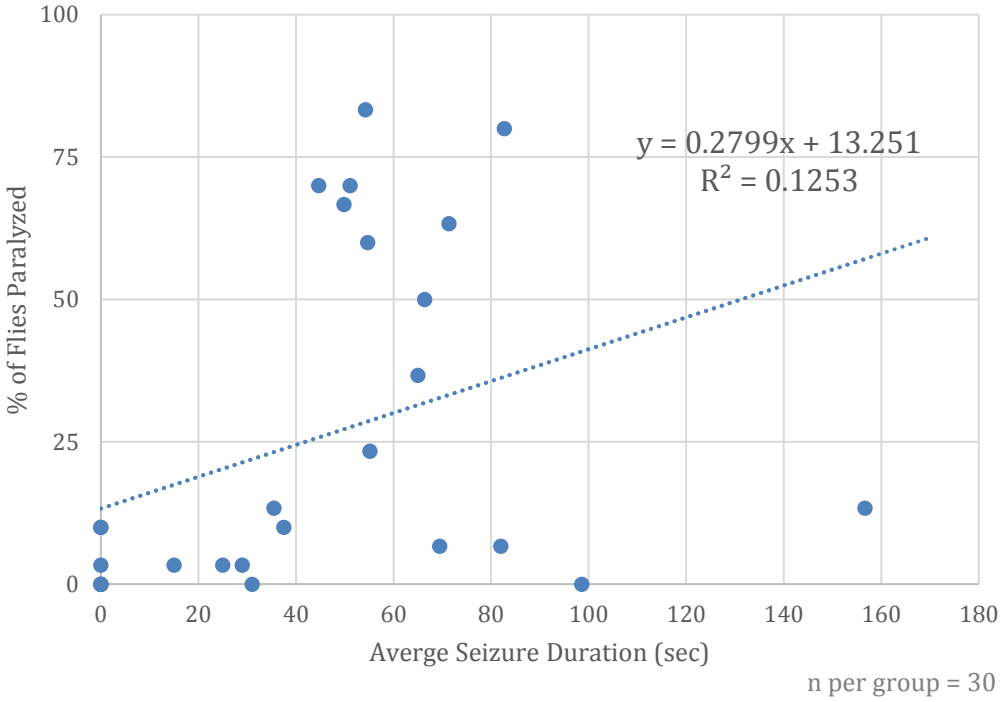


Figure 8: There are positive correlations between seizure duration and seizure/paralysis frequency.



A. Seizure duration and % SLA are positively correlated ($p < 0.05$), though the association ($R^2 = 0.1604$) is weak.



B. Seizure duration and % paralyzed are positively correlated ($p < 0.05$), though the association ($R^2 = 0.1253$) is weak.

DISCUSSION

In this study, it was shown that KD counteracts the effects of caffeine on seizure susceptibility in *Drosophila melanogaster*. Additionally, for flies on normal media or DR, chronic caffeine exposure resulted in lower seizure susceptibility compared to flies that were acutely exposed. The inherent seizure susceptibility of *eas* strain fruit flies, the tendency of caffeine to promote seizure phenotype, and the protective effects of KD in the absence of caffeine were all confirmed in this study. Taken together, this study was the first to inspect the concurrent effects of dietary restriction and caffeine exposure, and to test DR as an alternative to KD.

It was predicted that KD would mediate the effects of caffeine in a *Drosophila* epilepsy model due to the past success of dietary restriction in counteracting the physiological effects of caffeine (Mashal, 2016) and the long history of KD as a treatment for epilepsy and seizures (Hartman & Vining, 2007; Yudkoff et al., 2007; Li, O'Leary, & Tanner, 2017). The data presented here suggest that KD does counteract the inductive effect of caffeine, as *eas* strain flies had lower caffeine-induced susceptibilities when on KD relative to those on the normal media. Caffeine functions as a stimulant by promoting glutamate release (John, Kodama, & Siegel, 2014). Because KD contributes to the increased metabolism of glutamate into GABA (Yudkoff et al., 2007), the findings of this study can be explained by the opposing impacts of KD and caffeine on glutamate levels in the brain. By counteracting caffeine-induced glutamate release, KD can significantly impede the effects of caffeine on seizure susceptibility. Thus, this finding is novel because it implies that any glutamate inhibitor could be successful when modulating caffeine.

This study also aimed to clarify the potential protective effects of chronic (as opposed to acute) caffeine exposure. The protective effects of chronic caffeine have been investigated previously in mouse epilepsy models (Germé et al., 2015), but never in flies, or in relation to dietary restriction. Chronic caffeine did not have a significant protective effect in KD flies; this could be explained by the fact that KD-mediated increases in lipid supply can counteract caffeine-induced glutamate increases, regardless of exposure frequency (since ketosis is accomplished through chronic exposure) (Kossoff & McGrogan, 2005). However, in both seizure susceptible flies that were raised on normal media and flies that were raised on DR media, flies that were chronically exposed had significantly lower % SLA than flies who were

acutely exposed. This could be related to the ability of chronic caffeine exposure to protect the brain from injury during severe or prolonged seizures (Rigoulot et al., 2003). The fact that the protective effect was present in this study, where seizure induction was performed only once on each subject, implies that there is an underlying mechanism affected by chronic exposure that causes a decrease in both seizure susceptibility and brain damage during seizures.

It was also hypothesized that DR would be an effective alternative to KD when treating epilepsy. DR, due to its impacts on TOR signaling, has been associated with many health benefits, from increased lifespan (Lee & Longo, 2016) to modulation of drug addiction (Mashal, 2016). Because of its ability to counteract the effects of drugs such as caffeine, this study tested DR as a new strategy for epilepsy treatment. However, DR had no overall impact on seizure susceptibility *in vivo*. Because of this, no tests were done on the effect of DR and caffeine concurrently on seizure susceptibility. DR is proposed to function primarily via its effects on TOR signaling pathway (Katewa & Kapahi, 2010), whereas KD functions through manipulation of lipid availability (Bough & Dingledine, 2009). This difference between the two modes of dietary restriction can explain why DR did not act similarly to KD in seizure suppression. Thus, it is proposed that any alternate form of anticonvulsant dietary restriction should impact glutamate (or βHB) levels in a way that is similar to that of KD in order to have the desired effects. Since both KD and DR have well documented health benefits, it may be tempting to equivalate the two diets and their effects. The overall lack of success of DR in modulating seizure susceptibility indicates the perils of this mode of thinking.

It was hypothesized that seizure duration (the measure of seizure intensity used in this study) would be impacted by dietary restriction and caffeine exposure in a similar fashion to which seizure susceptibility was. There was no significant relationship between seizure duration, diet, and caffeine exposure, suggesting that nutritional profile is not a predictor for seizure intensity as was hypothesized. However, it was found that both seizure and paralysis susceptibility are positively correlated with seizure duration, indicating that susceptibility can be a predictor of intensity.

The use of *Drosophila* as a model organism in this study proved to be highly advantageous. Firstly, due to the high frequency of reproduction of fruit flies (Lutz, 1948), it was easy to obtain the near 1,000 flies needed for the experiment. Additionally, the *eas* fly strain is a reliable epilepsy model, and due to its bang-sensitivity, seizure induction was readily

accomplished via a vortex (Pavlidis, Ramaswami, & Tanouye, 1994; Li, 2016). Lastly, because this study involved extensive dietary restrictions, unhealthy levels of caffeine exposure, and seizure induction, the use of an invertebrate organism ensured an absence of cruelty in the experimental design (Lagadic & Caquet, 1998). Both mouse and fruit fly studies allow human diseases to be modeled and studied in less complex organisms; however, fruit fly studies should be performed as preliminaries prior to mouse studies, due to the lower costs, larger sample sizes, and lesser degree of ethical concerns present in fly studies.

However, (Pavlidis, Ramaswami, & Tanouye, 1994) noted a difficulty in obtaining viable *eas* strain males, and this study also faced that difficulty; the ratio of males to females was roughly 1:10 across all seizure susceptible groups. For this reason, the study only collected seizure induction data for female flies. Studies including both male and female flies are highly desirable, and thus future research should aim to replicate the results outlined in this study in males as well. This is especially relevant in light of discoveries which suggest that, in many cases, males and females respond differently to drug treatments (Woosley, 1998; Miller, 2001; Wizemann & Pardue, 2001).

Secondly, seizures were not analyzed for average velocity or average distance traveled due to incompatibility between the Fly Analysis Excel Visual Basic program and the equipment used to record the seizures. In the future, fly locomotion paths during SLA should be analyzed to provide a fuller picture of the combined effects of KD and caffeine; for example, a locomotion path with many sharp turns in a very close proximity would indicate a more intense seizure. Improvements to the analysis software such as the development of an algorithm that would combine distance traveled during SLA, seizure duration, and seizure velocity values into one *Drosophila*-specific seizure severity index would also be a valuable tool in studying seizure susceptible *Drosophila*.

Additionally, caution should be exercised when interpreting the significance of % SLA results due to the fact that two proportion z-tests were performed to test each relationship, and the probability of committing a Type I error increases with every statistical test performed; however, to combat this source of error, each group contained a minimum of 30 subjects, age and sex were constant amongst every subject, and groups of interest were outlined prior to the performance of statistical tests (no tests were performed outside of those previously-outlined groups).

Lastly, this study was restricted to comparing the effects of two dietary regimes with pre-established protective effects; flies with genetically manipulated TOR signaling were not included in the study, and metabolism assays were not performed. To confirm what the data suggest concerning the combined effects of KD and caffeine on seizure susceptibility and glutamate levels, biomolecular research techniques should be used to study the interactions of molecules such as glutamate, GABA, βHB, and acetyl-CoA, both on the cellular and organismal level. Additionally, further research should be done to clarify the biochemical pathways that contribute to the protective effects of chronic caffeine exposure.

The findings of this study suggest that patients who suffer from seizures and are on KD are at least partially safeguarded from the effects of caffeine and other glutamate promoters. This can impact the quality of life and the nutritional decisions of those diagnosed with epilepsy.

CONCLUSION

This is the first study to investigate the concurrent effects of dietary restrictions and caffeine exposure on seizure susceptibility. It is also the first study to test DR as an alternative to the ketogenic diet. It was found that KD modulates the effects of caffeine on seizure susceptibility in *Drosophila* and that the protective effects of chronic caffeine exposure extend to models in which dietary restriction is a factor. These findings hold implications for the quality of life and nutritional counseling of epilepsy patients and imply that KD may be an effective safeguard against glutamate-promoter-induced seizure susceptibility increases.

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REFERENCES

Alshuaib, W. B., & Mathew, M. V. (2006). Caffeine modulates potassium currents in *Drosophila* neurons. *International Journal of Developmental Neuroscience*, *24*(4), 249–253. doi: 10.1016/j.ijdevneu.2006.03.002

Aso, Y., Hattori, D., Yu, Y., Johnston, R. M., Iyer, N. A., Ngo, T.-T., … Rubin, G. M. (2014). The neuronal architecture of the mushroom body provides a logic for associative learning. *ELife*, *3*. doi: 10.7554/elife.04577

Ben-David, G., Miller, E., & Steinhauer, J. (2015). *Drosophila* spermatid individualization is sensitive to temperature and fatty acid metabolism. *Spermatogenesis*, *5*(1). doi: 10.1080/21565562.2015.1006089

Bough, K., & Dingledine, R. (2009). KETOGENIC DIET | Anticonvulsant Mechanisms of a Ketogenic Diet. *Encyclopedia of Basic Epilepsy Research*, 681–687. doi: 10.1016/b978-012373961-2.00030-8

Chippindale, A. K., Leroi, A. M., Kim, S. B., & Rose, M. R. (1993). Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. Nutrition and the cost of reproduction. *Journal of Evolutionary Biology*, *6*(2), 171–193. doi: 10.1046/j.1420-9101.1993.6020171.x

Dill, L. J. (2013). Seizure disorder. *Journal of the American Academy of Physician Assistants*, *26*(7), 49–50. doi: 10.1097/01.jaa.0000431518.51314.3d

During, D. (1993). Extracellular hippocampal glutamate and spontaneous seizure in the conscious human brain. *The Lancet*, *341*(8861), 1607–1610. doi: 10.1016/0140-6736(93)90754-5

Emran, S., Yang, M., He, X., Zandveld, J., & Piper, M. D. W. (2014). Target of rapamycin signalling mediates the lifespan-extending effects of dietary restriction by essential amino acid alteration. *Aging*, *6*(5), 390–398. doi: 10.18632/aging.100665

Faivre, J.-F., & Findlay, I. (1990). Action potential duration and activation of ATP-sensitive potassium current in isolated guinea-pig ventricular myocytes. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, *1029*(1), 167–172. doi: 10.1016/0005-2736(90)90450-3

Fisher, R. S., Boas, W. V. E., Blume, W., Elger, C., Genton, P., Lee, P., & Engel, J. (2005). Epileptic seizures and epilepsy: Definitions proposed by the International League Against Epilepsy (ILAE) and the International Bureau for Epilepsy (IBE). *Epilepsia*, *46*(4), 470–

472. doi: 10.1111/j.0013-9580.2005.66104.x

Fogle, K. J., Hertzler, J. I., Shon, J. H., & Palladino, M. J. (2016). The ATP-sensitive K channel is seizure protective and required for effective dietary therapy in a model of mitochondrial encephalomyopathy. *Journal of Neurogenetics*, *30*(3-4), 247–258. doi: 10.1080/01677063.2016.1252765

Germé, K., Faure, J.-B., Koning, E., & Nehlig, A. (2015). Effect of caffeine and adenosine receptor ligands on the expression of spike-and-wave discharges in Genetic Absence Epilepsy Rats from Strasbourg (GAERS). *Epilepsy Research*, *110*, 105–114. doi: 10.1016/j.eplepsyres.2014.11.022

Hartman, A. L., & Vining, E. P. G. (2007). Clinical aspects of the ketogenic diet. *Epilepsia*, *48*(1). doi: 10.1111/j.1528-1167.2007.00914.x

John J, Kodama T, Siegel JM. (2014). Caffeine promotes glutamate and histamine release in the posterior hypothalamus. *Am J Physiol Regul Integr Comp Physiol*. 2014;307(6):R704–R710. doi:10.1152/ajpregu.00114.2014

Karmen, A., Wróblewski, F., & Ladue, J. S. (1955). Transaminase activity in human blood. *Journal of Clinical Investigation*, *34*(1), 126–133. doi: 10.1172/jci103055

Katewa, S. D., & Kapahi, P. (2010). Dietary restriction and aging, 2009. *Aging Cell*, *9*(2), 105–112. doi: 10.1111/j.1474-9726.2010.00552.x

Kossoff, E. H., & McGrogan, J. R. (2005). Worldwide Use of the Ketogenic Diet. *Epilepsia*, *46*(2), 280–289. doi: 10.1111/j.0013-9580.2005.42704.x

Lagadic, L., & Caquet, T. (1998). Invertebrates in Testing of Environmental Chemicals: Are They Alternatives? *Environmental Health Perspectives*, *106*, 593. doi: 10.2307/3433810

Lee C, Longo V. Dietary restriction with and without caloric restriction for healthy aging. *F1000Res*. 2016;5:F1000 Faculty Rev-117. Published 2016 Jan 29. doi:10.12688/f1000research.7136.1

Lewis, E. B. (1960). A new standard food medium. *Drosophila Information Service*, *34*(117), 1-55.

Li, J. (2016). *Ketogenic diet and seizure susceptibility in a whole-animal Drosophila melanogaster model: Effects and mechanisms* (Unpublished bachelor's thesis). Mount Holyoke College, South Hadley, MA.

Li, J., O'Leary, E. I., & Tanner, G. R. (2017). The ketogenic diet metabolite beta-

hydroxybutyrate (β-HB) reduces incidence of seizure-like activity (SLA) in a K_{ATP} - and GABA b -dependent manner in a whole-animal *Drosophila melanogaster* model. *Epilepsy Research*, *133*, 6–9. doi: 10.1016/j.eplepsyres.2017.04.003

Lutz, F. E. (1948). *Field book of insects: of the United States and Canada, aiming to answer common questions*. New York: G.P. Putnam's Sons.

Mashal, R. (2016). *Development of a caffeine addiction paradigm to examine how dietary restriction and level of TOR signaling modulate the effects of drugs.* Unpublished manuscript.

Matsagas, K., Lim, D. B., Horwitz, M., Rizza, C. L., Mueller, L. D., Villeponteau, B., & Rose, M. R. (2009). Long-term functional side-effects of stimulants and sedatives in *Drosophila melanogaster*. *PLoS ONE*, *4*(8). doi: 10.1371/journal.pone.0006578

McNally, M. A., & Hartman, A. L. (2012). Ketone bodies in epilepsy. *Journal of Neurochemistry*, *121*(1), 28–35. doi: 10.1111/j.1471-4159.2012.07670.x

Melø, T. M., Nehlig, A., & Sonnewald, U. (2006). Neuronal–glial interactions in rats fed a ketogenic diet. *Neurochemistry International*, *48*(6-7), 498–507. doi: 10.1016/j.neuint.2005.12.037

Metaxakis, A., & Partridge, L. (2013). Dietary Restriction Extends Lifespan in Wild-Derived Populations of Drosophila melanogaster. *PLoS ONE*, *8*(9). doi: 10.1371/journal.pone.0074681

Miller, M. A. (2001). Gender-Based Differences in the Toxicity of Pharmaceuticals—The Food and Drug Administrations Perspective. *International Journal of Toxicology*, *20*(3), 149–152. doi: 10.1080/109158101317097728

Neal, E. G., Chaffe, H., Schwartz, R. H., Lawson, M. S., Edwards, N., Fitzsimmons, G., … Cross, J. H. (2008). The ketogenic diet for the treatment of childhood epilepsy: A randomised controlled trial. *The Lancet Neurology*, *7*(6), 500–506. doi: 10.1016/s1474-4422(08)70092-9

Parker, L., Howlett, I. C., Rusan, Z. M., & Tanouye, M. A. (2011). Seizure and epilepsy: Studies of seizure disorders in *Drosophila*. *International Review of Neurobiology Recent Advances in the Use of Drosophila in Neurobiology and Neurodegeneration*, 1–21. doi: 10.1016/b978-0-12-387003-2.00001-x

Pascual, A., Chaminade, M., & Préat, T. (2005). Ethanolamine kinase controls neuroblast

divisions in Drosophila mushroom bodies. *Developmental Biology*, *280*(1), 177–186. doi: 10.1016/j.ydbio.2005.01.017

Pavlidis, P., Ramaswami, M., & Tanouye, M. A. (1994). The Drosophila easily shocked gene: A mutation in a phospholipid synthetic pathway causes seizure, neuronal failure, and paralysis. *Cell*, *79*(1), 23–33. doi: 10.1016/0092-8674(94)90397-2

Petroff, O. A. C. (2002). Book review: GABA and glutamate in the human brain. *The Neuroscientist*, *8*(6), 562–573. doi: 10.1177/1073858402238515

Petroff, O. A. C., Rothman, D. L., Behar, K. L., & Mattson, R. H. (1996). Low brain GABA level is associated with poor seizure control. *Annals of Neurology*, *40*(6), 908–911. doi: 10.1002/ana.410400613

Rigoulot, M.-A., Leroy, C., Koning, E., Ferrandon, A., & Nehlig, A. (2003). Prolonged Low-dose Caffeine Exposure Protects Against Hippocampal Damage but Not Against the Occurrence of Epilepsy in the Lithium-pilocarpine Model in the Rat. *Epilepsia*, *44*(4), 529–535. doi: 10.1046/j.1528-1157.2003.50502.x

Roca, D. J., Schiller, G. D., & Farb, D. H. (1988). Chronic caffeine or theophylline exposure reduces gamma-aminobutyric acid/benzodiazepine receptor site interactions. *Molecular Pharmacology*, *33*(5), 481–485.

Saras, A., Wu, V. V., Brawer, H. J., & Tanouye, M. A. (2017). Investigation of seizure-susceptibility in a *Drosophila melanogaster* model of human epilepsy with optogenetic stimulation. *Genetics*, *206*(4), 1739–1746. doi: 10.1534/genetics.116.194779

Solinas, M., Ferré, S., You, Z.-B., Karcz-Kubicha, M., Popoli, P., & Goldberg, S. R. (2002). Caffeine induces dopamine and glutamate release in the shell of the nucleus accumbens. *The Journal of Neuroscience*, *22*(15), 6321–6324. doi: 10.1523/jneurosci.22-15-06321.2002

Stafstrom, C. E., & Carmant, L. (2015). Seizures and epilepsy: An overview for neuroscientists. *Cold Spring Harbor Perspectives in Medicine*, *5*(6). doi: 10.1101/cshperspect.a022426

Tennessen, J. M., Barry, W. E., Cox, J., & Thummel, C. S. (2014, June 15). Methods for studying metabolism in *Drosophila*. Retrieved from https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4048761/

Thiele, E. A. (2003). Assessing the efficacy of antiepileptic treatments: The ketogenic diet. *Epilepsia*, *44*, 26–29. doi: 10.1046/j.1528-1157.44.s7.4.x

Turturro, A., Witt, W. W., Lewis, S., Hass, B. S., Lipman, R. D., & Hart, R. W. (1999). Growth Curves and Survival Characteristics of the Animals Used in the Biomarkers of Aging Program. *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, *54*(11). doi: 10.1093/gerona/54.11.b492

Weindruch, R., & Walford, R. L. (1990). The Retardation of Aging and Disease by Dietary Restriction. *The Quarterly Review of Biology*, *65*(2), 256–256. doi: 10.1086/416808

Wiegand, G., & Remington, S. J. (1986). Citrate synthase: Structure, control, and mechanism. *Annual Review of Biophysics and Biophysical Chemistry*, *15*(1), 97–117. doi: 10.1146/annurev.bb.15.060186.000525

Wizemann, T. M., & Pardue, M. L. (2001). *Exploring the biological contributions to human health does sex matter?* Washington, D.C.: National Academy Press.

Woosley, R. L. (1998). From Bench to Bedside: Role of Gender-Based Therapeutics in the Clinical Care of Women. *Journal of Women's Health*, *7*(1), 21–23. doi: 10.1089/jwh.1998.7.21

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. *Journal of Neuroscience*, *29*(35), 11029–11037. doi: 10.1523/jneurosci.1653-09.2009

Yudkoff, M., Daikhin, Y., Melø, T. M., Nissim, I., Sonnewald, U., & Nissim, I. (2007). The ketogenic diet and brain metabolism of amino acids: Relationship to the anticonvulsant effect. *Annual Review of Nutrition*, *27*(1), 415–430. doi: 10.1146/annurev.nutr.27.061406.093722