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Correlation between sleep and lifespan in drosophila melanogaster

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CORRELATION BETWEEN SLEEP AND LIFESPAN IN *DROSOPHILA MELANOGASTER*

by

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ABSTRACT

Sleep has previously been associated with lifespan. Monitoring sleep in any given fly over their lifetime facilitates the ability to predict the lifespan of that given fly. Using this estimate, lifespan can potentially correlate with biological age to identify when health parameters have declined.

To confirm that the prediction algorithm could identify short and long-lived flies, glutathione levels in heads and bodies were compared between two groups. The results showed this to be consistent in the bodies of wild-type *Canton S* male flies, and showed that glutathione was decreased in the predicted biologically older flies. These data show that glutathione levels may provide a mechanism that links biological aging with lifespan. These novel methods provide a process by which lifespan can be estimated in alive flies to be used to identify factors that correlate with biological aging and test interventions that may increase lifespan.

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1. INTRODUCTION

Sleep is essential for all animal species, but it is unclear why such negative effects are felt from sleep deprivation and sleep loss. Prolonged sleep deprivation is capable of leading to death in flies (Shaw, Tortoni, Greenspan, & Robinson, 2002) rats (Rechtschaffen, Everson, & Toth, 2001) and humans (Schenkein & Montagna, 2006), but there is no significant research to show any sleep associated biological changes leading up to death.

Drosophila share a majority of its sleep homeostatic response with the mammalian sleep homeostatic response and also displays molecular, behavioral, and genetic characteristics similar to mammalian sleep, which makes it a justifiable model to research for sleep (Hendricks et al., 2000; Huber et al., 2004; Shaw, Cirelli, Greenspan, & Tononi, 2000). Unlike many mammalian model organisms, *Drosophila'*s short lifespan also permits correlating the form of sleep with longevity (Bushey, Hughes, Tononi, & Cirelli, 2010; G.R. et al., 2014). It is known that sleep fragmentation occurs in aging *Drosophila* as well as aging humans and is associated with the loss of sleep consolidation (Koh, Evans, Hendricks, & Sehgal, 2006). As humans age, sleep changes, and these changes could affect survival. Data suggest that sleep is significantly associated with lifespan variation and it may even affect longevity in *Drosophila* (Bushey et al., 2010).

Predicting lifespan could have many implications in the research setting, but no research has been found to successfully correlate a certain sleep

parameter with lifespan to make such statistical predictions. The ability to predict how long an individual can live could potentially change the way health is perceived. Using these predictions and potentially finding differences dependent on how long lifespan is predicted per individuals, specific biomarkers could be tested to find how they differ between the different individuals. Once a potential biomarker is found, new applications could be created to extend lifespan.

1.1. *DROSOPHILA* AS A MODEL ORGANISM

Drosophila and mammals share a majority of their sleep homeostatic response, which makes *Drosophila* a valid model to use in measuring sleep behavior and other sleep aspects (Cirelli, 2009; Hendricks et al., 2000; Huber et al., 2004; Shaw et al., 2000). It is also known that unlike many mammalian model organisms, *Drosophila* have a short enough lifespan which allows monitoring of sleep over the life as well as assessment of sleep patterns on longevity (Bushey et al., 2010; G.R. et al., 2014). Circadian rhythms which affect sleep and wake cycles also play a large role in sleep for both humans and flies (Dijk & Lockley, 2002; Huang et al., 2002; Koh et al., 2006). As humans age, sleep changes, and these changes could affect survival. It has been suggested that sleep is significantly associated with lifespan variation and it may even affect longevity in *Drosophila* (Bushey et al., 2010; Koh et al., 2006; Ohayon, Carskadon, Guilleminault, & Vitiello, 2004). However, there is only minimal research in which sleep is correlated with longevity, and even then, it just shows the quantity and quality of the sleep changes leading up to death (Cirelli, 2012).

Previous literature has found biological markers that correlate with sleepiness in both humans and flies (Cirelli & Tononi, 2001; Seugnet, Boero, Gottschalk, Duntley, & Shaw, 2006). Biomarkers correlating with aging have also been found, such as changes in sleep reliability and variability (Koudounas, Green, & Clancy, 2012), levels of alpha-2-macroglobulin in the blood (Ma, Li, Zhang, & Tong, 2004), and p16 protein which is a tumor suppressor protein (Krishnamurthy et al., 2004; Ressler et al., 2006). However, there is no successful research relating sleep parameters associated with lifespan to a particular biomarker.

1.2. MATHEMATICAL MODELING

Previously, there are only minimal methods adopted that can predict lifespan. Measuring the motor activity in C. elegans can estimate when death will occur in individuals (Hsu, Feng, Hsieh, & Xu, 2009). With these methods, C. elegans have to be monitored over life to determine when motor activity starts to decline and when death will occur (Hsu et al., 2009). Mitoflash (mitochondrial flash) is a frequency-coded optical readout which measures energy metabolism and free-radical production, and can also predict lifespan in C. elegans at day 3 of life (Shen et al., 2014). Although this shows success in predicting lifespan, there is no statistical model that associates with sleep which can estimate potential length of life.

Previous literature in *Drosophila* attempted statistical modeling of sleep parameters with lifespan, specifically longest sleep duration. The R^2 for longest

daily sleep duration over lifetime ranged from 0.04-0.316 (Koudounas et al., 2012). However, when predicting lifespan in flies was implemented and measured, mean lifespans for the top and bottom quartiles of the longest sleep duration were not significantly different (Koudounas et al., 2012). This showed that the flies could not be statistically predicted to have different lifespans from one another.

Linear regression analysis is a data modeling approach in which a quantitative response and multiple quantitative predictor variables are required (Kutner, 2005). The log of the survival can be taken to improve the normality and constant variance assumptions of the model (Kutner, 2005). There are various ways to select a model, with one common method is based on the highest adjusted R-square with low AIC (akaike information criterion) value (Rosner, 2010).

All methods adopted for the statistical predictive model came from original work completed by Gayla R. Olbricht within the Department of Mathematics and Statistics at Missouri University of Science and Technology.

1.3. MEASURING SLEEP OVER A LIFETIME

Sleep is essential for all animal species, and yet even with extensive research, sleep is not completely understood. It is widely known sleep is essential on the daily basis to function, and lack of quality sleep can cause a decrease in health in any organism (Kripke, Garfinkel, & Wingard, 2002; Schenkein & Montagna, 2006; Shaw et al., 2002). Prolonged sleep deprivation is

even capable of leading to death in flies (Shaw et al., 2002) and humans (Schenkein & Montagna, 2006).

Insufficient sleep may lead to negative health consequences and eventually mortality (Kripke et al., 2002), but there are only short term experimentation supporting this actuality. Survival experiments have been completed in *Drosophila* to correlate sleep changes to lifespan (Bushey et al., 2010; Cirelli, 2012) and to compare lifespan with physical deterioration and disease (Linford, Bilgir, Ro, & Pletcher, 2013), but no longevity experiments have been used to find a biological marker that may potentially affect the sleep that leads up to death. Lifespan is always sought to be increased in any organism, and in flies, many studies have been capable of doing so (Min, Flatt, Kulaots, & Tatar, 2007; Peng et al., 2012; Vaiserman et al., 2008), but the ability to know a specific biomarker associated with sleep which could increase lifespan could be incredible for potentially all organisms.

1.4. ANTIOXIDANT LEVELS

Reactive oxygen species are chemically reactive molecules which contain oxygen, and although a natural byproduct of metabolism, in large quantities can cause damage to cell structures leading the body to oxidative stress (Bayir, 2005; Kandola, Bowman, & Birch-Machin, 2015). Previous studies have found that increased oxidative stress is linked to chronological aging (Brown & Naidoo, 2010), and with increased oxidative stress with age, sleep becomes negatively affected (Brown & Naidoo, 2010; Koh et al., 2006; Zou, Meadows, Sharp, Jan, &

Jan, 2000). When flies are treated with paraquat, an oxidative stress producing chemical, the sleep and wake cycles began to diminish and mirror that of aging sleep. Since oxidative stress is associated with aging, sleep affected by oxidative stress looks like sleep affected by aging. This showed that oxidative stress levels increase with age which is what affects the sleep and rhythms in *Drosophila* (Bonilla, Medina-Leendertz, Villalobos, Molero, & Bohórquez, 2006; Koh et al., 2006). Loss of the circadian rhythm in *Drosophila* also resulted in increased oxidative damage, supporting the hypothesis that oxidative stress is associated with sleep disruption (Beaver et al., 2012). The link between aging, oxidative stress, and sleep affect is unclear.

Aging correlates with an accumulation of reactive oxygen species (Berlett & Stadtman, 1997; Cadenas & Davies, 2000; Rusu, 1997; Sohal & Weindruch, 1996). The body's natural protection to increased levels of reactive oxygen species is antioxidants (Kandola et al., 2015), specifically glutathione (GSH) and its thiol component, cysteine (S. C. Lu, 2009; S.C. Lu, 2013). Glutathione is an important antioxidant which protects the body from reactive oxygen species, elements which can cause damage to cell structures. Cysteine is an amino acid which serves a structural role by being one amino acid in the tripeptide which makes up glutathione (S.C. Lu, 2013). Usually, oxidative stress is prevented through the action of antioxidants, but when there is an excess of reactive oxygen species, these natural defenses start to fail and oxidative stress occurs (Kandola et al., 2015). Aging seems to increase the levels of reactive oxygen species in the body due to a reduction of antioxidant levels which naturally

occurs with age from natural body processes (Dröge, 2003; Sohal & Weindruch, 1996). Other components which increase oxidative stress in the body include alcohol (Wu & Cederbaum, 2003) and smoking cigarettes (Rahman & MacNee, 1999).

Glutathione has previously been associated with fluctuations in the circadian rhythm in *Drosophila* and the biosynthesis of glutathione was found to be controlled by the circadian rhythm system (Beaver et al., 2012; Klichko et al., 2015). When an organism ages, their circadian rhythm weakens, and so with that, any associated biomarkers should be affected (Klichko et al., 2015). Evidence from previous studies found that certain antioxidants, such as methionine and cysteine-glycine, were both associated with decreasing with age in the head, but that glutathione and cysteine did not correlate with aging in the head (Klichko et al., 2015). Results from the head consist mainly of the brain, but may also include other components of the head such as the eyes. However, when certain tissues from the main organs, liver, kidney and heart, were measured in aging mice, it was concluded that glutathione decreased with age (Hazelton & Lang, 1980; Pouget et al., 2015). More research has to be completed in this field with *Drosophila* to determine if the aging glutathione pattern is conserved across organisms.

1.5. CHRONOLOGICAL VERSUS BIOLOGICAL AGING

Chronological age is actual age in years, whereas biological age is the rate at which your body ages (Figure 1.1) (Dontsov & Krut'ko, 2015; Ries &

Pöthig, 1984). This includes the age at which the organs, cells, and systems age and deteriorate (Dontsov & Krut'ko, 2015; Ries & Pöthig, 1984). Chronological age is much easier to measure; however you usually can't measure biological age since by the time you know the actual biological age of a model the organism has already died, which is why it important to be able to predict biological age. In the past, biological markers have been measured by using various statistical procedures, but none have been found to correlate with sleep and lifespan (Lebreton, Burnham, Clobert, & Anderson, 1992; Ludwig & Smoke, 1980).

1.6. ALZHEIMER'S DISEASE IN *DROSOPHILA*

Alzheimer's disease is a human neurodegenerative disease. Two major components of the disease include amyloid precursor protein (APP) and beta-secretase (BACE), the two proteins manipulated to create the Alzheimer's model in *Drosophila* (Greeve et al., 2004). Using *Drosophila* as a model organism is ideal in understanding the changes that occur in sleep prior to onset of Alzheimer's disease and the changes that continue as the disease progresses over lifetime (Bilen & Bonini, 2005).

The Gal4/UAS system is widely used to genetically create an Alzheimer's model in *Drosophila* (Gerstner, Lenz, Chan, Pfeiffenberger, & Pack, 2014). In the instance of the genotype used, Gal4 is a transcription factor which binds to the upstream activating sequence (UAS) and regulates the specific DNA expression (Duffy, 2002). GeneSwitch is a protein which activates the Gal4 system when initiated with Mifepristone (Osterwalder, Yoon, White, & Keshishian, 2001). The

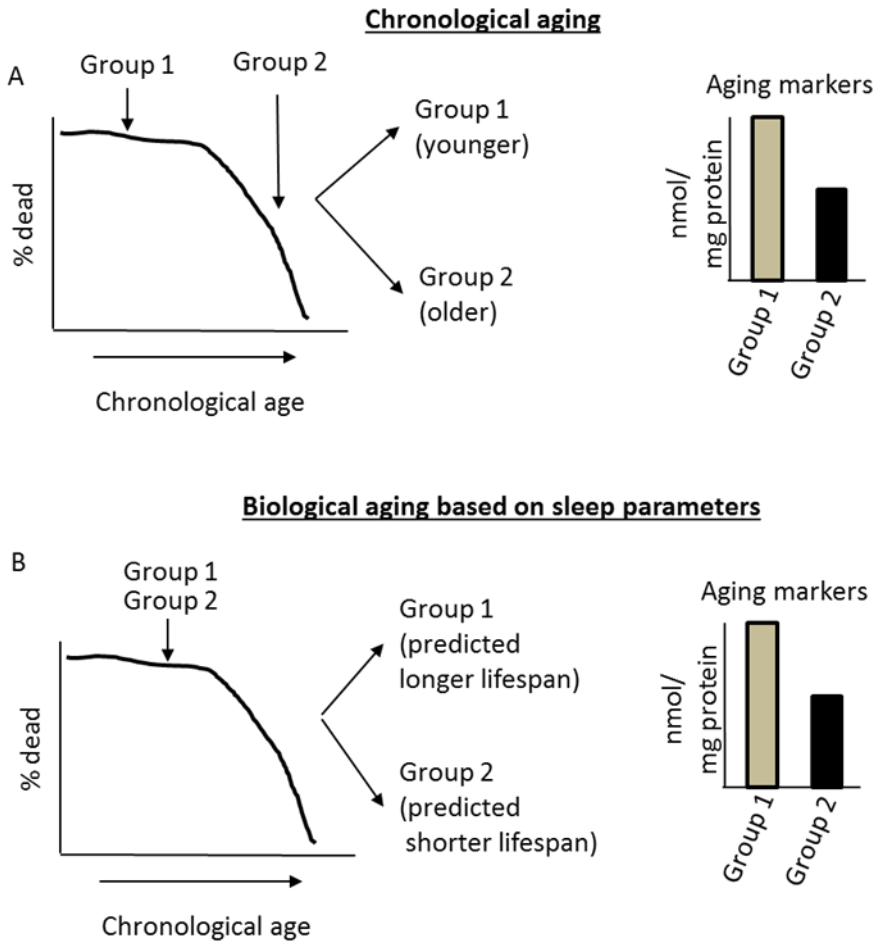


Figure 1.1. Protocol for testing for aging markers in flies. (A) Chronological aging shows differences in metabolic factors associated with aging. Such a factor could be molecules involved in neutralizing reactive oxygen species. (B) The scheme proposed here takes flies at the same chronological age.

GeneSwitch protein is tissue-specific to specify where the action of the Gal4/UASsystem will take place (Osterwalder et al., 2001). Amyloid precursor protein (APP) and β-secretase (BACE) both play a role in Alzheimer's disease (Greeve et al., 2004). Amyloid precursor protein plays an important role in the pathophysiology is Alzheimer's disease and β-secretase is an APP cleaving enzyme (Greeve et al., 2004). The γ-secretase cleavage of BACE is important in

only cleaving a single protein (Greeve et al., 2004). APP and BACE transgenic flies can be crossed with Gal4 GeneSwitch flies to create progeny, DaGSw>UAS-APP^{42}, UAS-BACE. When fed Mifeprostone, the Gal4 GeneSwitch protein binds to APP and BACE and Alzheimer's disease is induced (Greeve et al., 2004; Mhatre et al., 2014).

Sleep disruptions, although not sleep quantity, seem to associate with preclinical Alzheimer's disease (Ju et al., 2013) and individuals with increased sleep fragmentation have an increased risk of developing Alzheimer's disease during their lifetime (Lim, Kowgier, Yu, Buchman, & Bennett, 2013). These sleep disruptions also show evidence of contributing to formation of the amyloid-β plaques that are highly associated with Alzheimer's disease (Kang et al., 2009).

1.7. METABOLIC ENERGY LEVELS

The restoration of energy stores in the brain, normally reduced during wake, is thought to be a large purpose of sleep (Benington & Craig Heller, 1995; Zimmerman et al., 2004). Glucose, glycogen, ketone bodies, and triglycerides are all such energy stores available to be measured in *Drosophila* (Tennessen, Barry, Cox, & Thummel, 2014). Glucose, ketone bodies, and triglyceride levels have all been shown to associate with sleep (Chikahisa, Shimizu, Shiuchi, & Séi, 2014; Gottlieb et al., 2005; Telliez, Bach, Dewasmes, Leke, & Libert, 1998), but it has been shown that gene expression directly related to brain glycogen metabolism displays variation during sleep and wake (Petit, Tobler, Allaman,

Borbély, & Magistretti, 2002) and there are changes in glycogen levels associated with sleep and wake (Zimmerman et al., 2004).

1.8. CLIMBING ASSAY IN *DROSOPHILA*

Drosophila naturally climbs upwards against gravity after being tapped or pushed down (Kamikouchi et al., 2009), and staying healthy is dependent on this normal physical activity (Balasubramani et al., 2014). As flies age, physical activity decreases, and so does the ability to climb upwards (Balasubramani et al., 2014). Even when tested at various gravity levels, climbing activity of flies became impaired with age (Balasubramani et al., 2014).

1.9. CALORIC RESTRICTIONS AND SLEEP

Studying caloric restriction in model organisms dates back to as early as the 1930's (McCay, Crowell, & Maynard, 1935). Early data pertaining to caloric restriction was completed in rodents (McCay et al., 1935), and has shown that a nutritional restriction of diet will successfully increase lifespan in rodents (Yu, Masoro, & McMahan, 1985) and subsequently in yeast (Jiang, Jaruga, Repnevskaya, & Jazwinski, 2000), *C. elegans* (Klass, 1977; Lee, Klopp, Weindruch, & Prolla, 1999), and even monkeys (Colman et al., 2014). More recent data has been acquired in *Drosophila* (Min et al., 2007; Piper, Mair, & Partridge, 2005; Sohal & Weindruch, 1996). Further research has shown that high yeast diets actually increase the age-associated sleep issues that occur in *Drosophila* (Yamazaki et al., 2012).

Previous research has shown that for a lower calorie diet, it's not necessarily the lower caloric intake which increases lifespan, but rather the other components that are decreased during caloric decrease which affect lifespan such as protein or amino acids (Mair, Piper, & Partridge, 2005; Piper et al., 2005). However, other literature supports that it is the low yeast concentration that increases lifespan (Min et al., 2007). It has been found that for the *Drosophila* diet, yeast concentration and caloric intake are correlated, which is why the caloric differences in the diet are based off of levels of yeast (Min et al., 2007). For these reasons, the diet used was based off of purely yeast concentration to see if lifespan could be extended (Min et al., 2007).

1.10. GOALS OF THESIS

The goals presented in this thesis were to overall find a biomarker that correlates lifespan with sleep. The initial goal was to collect lifespan data in different genotypes, wild-type and circadian rhythm mutants, and manipulate the data to find a parameter to statistically model for lifespan prediction. Parallel to these experiments, other experiments were completed to determine if anything else could be correlated with sleep and lifespan (climbing assay, caloric restriction assay). Once sleep data was collected, this allowed the focus to be lifespan prediction to measure potential biological markers which could associate sleep and aging.

2. METHODS AND MATERIALS

2.1. *DROSOPHILA MELANOGASTER* GENETICS

Three genotypes of *Drosophila melanogaster* were selected for this study, a wild-type, *Canton S (CS),* and two circadian rhythm mutants. The *period* (*per*^{01}) gene plays an important role in determining the period length for circadian rhythms (Plautz et al., 1997), and the *per*^{iso} genotype, a circadian rhythm mutant, is a variant of the *period* mutant with an isogenized (genetically identical) X chromosome. The *cycle* gene is involved in the origination of biological rhythms by acting as a transcription factor that binds to RNA to activate transcription of *period* and *timeless* (*tim*) genes (Rutila et al., 1998). The *cycle* (*cyc*^{01}) genotype is the second circadian rhythm mutant used in the subsequent studies.

2.2. DESCRIPTION OF *DROSOPHILA MELANOGASTER* HUSBANDRY

Drosophila Melanogaster were placed in standard 75mm x 25mm diameter polystyrene vials (Figure 2.1) with up to 30 flies per vial on a standard sucrose, agar, yeast, molasses, and corn syrup diet. The flies were housed in an enclosed incubator at 25°C on a 12:12 light:dark (LD) schedule.

2.3. SLEEP MEASUREMENTS AND ANALYSIS

Males and virgin females were collected for three days and housed separately in vials of 15-25 flies per vial. Flies aged 4-7 days old were placed into individual glass tubes measuring 3mm x 65mm long and loaded into

monitors for data collection to commence at 9 A.M. the following morning in constant darkness (DD) or a 12 hour light:dark (LD) schedule. Each monitor holds 32 individual flies and using an infrared beam to identify movement, records activity of each fly for every minute (Figure 2.2). Food tubes were

Figure 2.1. Polystyrene vials used to house the *Drosophila melanogaster* in the laboratory.

changed every 3-4 days and for flies in DD conditions, food was changed under low red lights to avoid light induced behaviors in the flies. Activity is measured using the DAM2 system by Trikinetics (Trikinetics, Waltham, MA, USA) as described previously (Hendricks et al., 2000; Shaw et al., 2000). Total activity is recorded in 1 minute bins 24 hours a day, and each minute of data is then downloaded using an in-house program then converted into either a 1 (wake) or 0 (sleep) (sleep-wake data) by another in-house program. Sleep is empirically defined as five continuous minutes of inactivity (Shaw et al., 2000). Data

collection occurs until time deemed for each experiment. Raw data was converted into daily sleep and sleep statistics using an in-house program. This program converts raw data from the Trikinetics system to hourly activity and determines sleep parameters for each fly on a daily basis: total sleep, average day bout duration, average night bout duration, maximum day bout duration, maximum night bout duration, amount of day bouts, amount of night bouts, total daytime sleep, and total nighttime sleep.

Figure 2.2. *Drosophila* Activity Monitor used to record activity in individual flies for every minute.

2.4. SURVIVAL IN COMPARISON TO SLEEP IN *DROSOPHILA*

Males and virgin females for each of the three genotypes mentioned (*CS*, per^{iso}, per^{01} and cyc^{01}) were loaded into monitors and kept in individual tubes with sleep being recorded until death. Both the DD environment, using approximately 96 flies per gender, and LD environment, using approximately 64 flies of each

gender for *CS* and approximately 96 flies per gender for per^{iso} and cyc^{01}, was completed for survival data. Tubes were changed with new food every 3-4 days, and all deaths were recorded. Sleep data was checked daily and a survival curve was created to compare deaths between genders and environments.

2.5. STATISTICAL ANALYSIS FOR COMPARING ACTUAL TO PREDICTED LIFESPAN

Using the converted individual 0-1 data, Dr. Gayla R. Olbricht from the Department of Mathematics and Statistics created a statistical model, specifically using multiple linear regression, to predict lifetime in individual flies. Multiple linear regression is the modeling of two or more explanatory variables (predictors) and a response variable (current sleep bout). The transition probabilities for staying awake, or the likelihood that a fly will stay awake at any given minute (a_11) as well as transition probabilities for staying asleep, or the likelihood a fly will stay asleep at any given minute (a_00) were calculated for each day the fly was alive. The overall proportion of time spent asleep (pi_0) and overall proportion of time awake (pi_1) were also calculated (proportion of time asleep (pi_0)=(number of minutes asleep)/total number of minutes alive, proportion of time awake (pi_1)=(number of minutes awake)/total number of minutes alive, transition probability of staying asleep (a_00)=(number of times a transition from sleep at time i to sleep at time i+1) / total number of times a transition out of the sleep state, transition probability of staying awake (a_11)=(number of times a transition from wake at time i to wake at time i+1) / total number of times a transition out of the wake state). These parameters were

run through a scatterplot matrix in the JMP statistical package to determine predictors for a sleep model. Using these predictors, akaike information criterion (AIC) is used to determine the best model to use for the data using the adjusted R^2. (G.R. et al., 2014) (methods adopted from Dr. Gayla Olbricht in the University of Missouri Department of Mathematics and Statistics).

2.6. STATISTICALLY PREDICTING LIFESPAN IN *DROSOPHILA*

Using the transition probabilities initially calculated with the 0-1 data in previous methods (pi_0, a_00, and a_11), as well as the transition probabilities square terms, a model is created dependent on the mean of first differences of daily transition probabilities, standard deviation of first differences of daily transition probabilities, as well as the probabilities of sleep over the lifetime up to a given day (X). The first differences of these transition probabilities were calculated by subtracting values from one day from the previous day over the fly's lifetime. This model can then be used to predict lifespan in the given flies at any certain day (X).

2.7. ANTIOXIDANT LEVELS FOUND IN *DROSOPHILA*

For detecting GSH, Cysteine, and other antioxidants in *Drosophila*, heads were detached from bodies in *CS* flies and kept in -80° overnight. GSH standards were suspended in a serine-borate (SB) buffer, which was combined with a solution of N-(1-pyrenyl)-maleimide (NPM)/acetonitrile and HPLC-grade water for 5 minutes in a machine timed reaction. Hydrochloric acid was added to stop the

reaction and standards were refrigerated during sample preparation. Groups of 50 fly heads and bodies were homogenized, centrifuged, and supernatant extracted and processed according to the same protocol as the standard solutions. Samples were transferred to HPLC vials. Samples and standards were run on the HPLC system to quantify GSH. The HPLC system, Dionex Ultimate 3000, consisted of a Finnigan™ SpectraSYSTEM SCM1000 vacuum membrane degasser, a Finnigan™ SpectraSYSTEM P2000 gradient pump, a Finnigan™ SpectraSYSTEM AS3000 Autosampler, and a Finnigan™ SpectraSYSTEM FL3000 fluorescence detector (A-ex=330 nm and A-em=376 nm) (Thermo Electron Corp., Austin, TX, USA). A Bradford Assay was then completed to normalize results from the HPLC method. Bradford solution (Bio-Rad protein dye) was combined with protein standards and the same supernatant used for GSH detection, then poured into cuvettes and protein levels were measured using a spectrophotometer. The antioxidants measured were from the glutathione pathway and based off what the HPLC machine could read from the samples (Figure 2.3). (Methods adopted from Dr. Nuran Ercal of the Department of Chemistry at Missouri University of Science and Technology).

2.8. MEASURING ROS IN COMPARISION TO LIFE PREDICTION

Using a new set of male *CS* flies (either n=264 or n=512), sleep was monitored until day 30 of life based on a predicted model which gave an R^2 of 54.3%. The lifespan predictions were then used to split the flies into longer-lived and shorter-lived groups, and the heads and bodies of these two groups were

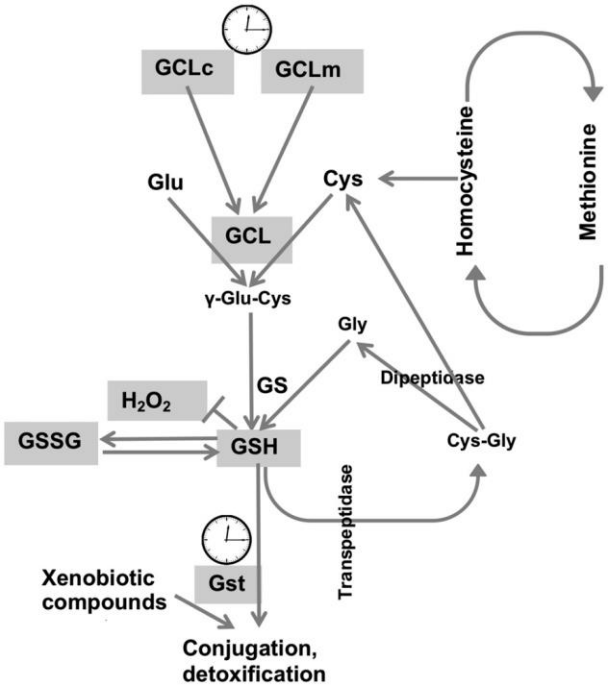


Figure 2.3. Synthesis and metabolism of glutathione and its regulation by the circadian system. GSH synthesis and Cysteine which is supplied via trans-sulfuration pathway and as a product of GSH degradation (Figure adopted from (Klichko et al., 2015))

used to measure glutathione and cysteine using the same methods as mentioned before.

2.9. INDUCTION OF ALZHEIMER'S IN *DROSOPHILA*

The Gal4/UAS system was used to genetically create an Alzheimer's model in *Drosophila* (Gerstner et al., 2014). In this instance, APP and BACE transgenic flies were crossed with Gal4 GeneSwitch flies to create the progeny, DaGSw>UAS-APP^{42}, UAS-BACE. When fed Mifeprostone, the Gal4 GeneSwitch protein binds to APP and BACE and Alzheimer's disease is induced (Greeve et al., 2004; Mhatre et al., 2014). Sixty-four flies of each gender were dosed with

0mg/mL, 12.5mg/mL, 25mg/mL, or 100mg/mL of mifepristone, and a survival experiment was continued until all deaths occurred in the flies. Sleep data was recorded for each day alive.

2.10. METABOLIC ENERGY LEVELS IN *DROSOPHILA*

Wild-type *CS* males and virgin females were collected and aged for 3-4 days, then loaded into LD for data collection to commence at 9 A.M. the next day. Baseline activity data was then collected for 24 hours, and the flies were starved and activity data for the starvation period was collected for another 24 hours. All fly tubes were labeled and frozen for 3-5 days at -80°C. Sleep data was analyzed as above and flies were clustered into three groups dependent on how sleep changed during starvation from baseline (Group 1: flies slept less when starved, Group 2: flies slept about the same starved as they did baseline, or Group 3: flies slept more when starved). The flies were split into proper groups and homogenized in phosphate buffered saline (PBS). Samples were centrifuged and supernatant was transferred into an Eppendorf tube. A well plate was setup using glucose infinity reagent to measure levels of glucose and glycogen (when mixed with starch reagent to break down the glucose polymer) in the samples, Wako Diagnostics total ketone bodies kit to test ketone levels in the samples, and triglyceride infinity reagent to measure triglyceride levels in the samples. The plate was incubated at 37°C for 5 minutes, centrifuged to rid of all bubbles, and all energy levels were measured using the BMG Labtech FLUOstar Omega plate reader (Tennessen et al., 2014; Zimmerman et al., 2004).

2.11. CLIMBING ASSAY COMPARATIVE TO SLEEP IN *DROSOPHILA*

The climbing assay consisted of 30 male and 30 virgin female wild-type *CS*; these flies were split in groups of 10 and placed between two polystyrene vials. The flies were tapped to the bottom of the vials and allowed 30 seconds to climb before being capped. Flies were counted in the lower and upper vials, and then loaded into tubes and monitors to have their sleep recorded for 4 days dependent on whether they climbed or not (Balasubramani et al., 2014; Feany & Bender, 2000). After four days of data recording, the flies were taken out and placed in vials of approximately 10 flies each until 21 days of age when the two groups had sleep recorded for another 4 days. After the second round of data recording, the flies were placed back in vials of approximately 10 and changed on new food every 2-3 days. Deaths were recorded for both groups until death of all flies.

2.12. CALORIC RESTRICTION AFFECTING SURVIVAL IN *DROSOPHILA*

For survival dependent on controlled diets, the same methods as all previous individual survival experiments were used for *CS* flies were kept in an LD environment until death. Diet of these flies consisted of either a high yeast, low sugar diet or a low yeast, high sugar diet, which reflected either high in calories (high yeast) or low in calories (low yeast) (Min et al., 2007). Diets also included corn meal and agar to gelatinize the food source, antibiotics (streptomycin and penicillin) to avoid ill flies, and tegosept and propionic acid as mold-inhibitors (Table 2.1).

Table 2.1. Different caloric diets used to show differences in how calories can affect lifespan; one consists of high yeast and the other low yeast.

	Yeast	Sucrose	Agar	Corn Meal	Antibiotics	Tegosept	Propionic acid	Distilled H_2O
High Yeast	8 g	2 g	0.3 g	2.6 g	50 µL	375 µL	375 µL	50 mL
Low Yeast	2 g	8 g	0.3 g	2.6 g	50 µL	375 µL	375 µL	51 mL

3. LIFESPAN PREDICTION AND A CORRELATING BIOMARKER RESULTS

3.1. SURVIVAL CURVES FOR DIFFERENT GENOTYPES

Data for lifespan was collected for all genotypes of interest including wild-type and circadian rhythm mutants. *Drosophila melanogaster* activity were monitored from day 3-5 of their life until death in both dark:dark (DD) or light:dark (LD) environments to observe the hour that deaths occurred. One wild-type genotype, *Canton S* skeath (n=55 for females in LD, n=54 for males in LD, n=192 for females in DD, n=192 for males in DD) (Figure 3.1), and *Canton S* from Paul Shaw's Lab (n=57 for females in LD and n=60 for males in LD) (Figure 3.2) were measured. Three circadian rhythm mutant genotypes, per^{iso} (n=89 for females in LD, n=89 for males in LD, n=184 for females in DD, n=192 for males in DD) (Figure 3.3), cyc^{01} (n=83 for females in LD, n=88 for males in LD, n=190 for females in DD, n=189 for males in DD) (Figure 3.4), and per^{01} (n=46 for females in LD and n=35 for males in LD) (Figure 3.5) were also measured. All deaths were documented and Kaplan Meiers were created to view the typical lifespan for each genotype in each environment. Log-rank tests were completed to test for significance. All genotypes showed a typical lifespan for *Drosophila*. Both genders of *Canton S* skeath and cyc^{01} died significantly quicker in the DD environment when compared to LD. In per^{iso} flies, an initial group of the individuals started dying significantly quicker in LD, then the rest of the individuals leveled out and died linearly in both environments about the same rate, although still significantly different from over the lifetime. The LD

environment provides light cues to the circadian rhythm mutants, which could initiate a change in lifespan. In the wild-type flies, the removal of light cues could repress the circadian rhythm, which would also affect lifespan.

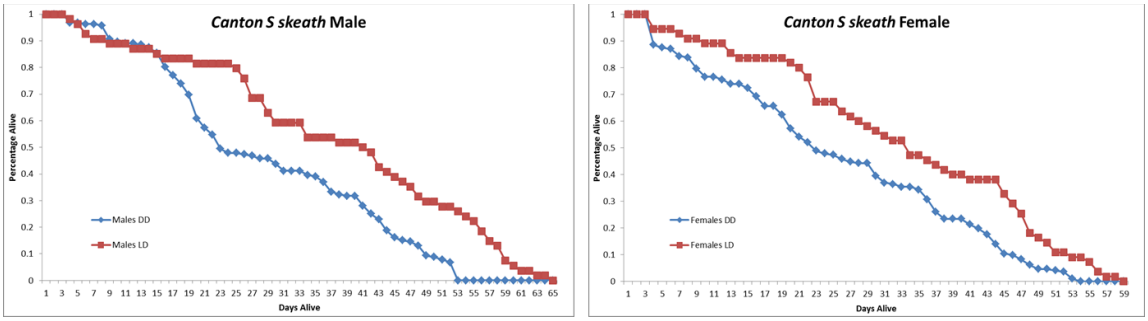


Figure 3.1. Survival in *Canton S* skeath flies between LD and DD environments. A Kaplan Meier between wild-type *Canton S* skeath in LD vs. DD environments with p-values < 0.001 by log-rank test.

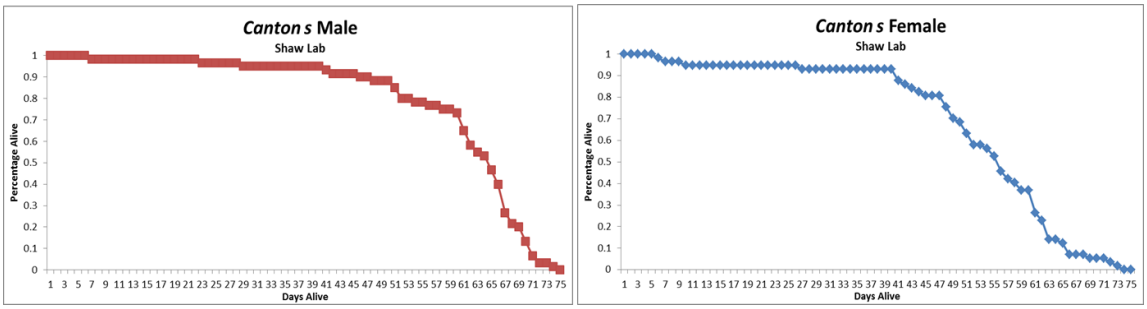


Figure 3.2. Survival in *Canton S* Shaw flies in LD. Survival curves of male and female *Canton S* flies from Paul Shaw's lab in LD environment.

3.2. CORRELATING SURVIVAL WITH SLEEP

Understanding how sleep changes with lifespan in the genotypes selected was important to use the sleep parameters to potentially predict lifespan. Sleep

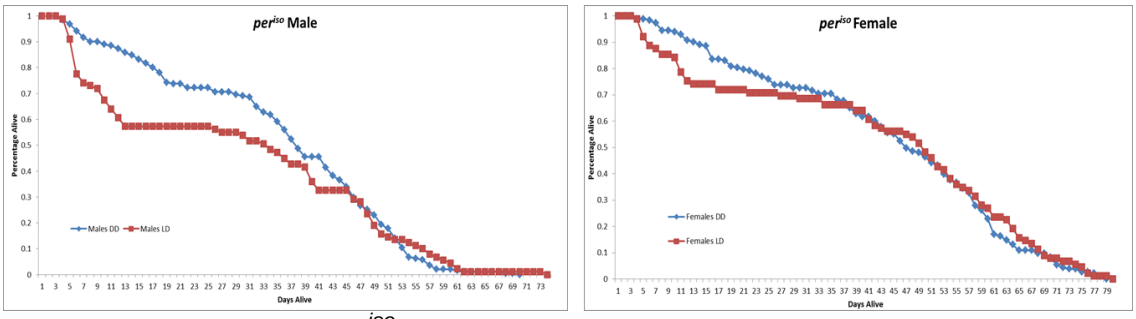


Figure 3.3. Survival in *per*^{iso} flies between LD and DD environments. A Kaplan Meier between *per*^{iso} flies in LD vs. DD environments with p-values < 0.05 by log-rank test.

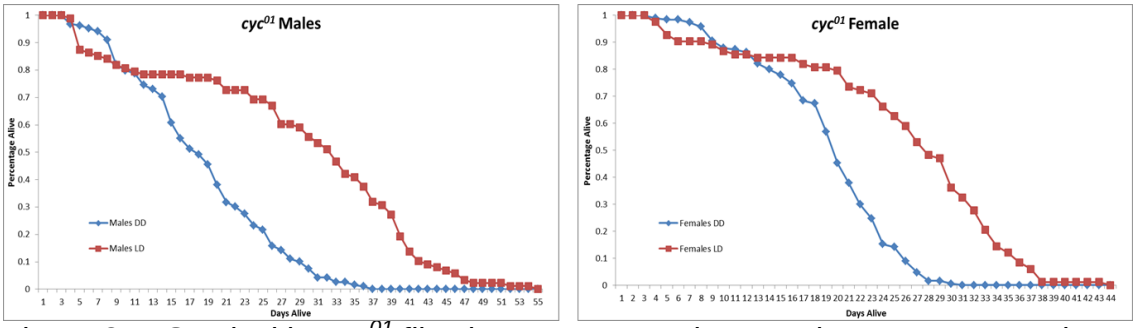


Figure 3.4. Survival in *cyc*^{01} flies between LD and DD environments. A Kaplan Meier between *cyc*^{01} flies in LD vs. DD environments with p-values < 0.05 by log-rank test.

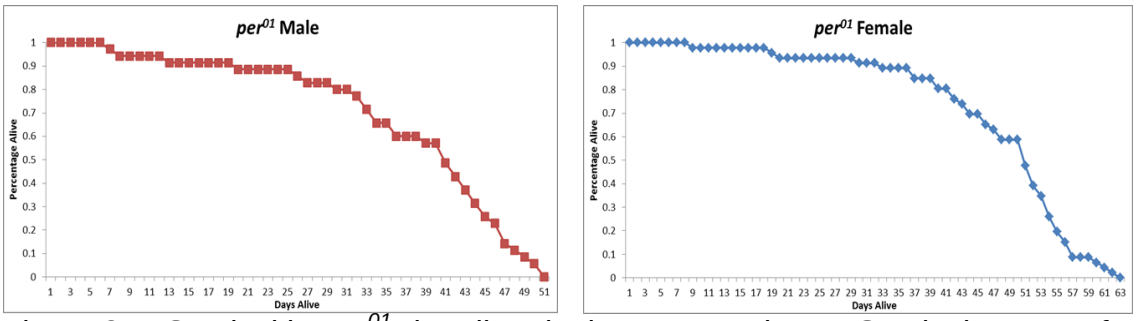


Figure 3.5. Survival in *per*^{01} circadian rhythm mutants in LD. Survival curves of male and female *per*^{01} flies in LD environment.

was measured over a lifetime in *Canton S*, per^{iso}, cyc^{01}, and per^{01} flies. Sleep in minutes during a 24 hour time period, averaged over three days, was graphed early in life (day 9-11) as well as later in life (day 54-56 for all genotypes except cyc^{01} at day 34-36 and per^{01} at day 44-46) to compare how sleep changes as the flies' age in the above genotypes. This was done in a light:dark environment to distinguish night from day. Sleep parameters were graphed to determine if they correlated with aging (Figure 3.6, 3.7, 3.8, 3.9, 3.10, 3.11, 3.12, 3.13, 3.14, and 3.15). In both genders for all genotypes, except per^{01} flies, sleep increased over the day with age. In *Canton S* Shaw males, cyc^{01} males and females, and per^{iso} males and females, night bout number and night bout duration significantly changed with age, showing a change in sleep consolidation. In these instances, the sleep either became more consolidated (longer sleep bout duration with fewer sleep bouts) or more fragmented (shorter sleep bout duration with more sleep bouts). In *Canton S* skeath males and females, and per^{01} males, there were some significant differences in the sleep parameters with age, which shows sleep does change with these particular genotypes and genders. However, *Canton S* Shaw females and per^{01} females showed no significant differences in sleep parameters with age, so there is no evidence to show their sleep changes.

3.3. STATISTICS CORRELATED WITH SURVIVAL

Using the mean of first differences, standard deviation of first differences, and sleep probabilities over the lifetime of each fly, multiple linear regression was used to predict lifespan. Actual lifespan was compared to predicted lifespan. A

statistical model was created, as described in methods, using sleep parameters over the lifespan of the genotypes mentioned above (*CS*, per^{iso}, per^{01} and cyc^{01}) to predict death in both 12:12 light:dark and 12:12 dark:dark conditions (Figure 3.16, 3.17, 3.18, 3.19, 3.20, 3.21, 3.22, and 3.23). This model works by comparing how sleep changes in each individual over a lifetime. The predicted death was then compared to the actual death of each fly to find accuracy of the model for each genotype represented by the R^2. A wider spread of flies between actual and predicted survival is easier to split the flies into two distinct groups (short and long-lived). The genotypes of the wider spread predictive graphs will

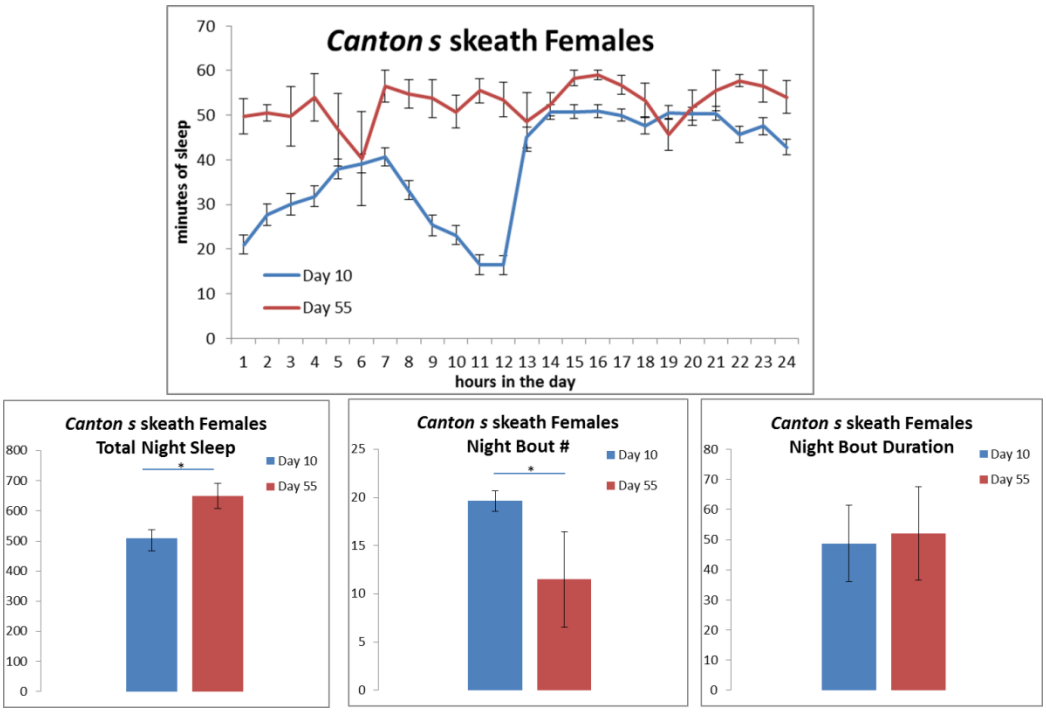


Figure 3.6. Comparative sleep and parameters as female *Canton S* skeath flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 55 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05 by student's t-test).

be selected for initial experimentation. For all genotypes tested environmentally in DD and LD, the predictive model in DD showed a wider spread of short versus long-lived flies, possibly due to larger sample sizes. Wild-type *CS* Shaw males and circadian rhythm mutant *cyc*^{01} males showed a statistically strong R^2 with a large spread of short and long-lived flies making them ideal candidates for predictive experimentation.

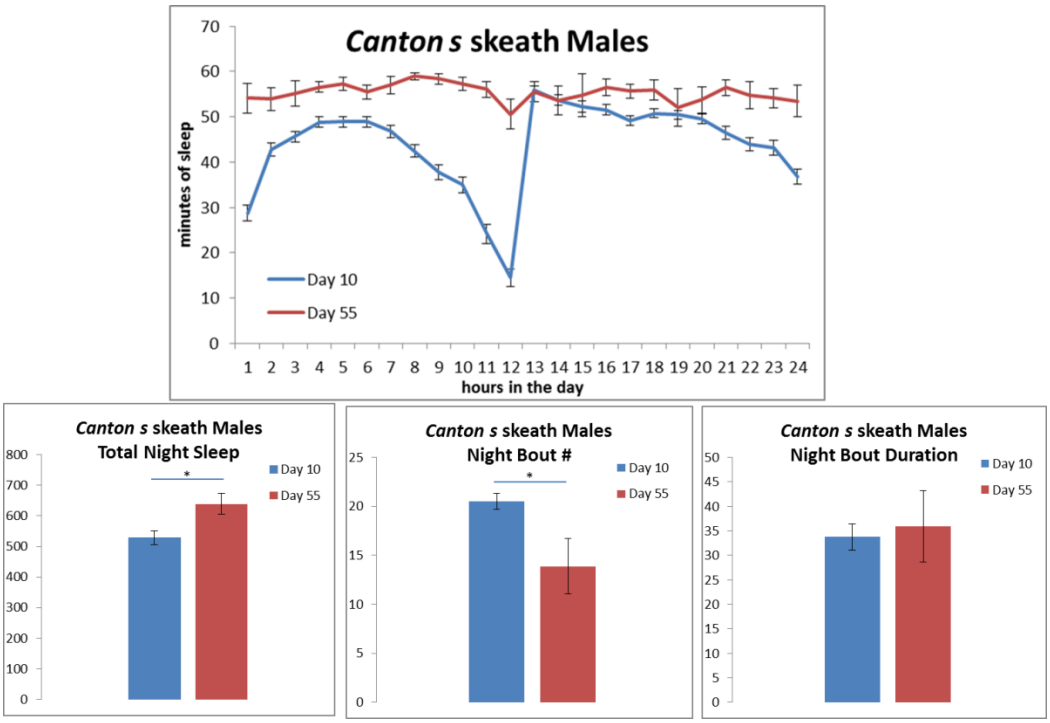


Figure 3.7. Comparative sleep and parameters as male *Canton S* skeath flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 55 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05 by student's t-test).

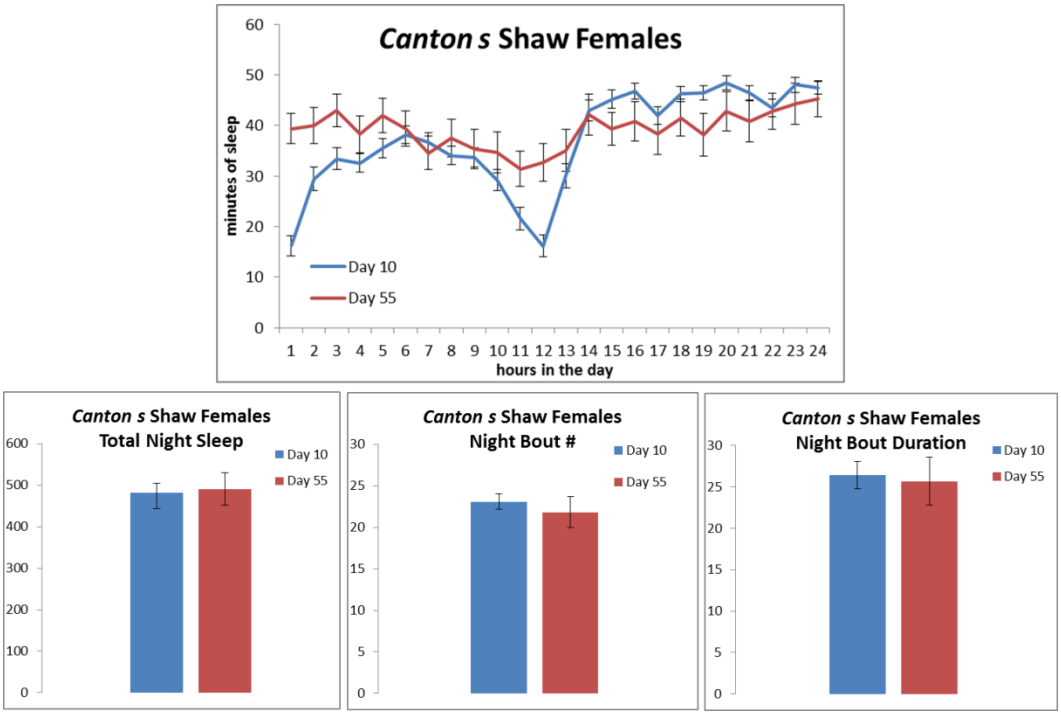


Figure 3.8. Comparative sleep and parameters as female *Canton S* Shaw flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 55 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (no significant differences).

3.4. RUNNING THE PREDICTIVE MODEL AT DIFFERENT DAYS

Once a descriptive model was successfully created for use over a lifetime, it was then possible to break the data up by certain days and test the flies' data up to that point to find how predictive it could be. With this, it was possible to determine at which point lifespan could be adequately predicted. With evaluation of sleep and the selection of a good model based off the R^2 and wide range of flies based on predicted lifespan versus actual lifespan, male wild-type *CS* Shaw flies as well as male circadian rhythm mutant cyc^{01} flies were initially selected for further biological testing. Using the model created by knowing the actual survival, a predictive model for each genotype selected was created at different time

points during the flies' life to determine at which point the model could most accurately predict death. It was determined that the model which most adequately predicted death in *CS* is at day 30 (R^2=39.4%) and in *cyc*^{01} is at day 15 (R^2=33.3%) to be able to do further biological testing (Figure 3.24). The most adequate model was based on a high R^2 with a wide range of flies between predicted and actual lifespan to accurately split the flies into short and long-lived groups.

3.5. ANTIOXIDANTS CORRELATED WITH CHRONOLOGICAL AGING

With previous literature showing significant differences in certain

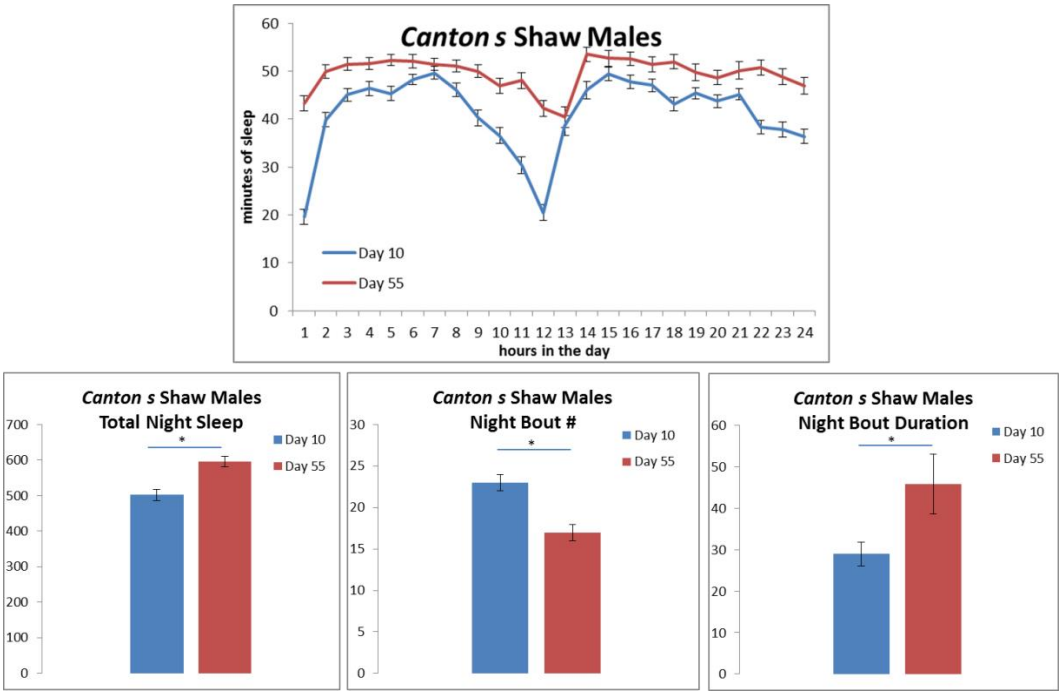


Figure 3.9. Comparative sleep and parameters as male *Canton S* Shaw flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 55 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05 by student's t-test).

antioxidant levels with age (Beaver et al., 2012), these experiments were replicated correlating these antioxidant levels with chronological age. Circadian rhythm mutant *cyc*^{01} flies were used to get a good reading of antioxidants based off of age and not circadian rhythm, since previous literature has proven that circadian rhythms affect GSH levels (Klichko et al., 2015). The flies were aged to either seven or twenty-one days and heads and bodies were analyzed separately. Using HPLC, derivitized antioxidant and aminothiol levels were measured. Three peaks showed a difference between young and old *cyc*^{01} flies. These peaks were identified as glutathione, cysteine, and an unknown peak. The

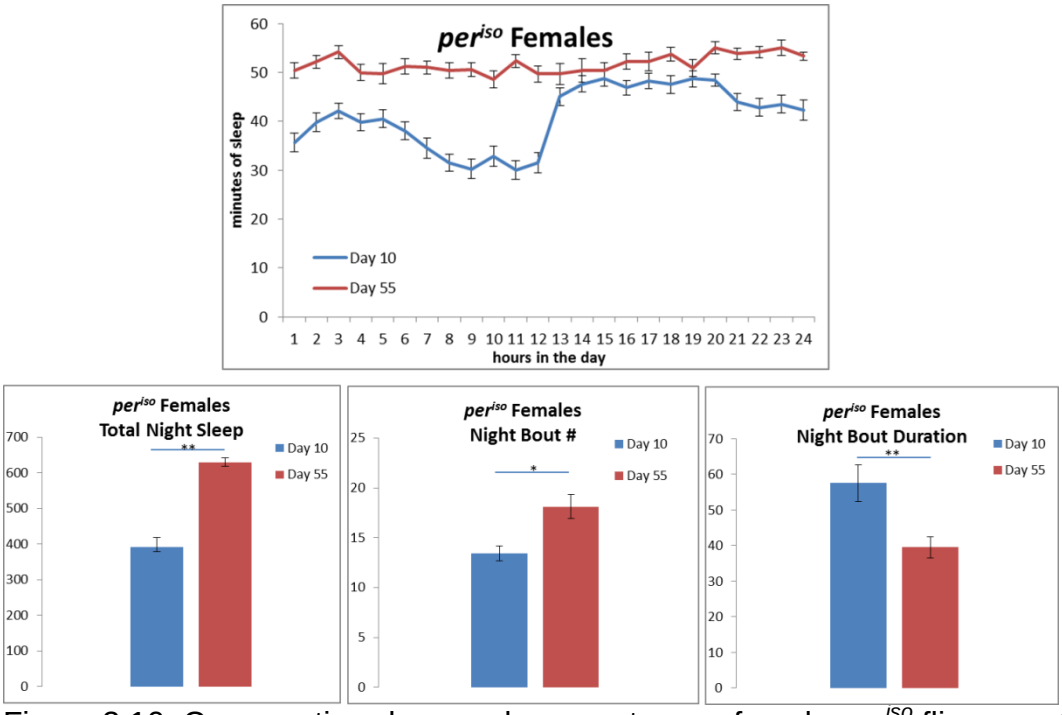


Figure 3.10. Comparative sleep and parameters as female *per*^{iso} flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 55 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05, **p-value<0.01 by student's t-test).

sample size was three biological replications with 25 heads or bodies per replication. There was a significant difference in glutathione in the different aged bodies (p-value<0.05), as well as a significant increase between the two groups in the heads for cysteine and the unknown peak (Figure 3.25).

3.6. ANTIOXIDANTS CORRELATED WITH BIOLOGICAL AGING

Successful results correlating chronological age with antioxidant levels allowed further experimentation correlating biological age with antioxidant levels. Male wild-type *Canton S* Shaw flies (n=180) and male circadian rhythm mutant

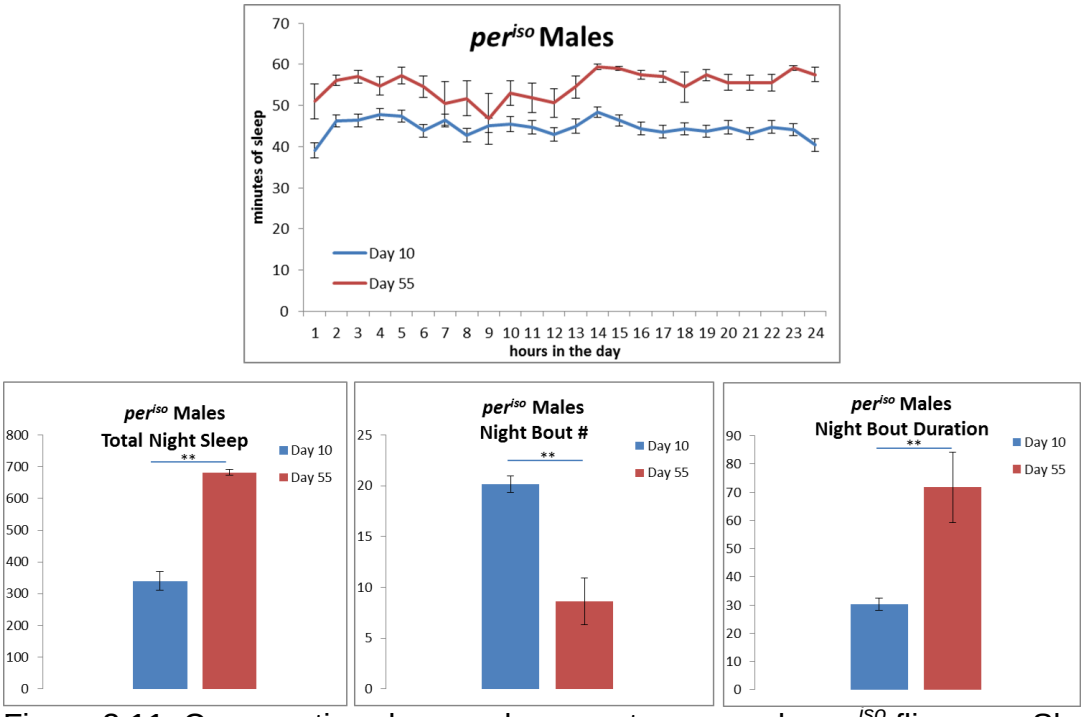


Figure 3.11. Comparative sleep and parameters as male *per*^{JSO} flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 55 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05, **p-value<0.01 by student's t-test).

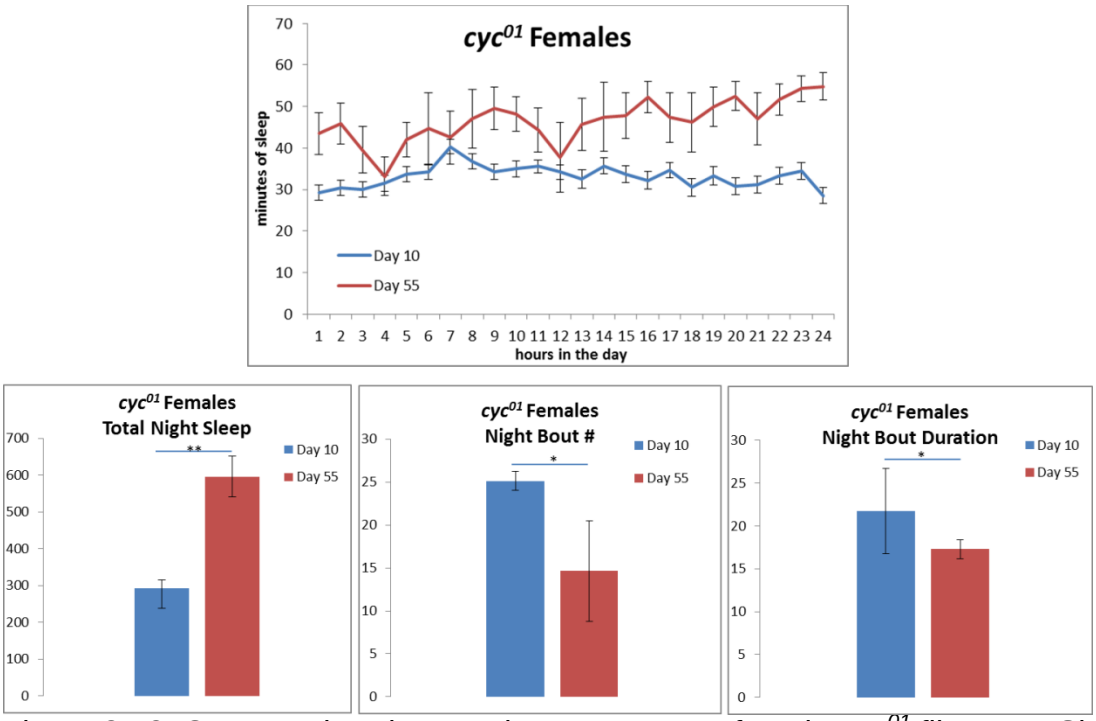


Figure 3.12. Comparative sleep and parameters as female *cyc*^{01} flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 35 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05, **p-value<0.01 by student's t-test).

cyc^{01} flies (n=203) were monitored over 30 and 15 days of life, respectively, as the statistical model shown previously (Figure 3.24) gave an R^2 of 39.4% and 33.3%, respectively. At those points in life, the flies were removed from monitoring and their sleep parameters were calculated and used in the model to estimate lifespan. The flies were split into predicted long-lived or short-lived dependent on the model outcome. Antioxidant levels of glutathione, cysteine, and an unknown peak (which was the same in both the *CS* and *cyc*^{01} flies) were all measured in the flies heads and bodies (Figure 3.26 and 3.27). Although no

significance was found in any antioxidant levels in the *cyc*^{01} flies, significant differences in glutathione and cysteine were found in the *CS* bodies.

3.7. ANTIOXIDANTS CORRELATED WITH BIOLOGICAL AGING IN *CANTON S* FLIES

Successfully finding that GSH and cysteine were decreased in predicted shorter living flies for *CS* males, the experiment was replicated in a larger sample size to validate earlier findings. Male wild-type *Canton S* Shaw flies (n=374) were monitored over 30 days of life. At 30 days of life, the flies were removed from

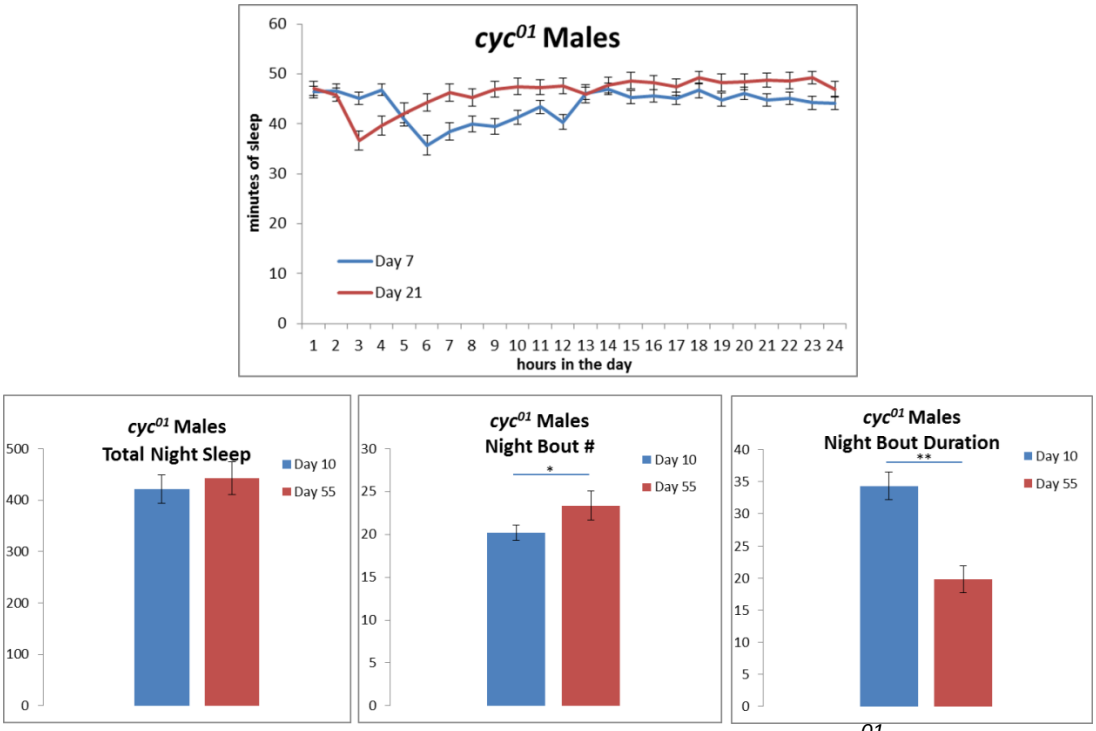


Figure 3.13. Comparative sleep and parameters as male *cyc*^{01} flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 35 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05, **p-value<0.01 by student's t-test).

monitoring and their sleep parameters were calculated and used in the model to predict their lifespan. The flies were split into long-lived or short-lived dependent on the model outcome. Antioxidant levels of glutathione, cysteine, and an unknown peak were all measured in the flies heads and bodies (Figure 3.28). No significance was found in either heads or bodies for cysteine and the unknown peak; however, there was a significant decrease in GSH for the flies had a shorter predicted lifespan.

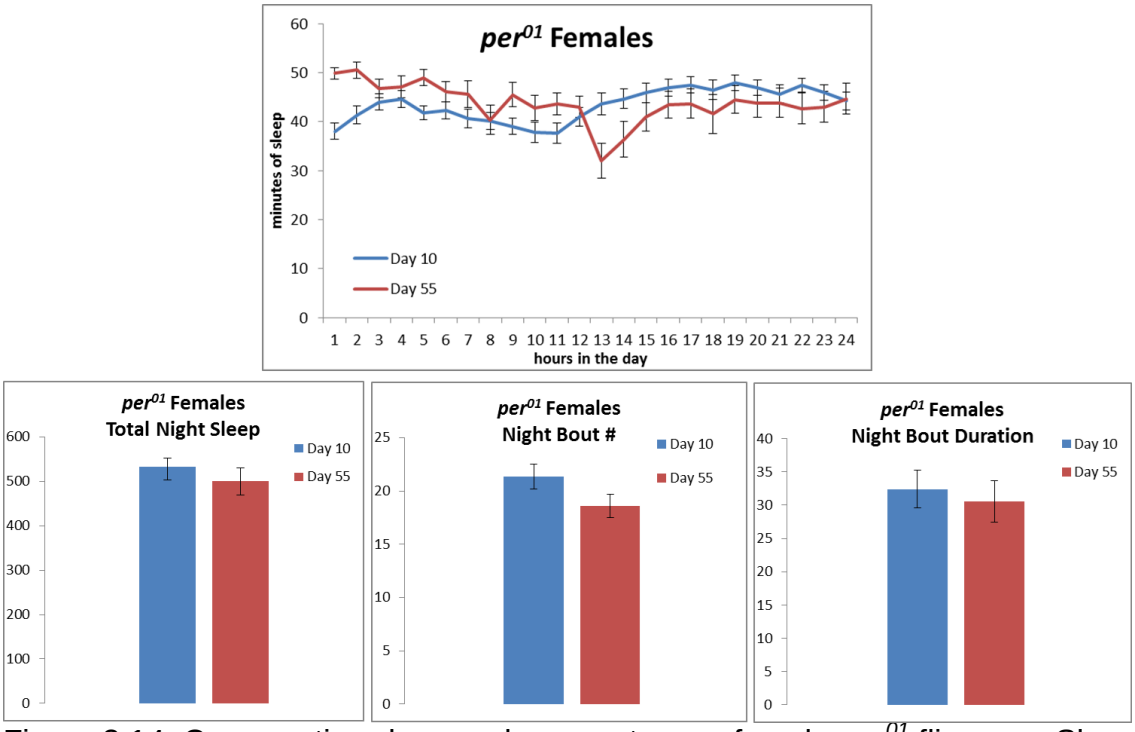


Figure 3.14. Comparative sleep and parameters as female *per*^{01} flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 45 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (no significant differences).

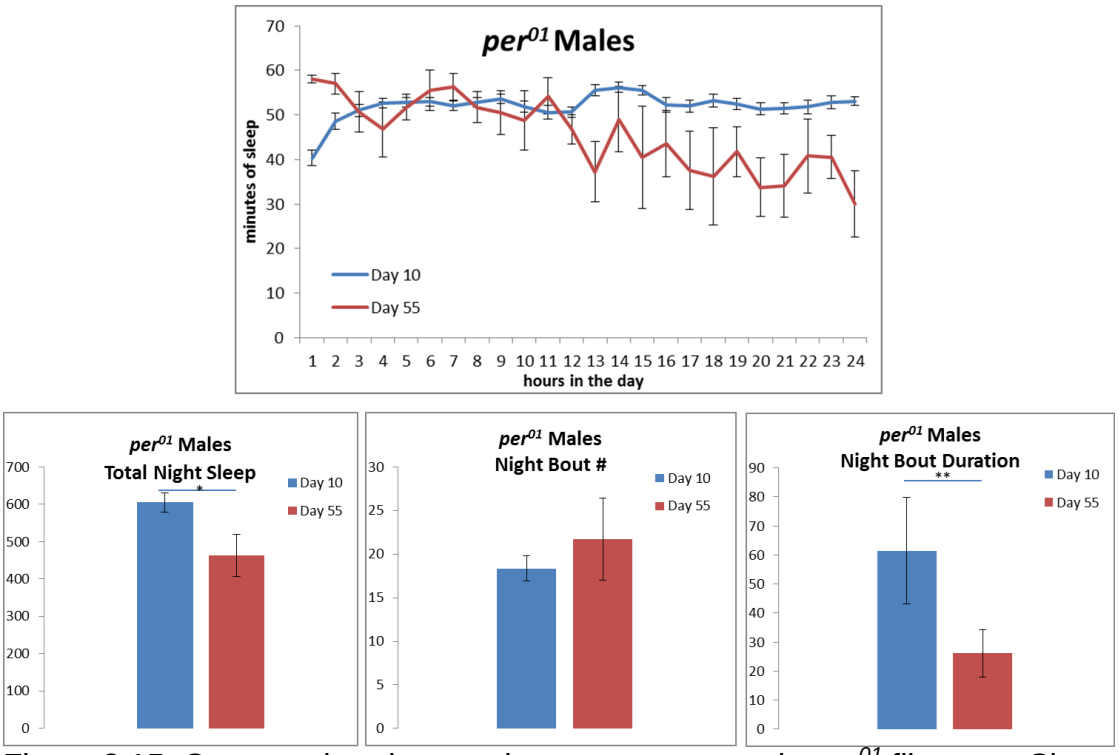


Figure 3.15. Comparative sleep and parameters as male *per*^{01} flies age. Sleep in LD environment over 24 hours at day 10 of life as well as day 45 of life to compare how sleep changes with age, as well as total night sleep, night bout number, and night bout duration (*p-value<0.05, **p-value<0.01 by student's t-test).

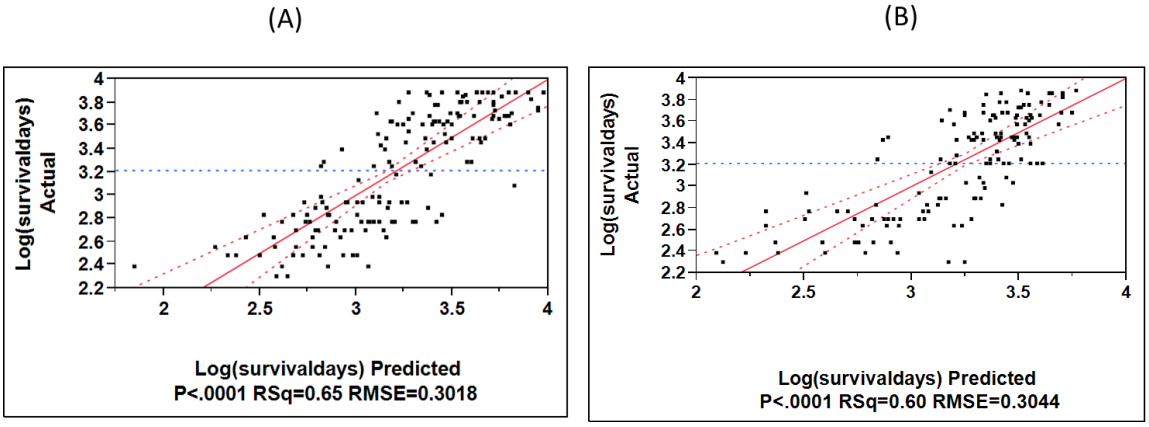


Figure 3.16. Mathematical modeling of sleep parameters for *Canton S* skeath in DD. A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *Canton S* skeath flies as well as (B) female *Canton S* skeath. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 dark:dark conditions. The predicted lifespan correlated significantly with the actual lifespan (p<0.0001).

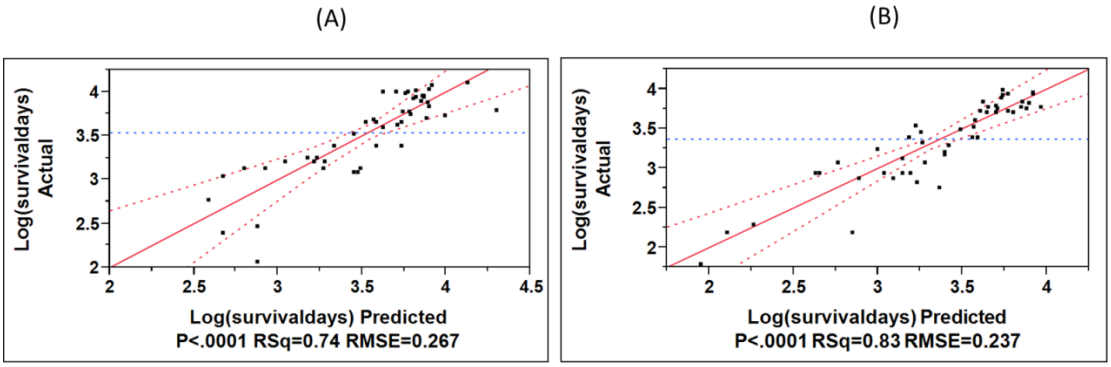


Figure 3.17. Mathematical modeling of sleep parameters for *Canton S* skeath in LD. A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *Canton S* skeath flies as well as (B) female *Canton S* skeath. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 light:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

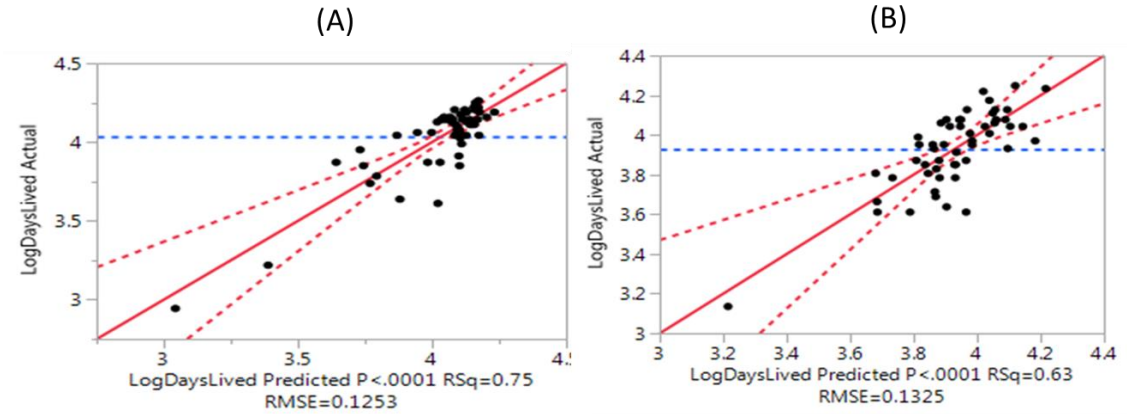


Figure 3.18. Mathematical modeling of sleep parameters for *Canton S* Shaw in LD. A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *Canton S* Shaw flies as well as (B) female *Canton S* Shaw. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 light:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

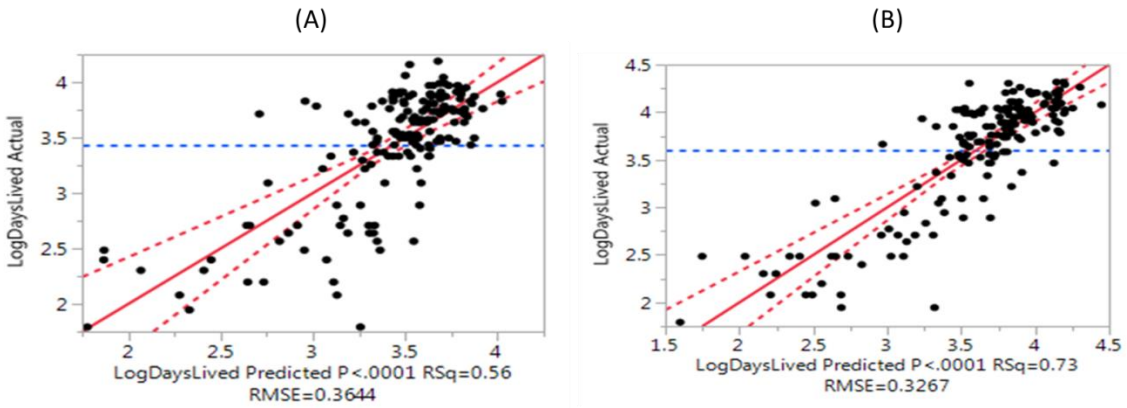


Figure 3.19. Mathematical modeling of sleep parameters for *per*^{iso} in DD. A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *per*^{iso} flies as well as (B) female *per*^{iso}. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 dark:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

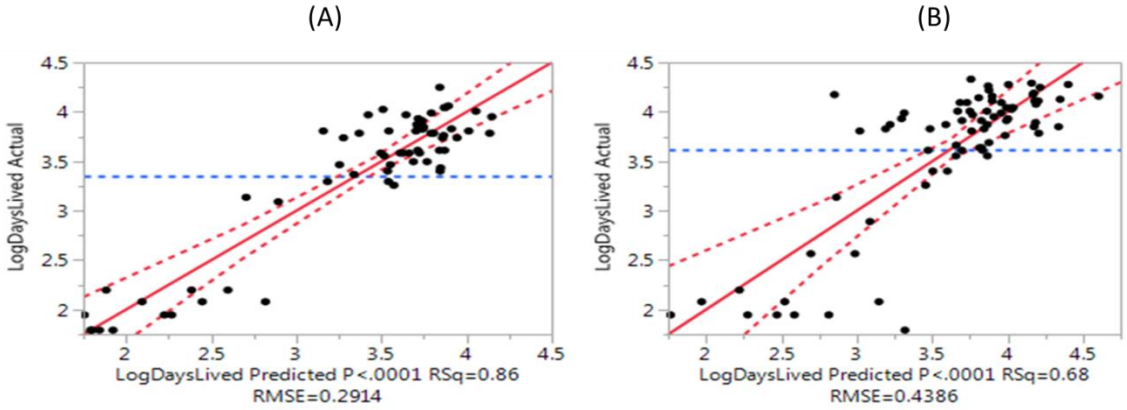


Figure 3.20. Mathematical modeling of sleep parameters for *per*^{iso} in LD**.** A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *per*^{iso} flies as well as (B) female *per*^{iso}. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 light:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

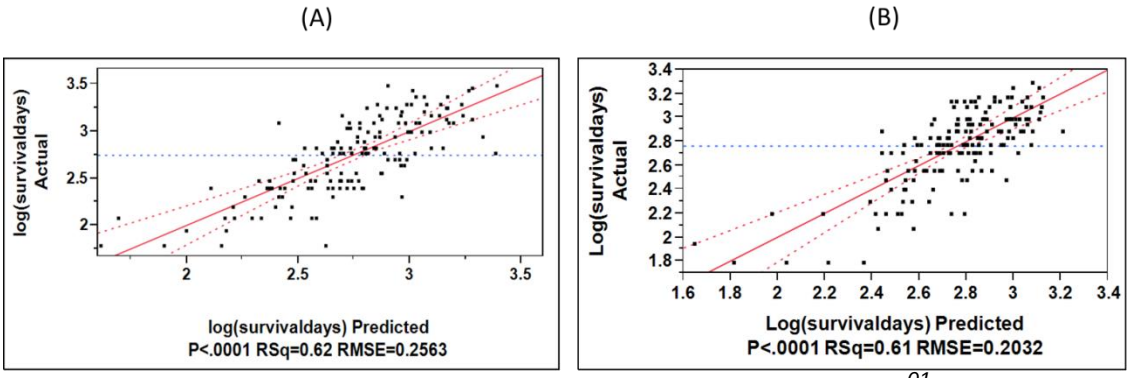


Figure 3.21. Mathematical modeling of sleep parameters for *cyc*^{01} in DD**.** A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *cyc*^{01} flies as well as (B) female *cyc*^{01}. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 dark:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

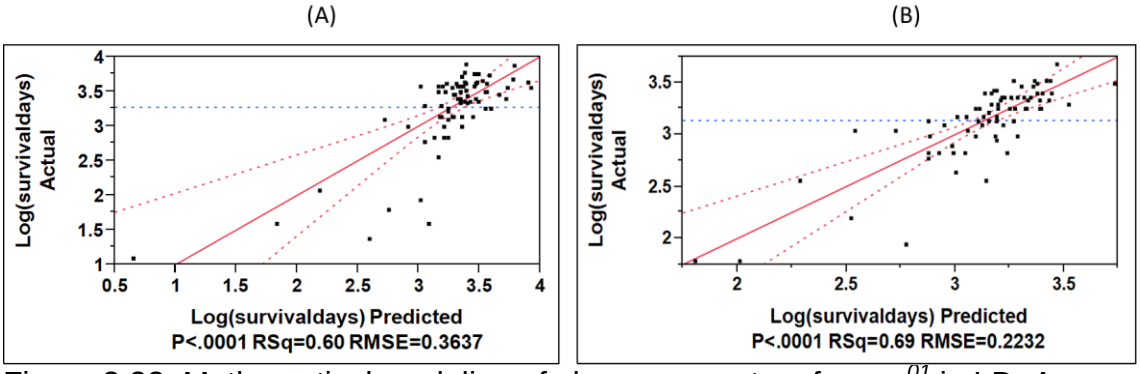


Figure 3.22. Mathematical modeling of sleep parameters for *cyc*^{01} in LD. A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *cyc*^{01} flies as well as (B) female *cyc*^{01}. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 light:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

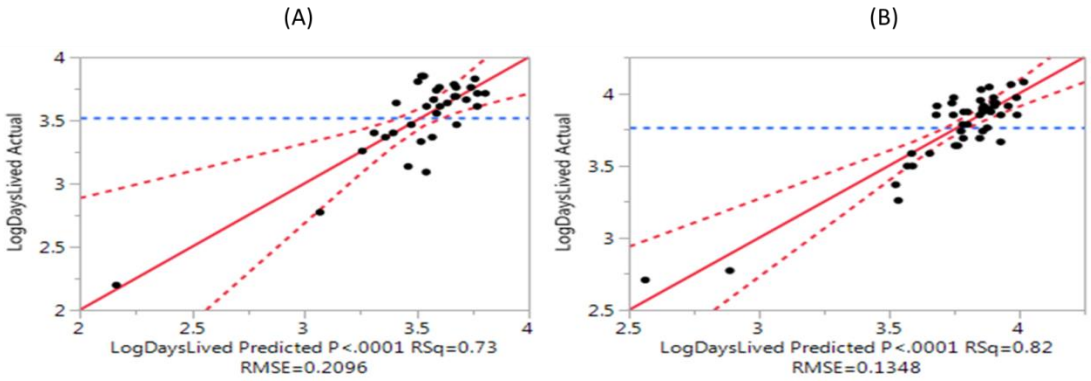


Figure 3.23. Mathematical modeling of sleep parameters for *per*^{01} in LD. A statistical model based off previous lifespan experiments can be used to predict lifespan in (A) male *per*^{01} flies as well as (B) female *per*^{01}. Model parameters were applied to the sleep and wake transitions in both genotypes housed under 12:12 light:dark conditions. The predicted lifespan correlated significantly with the actual lifespan ($p<0.0001$).

(A)

Model up to Day	Sample Size	R-quare FD MODEL
10*	57	40.10%
15*	57	43.90%
20*	57	56.90%
25	57	36.00%
30	57	39.40%
35	57	41.30%
40	55	56.30%
45	53	37.50%
50	48	38.20%
55	45	32.50%
PARAMETERS OVER LIFETIME *	57	66.20%

(B)

Model up to Day	Sample Size	R-square FD MODEL
5*	69	21.00%
10	69	42.90%
15	68	33.30%
20	64	31.60%
25	53	55.20%
30	41	47.90%
PARAMETERS OVER LIFETIME *	69	46.00%

Figure 3.24. Correlation of lifespan with increasing chronological age. For both (A) *Canton S* Shaw males and (B) *cyc*^{01} males, the number of days used for the correlation with final lifespan are listed in the first column. The sample size for each number of days is listed in the second column. The r^2 for the first differences (FD) model at that day is presented in the third column and the model using the absolute values of the differences in the right hand column for males under light:dark conditions. The correlation for the entire lifetime's worth of data is shown in the last row.

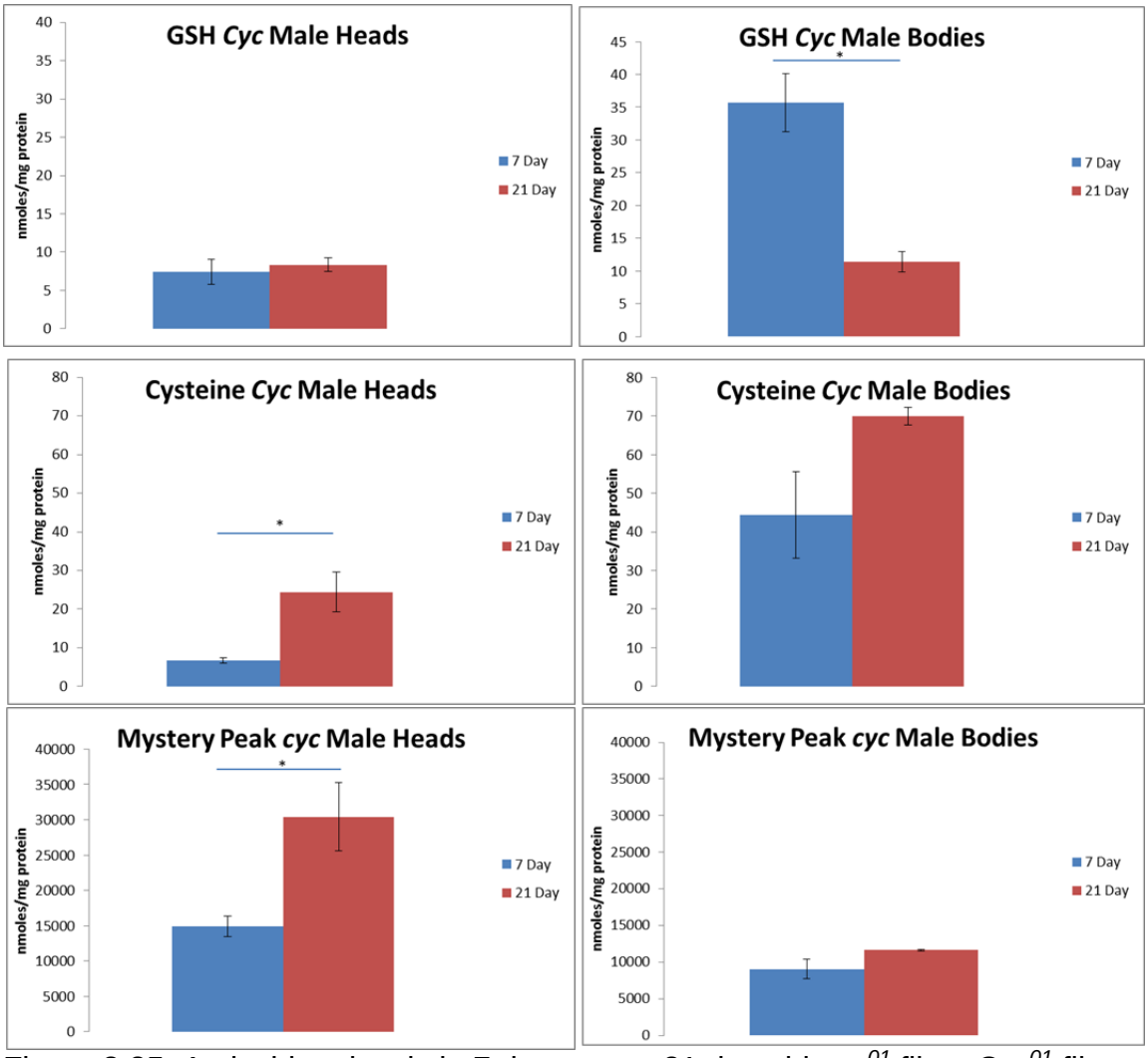


Figure 3.25. Antioxidant levels in 7 day versus 21 day old *cyc*^{01} flies. *Cyc*^{01} flies were aged to either 7 or 21 days and glutathione and cysteine levels as well as an unknown peak were quantified by HPLC in heads and bodies. (*p<0.05, by student's t-test, n=3 biological reps of 25 pooled flies for each condition).

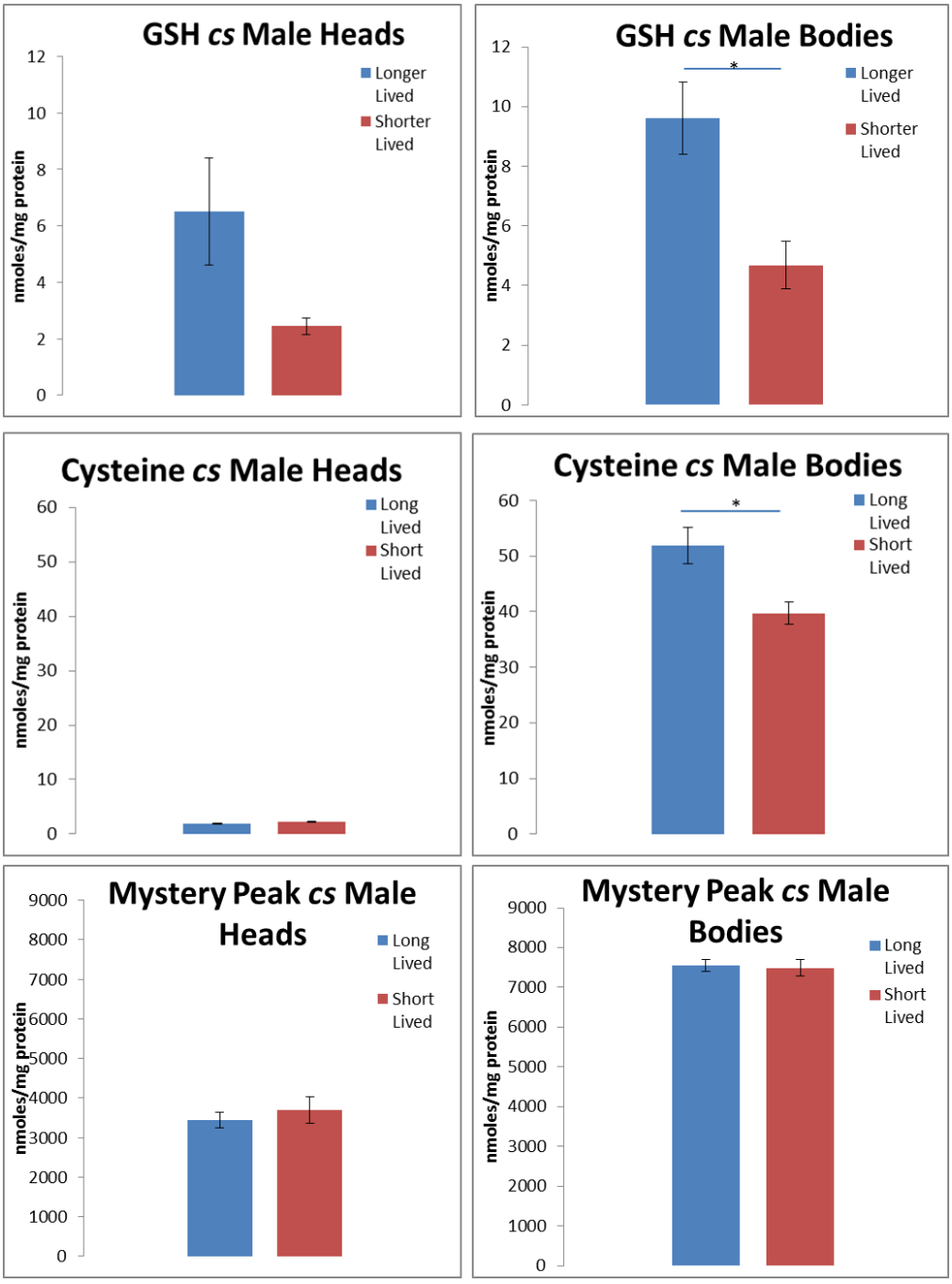


Figure 3.26. Antioxidant levels in predicted lifespan wild-type *Canton S* flies. Antioxidant levels of glutathione, cysteine, and an unknown peak, were measured in heads and bodies for short and long-lived flies as well as a control. Three replicates were completed for each group with 25 flies per replicate (*p-value<0.05 by students t-test).

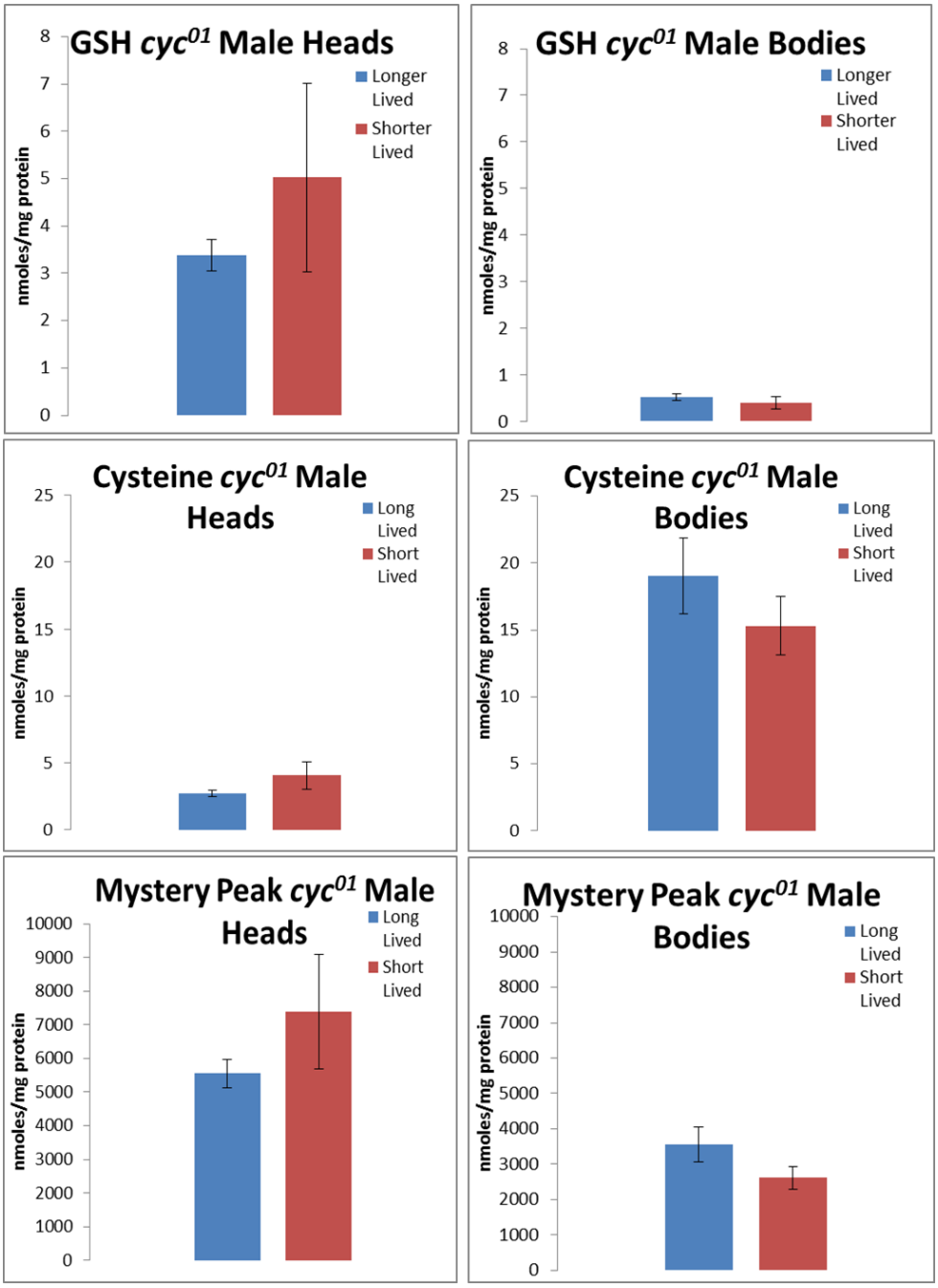


Figure 3.27. Antioxidant levels in predicted lifespan circadian rhythm mutant *cyc*^{01} flies. Antioxidant levels of glutathione, cysteine, and an unknown peak, were measured in heads and bodies for short and long-lived flies. Three replicates were completed for each group with 25 flies per replicate. No significance was found by student's t-test.

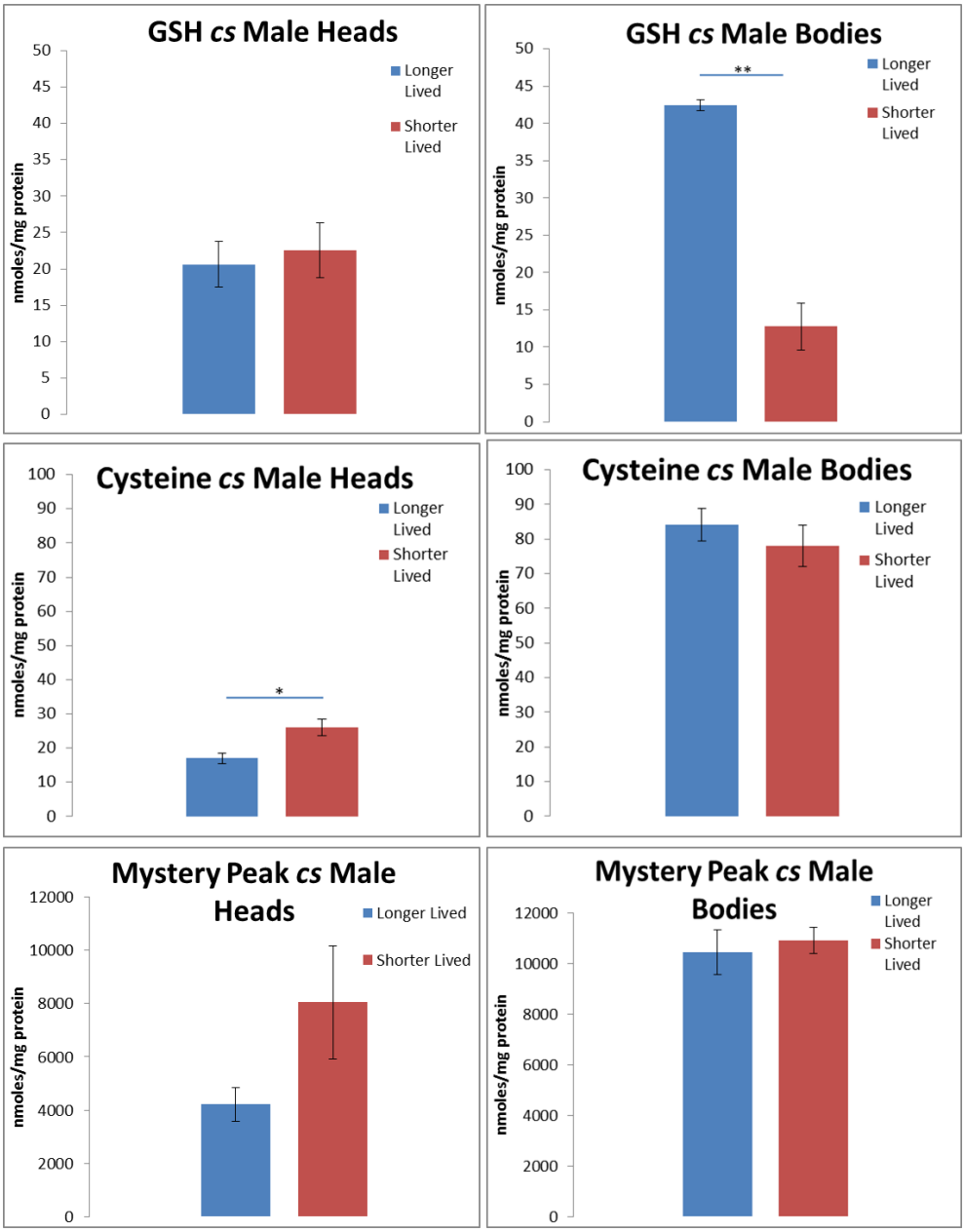


Figure 3.28. Antioxidant levels in wild-type *Canton S* flies repeated. Antioxidant levels of glutathione, cysteine, and an unknown peak, were measured in heads and bodies for short and long-lived flies as well as a control. Five replicates were completed for each group with 25 flies per replicate (*p-value<0.05, **p-value<0.01 by students t-test).

4. RESULTS RELATING TO OTHER POTENTIAL BIOLOGICAL MARKERS ASSOCIATED WITH AGING

4.1. ALZHEIMER'S IN *DROSOPHILA*

Alzheimer's is an aging disease in which the buildup of β-amylase and Tau proteins cause neurodegenerative effects (Bilen, 2005). The β-amylase *Drosophila* model was selected for preliminary data since they have been previously used in the laboratory. Preliminary data was measured to test if Alzheimer's could be induced and whether a higher dose of Mifepristone would advance this induction. Although this has been completed before, the goal was to determine a different dose curve (Greeve et al., 2004). Having a quicker induced versus slower induced model could potentially have applications in comparing mild versus extreme cases of Alzheimer's. Mifepristone was dosed to flies to induce the disease in the DaGSw>UAS-APP^{42}, UAS-BACE flies. The different dosages of Mifepristone (0 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL) were put in the food for sixteen DaGSw>UAS-APP^{42}, UAS-BACE flies of each gender per dose. The dose curve was used to determine the optimal dose of Mifepristone. It can also be used for mathematical modeling of fast and slow onset of Alzheimer's. Sleep was monitored in all flies from day 3-5 of their life until death in a 12:12 light:dark environment. All deaths were recorded and a survival curve was created to compare the four dosages for each gender. A log rank test between each dose of mifepristone in both males and females was calculated and it was determined that all doses (12.5 mg/mL, 25 mg/mL, and 50 mg/mL) were significantly different from the 0 mg/mL dose with p-values <0.001.

In both genders, the lifespan of the 50 mg/mL dose flies were significantly different from the 12.5 mg/mL dose flies, but not the 25 mg/mL flies. The flies from the 12.5 mg/mL dose also lived significantly longer than the flies in the 25 mg/mL dose in both genders (Figure 4.1). Sleep changes early in life compared to later in life between dosages shows how sleep differs between groups and how the mifepristone affects sleep with age (Figure 4.2). Sleep parameter changes, specifically total amount of night sleep, sleep bout number, and night bout duration, were also recorded and changes between groups were graphed to show significant changes in sleep between 0 mg/mL and other doses as well as how the sleep parameters changed with age. Total night sleep significantly decreased with age in all doses in males and in the 0 mg/mL and 50 mg/mL dose in females. The number of night bouts significantly increased in all doses in males, but only in the 0 mg/mL dose for females. Night bout duration significantly decreased in all doses for the males, and in the 0 mg/mL and 12.5 mg/mL dose for females (Figure 4.3).

4.2. METABOLIC ENERGY LEVELS IN *DROSOPHILA*

The ability to regulate sleep could potentially be a component that correlates with lifespan, so preliminary data determined how flies regulate sleep differently when placed in the same experimental conditions. to determine how metabolic levels changed with starvation, wild-type *Canton S* flies (n=96) were monitored for 24 hours under a baseline condition then starved for 24 hours, and sleep between the two conditions was compared to split the flies into three

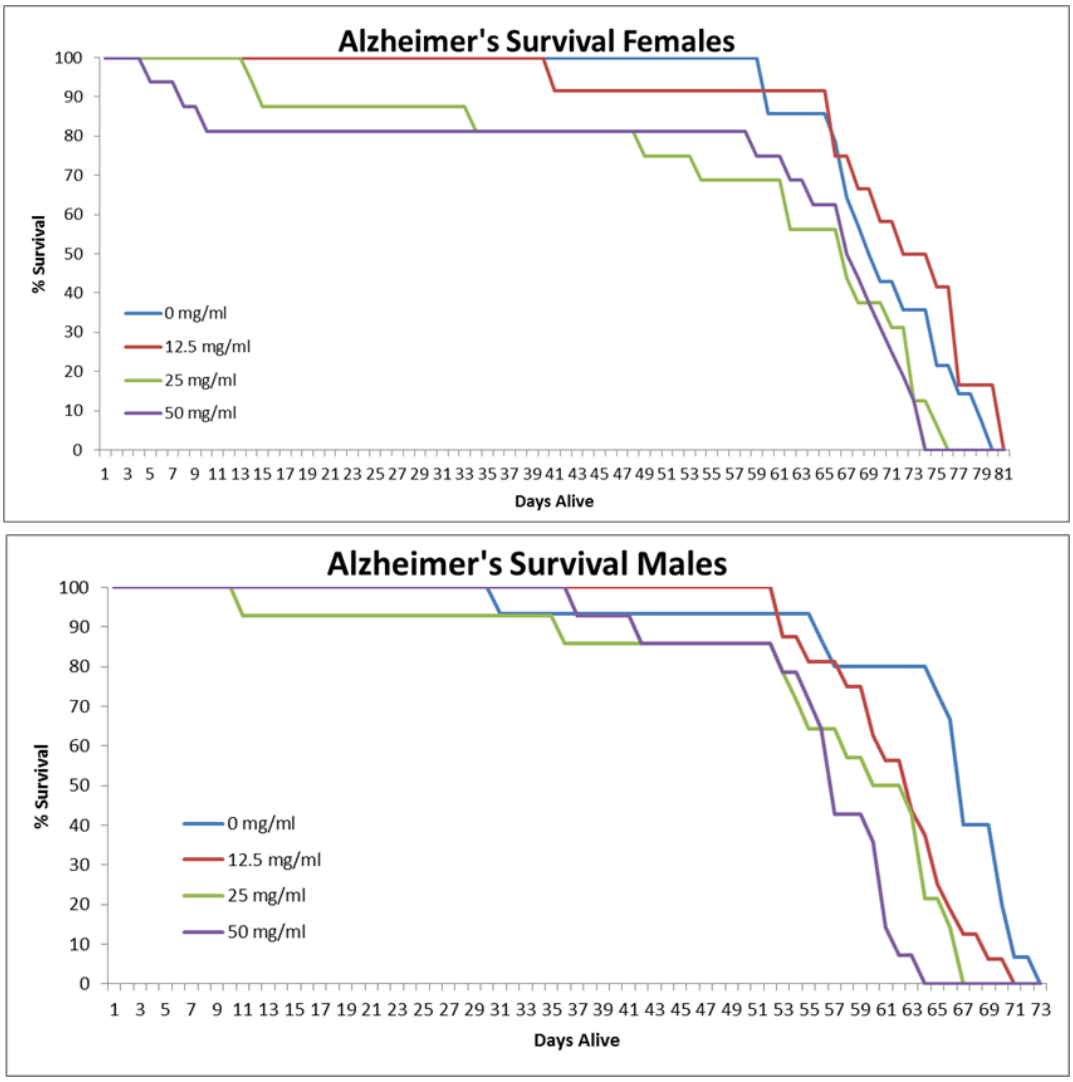


Figure 4.1. Survival curves of male and female Alzheimer's model flies. DaGSw>UAS-APP[42], UAS-BACE flies in an LD environment with different Mifepristone dosages. All dosages were significantly different from 0 mg/ml (p-value<0.001 by long-rank test).

different groups based on how much sleep changed during starvation from baseline. The average difference from baseline in *CS* males for group 1 was -34.723 (±3.38), for group 2 was 3.794 (±1.39) , and for group 3 was 25.759 (±3.71), with all groups significantly different from each other (Figure 3.36). The average difference from baseline in *CS* females for group 1 was -3.656 (±3.99),

for group 2 was 11.647 (±0.81), and for group 3 was 30.522 (±4.07), with all groups significantly different from each other (Figure 4.4). Metabolic levels of glucose, glycogen, ketone bodies, and triglycerides were then measured in the three separate groups and a control in both males and females to compare how levels change compared to sleep changes between baseline and starvation conditions (Figure 4.5). Triglyceride levels were undetermined and so deleted from analysis.

4.3. CLIMBING ASSAY

The ability to climb early in life has been correlated with lifespan (Le Bourg & Lints, 1992), and so experimental data was completed to determine if this

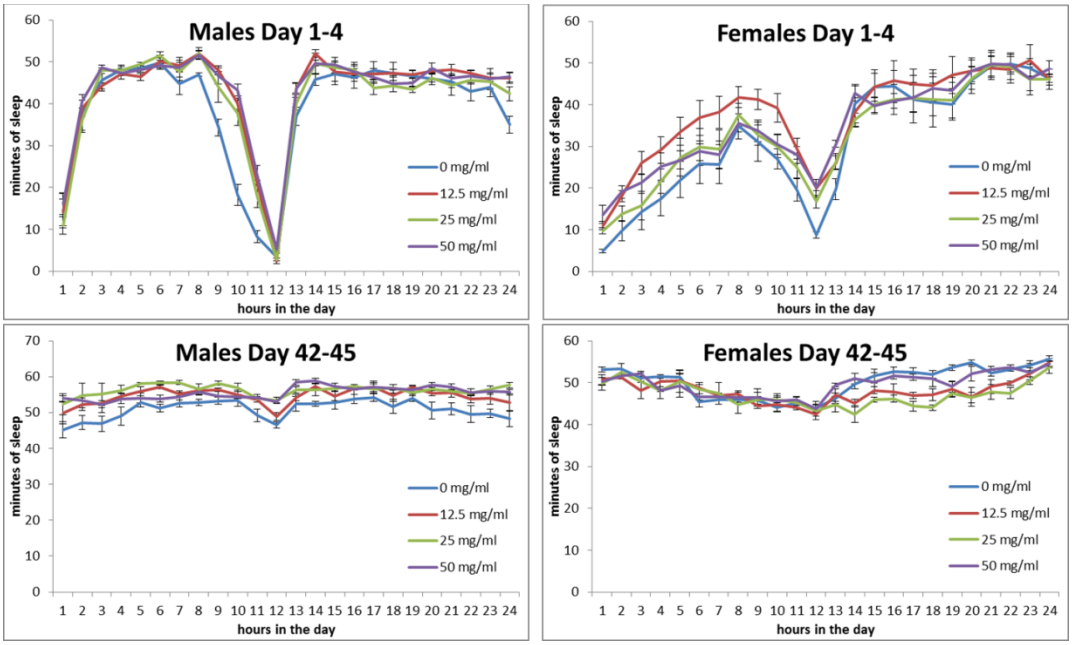


Figure 4.2. Changes in sleep for Alzheimer's model flies. Sleep changes from an average of day 1-4 after being on mifepristone and an average of day 42-45 after being on mifepristone for both males and females in an LD environment was graphed.

could be replicated. If the data was replicated, this protocol could have been a potential marker for aging. A climbing assay was performed to determine if sleep and survival were dependent on a fly's ability to climb and use natural instincts early in life. Around fifty wild-type *Canton S* flies of each gender were used to complete a climbing assay differentiating two groups: flies that climbed up (n=24 for females, n=23 for males) and flies that didn't climb (n=27 for females, n=32

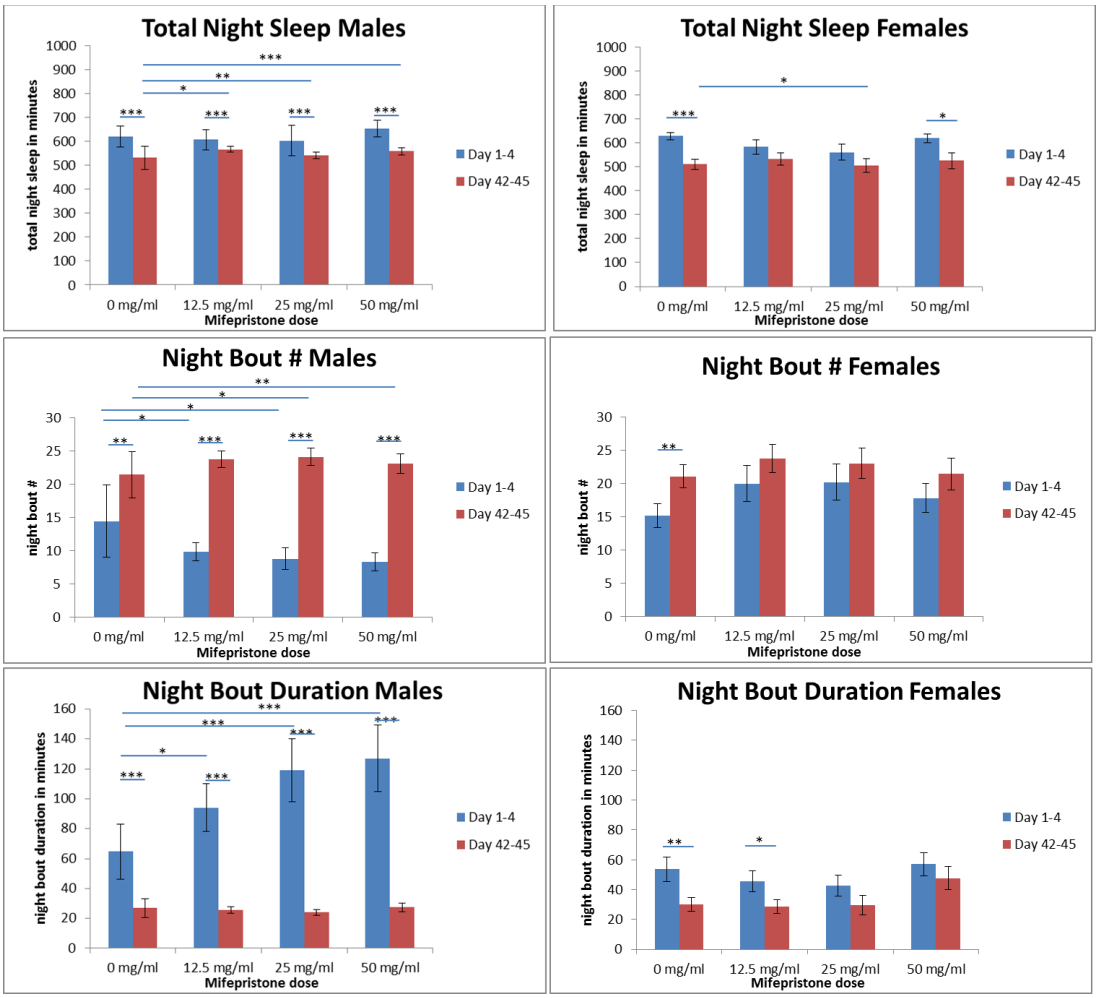
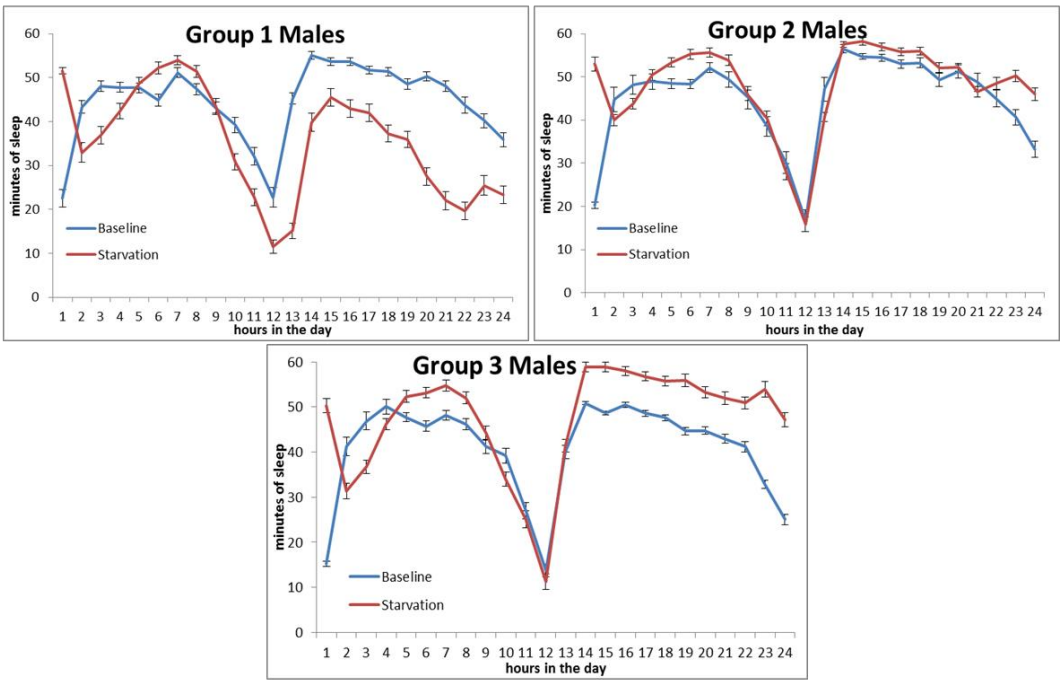


Figure 4.3. Changes in sleep parameters for aging Alzheimer's model flies. Significant sleep parameters were graphed to show how these parameters changed with age after being dosed with Mifepristone as well as how the sleep changes differed between different dosages (*p<0.05, **p<0.01, ***p<0.001 by an ANOVA).

(A)



(B)

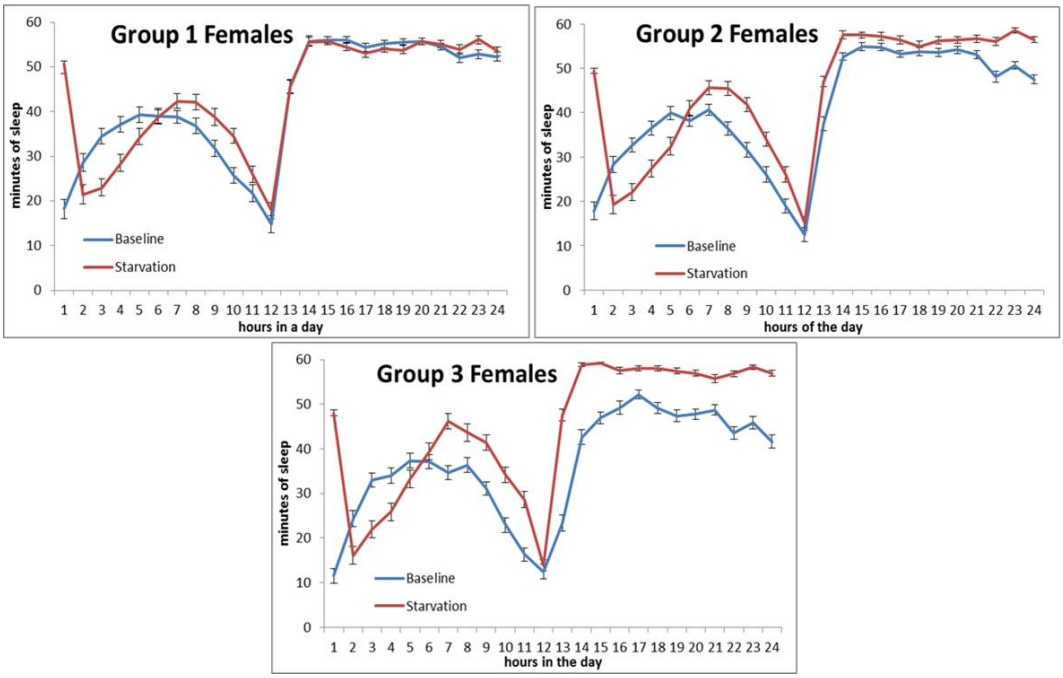


Figure 4.4. Sleep changes for three groups of flies dependent on difference between baseline and starvation. Wild-type *Canton S* (A) male and (B) female flies were split into three groups dependent on how they slept during starvation compared to baseline (n=4 biological reps of 8 flies for each condition).

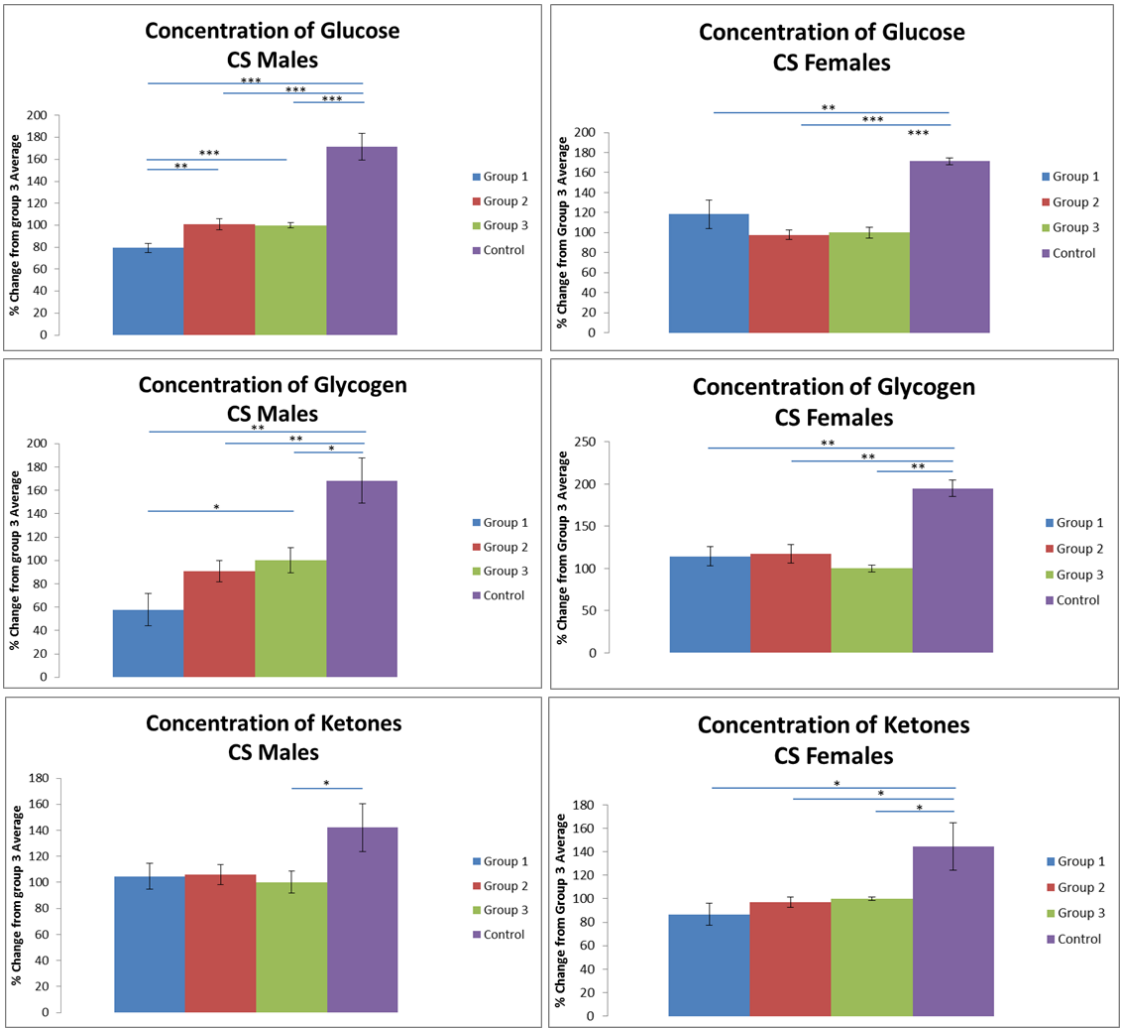


Figure 4.5. Metabolic levels in *Canton S* fly groups dependent on sleep changes. Metabolic levels of glucose, glycogen, and ketone bodies were all measured in both males and females of wild-type *Canton S* flies separated by the change in sleep compared to baseline after starvation (*p<0.05, **p<0.01, ***p<0.001 by an ANOVA).

for males). Sleep was recorded in these flies for four days and compared between the two groups for each gender (Figure 4.6). Sleep parameters were also calculated and although females had significant differences between both groups in total sleep, average day bout length, max day and night bout length, and amount of day and night bouts, the males showed no significant differences between the flies that climbed and the flies that didn't. After being removed from

sleep recording, the flies were monitored for the rest of their lives and Kaplan Meier survival curves were graphed to show any differences in lifespan between the two climbing groups, which the log-rank test deemed no significant difference in the groups for either gender (Figure 4.7).

4.4. CALORIC RESTRICTION

Caloric restriction in *Drosophila* has been shown to increase lifespan

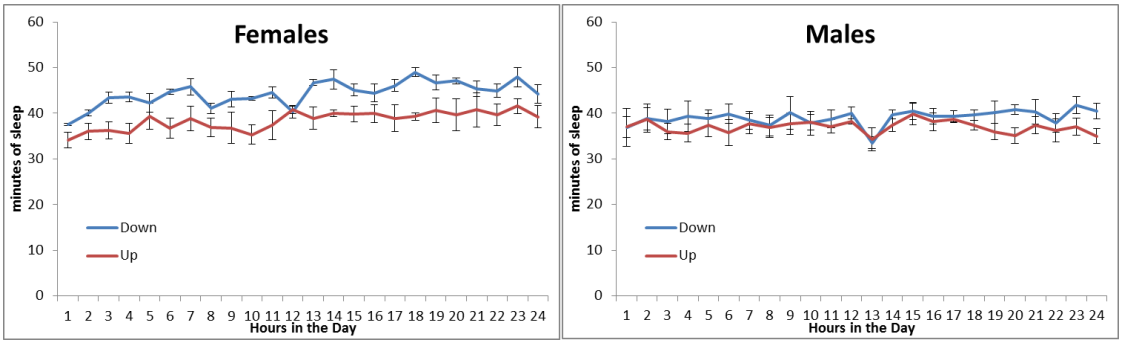


Figure 4.6. Sleep changed dependent on whether *Canton S* flies climb or not. Two groups of flies were compared depending on whether they initially climbed up or didn't move when given the opportunity. The sleep is significantly different between groups for each gender (p-values<0.05 by an ANOVA).

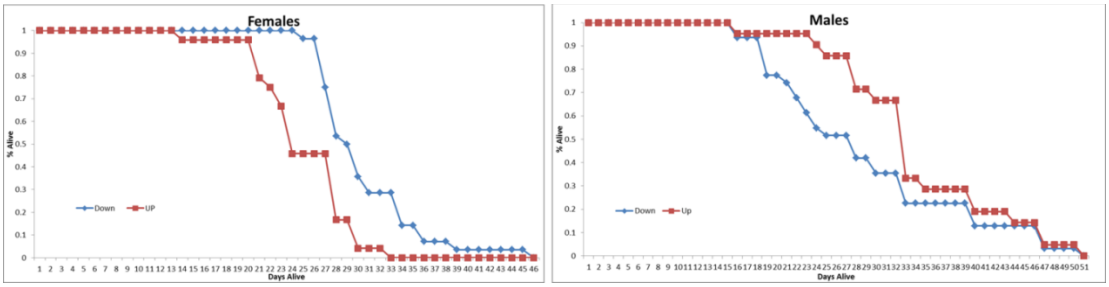


Figure 4.7. Survival curves for male and female *Canton S* flies dependent on if they climb when given the opportunity. Both genders showed differences in lifespan dependent on the groups, but neither showed significance between the two groups overall (ANOVA).

compared to flies fed a high caloric diet (Piper et al., 2005). Preliminary data to replicate these results was determined if this could potentially be a marker for aging, and if so, if changing the diet could potentially help to extend lifespan. Two groups of wild-type *Canton S* flies were distinguished by whether they were fed a high yeast or low yeast diet. Groups were monitored over their lifetime, all deaths were recorded, and Kaplan Meier survival curves were graphed. Log-rank tests were completed to find significance, which showed that survival was significantly

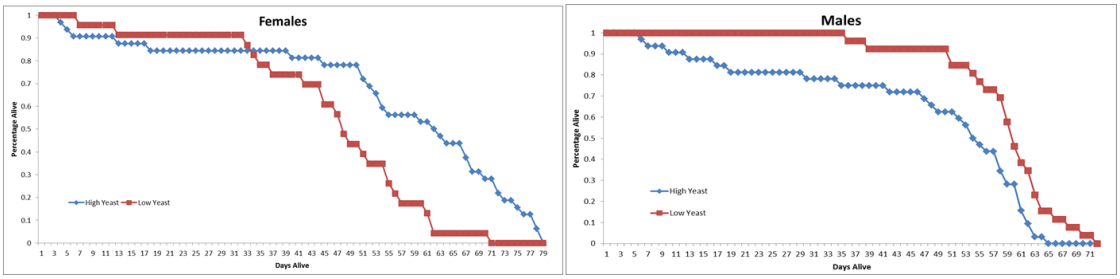


Figure 4.8. Kaplan Meier Survival Curves comparing lifespan of flies dependent on their diet. Wild-type *Canton S* flies were split into two groups and fed a high yeast and a low yeast diet over their lifetime. All deaths were recorded to determine differences in lifespan based off diet. (p-value for females <.001, p-value for males < 0.05).

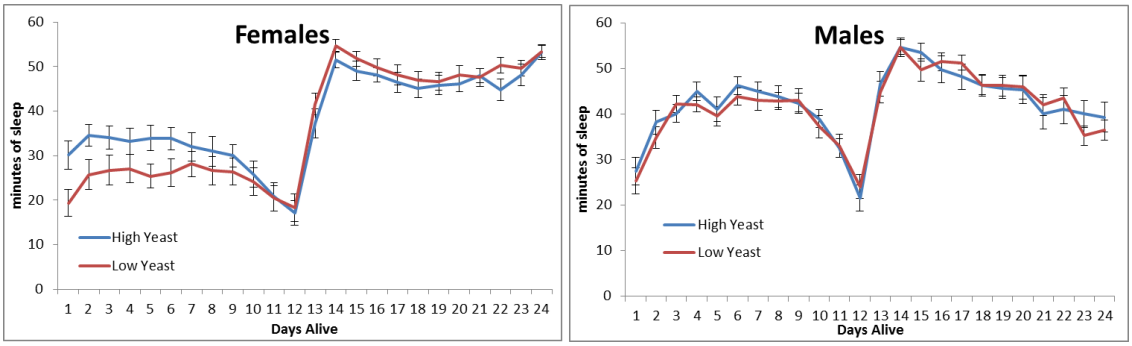


Figure 4.9. Sleep comparisons dependent on caloric diet. Wild-type *Canton S* flies comparing sleep on day 25 of life dependent on whether they were fed a high yeast or low yeast diet. In both genders, neither diet group was significantly different from the other (ANOVA).

different between flies feeding on high and low yeast diets for both genders. However, in females the high yeast dieters lived longer whereas in males, the low yeast dieters lived longer (Figure 4.8). Sleep was compared between the two groups for each gender at an average of day 24-26 of their life to show that even though lifespans were significantly different, sleep in the flies did not significantly change dependent on the different diets (Figure 4.9). Sleep parameters were also calculated for both genders and in females, both night bout number and night bout duration were significantly increased in low yeast flies whereas in males only total night sleep increased (Figure 4.10). This could be due to the different patterns of survival for opposite genders.

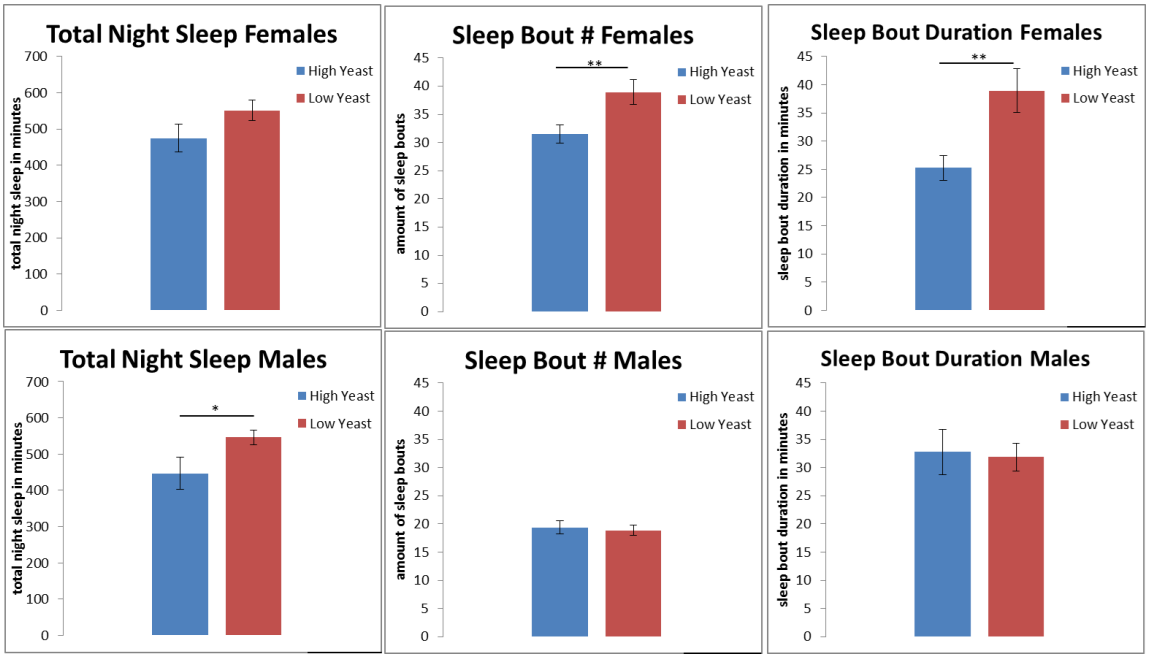


Figure 4.10. Sleep parameter comparisons dependent on caloric diet. Wild-type *Canton S* flies comparing sleep parameters on day 25 of life dependent on whether they were fed a high yeast or low yeast diet (*p-value<0.05, **p-value<0.01 by student's t-test).

5. THESIS DISCUSSION

5.1. SURVIVAL IN MULTIPLE GENOTYPES SHOWS THAT SLEEP CORRELATES WITH LIFESPAN

Very little research has been completed correlating survival with sleep other than understanding that sleep quality and quantity change with age (Cirelli, 2012), but further research to determine the mechanisms as to why this is occurring are unclear. To begin to research such mechanisms, sleep was measured from birth until death (Bushey et al., 2010; Koh et al., 2006). In the lab, these experiments were completed in both wild-type flies (*Canton S*) as well as circadian rhythm mutants (per^{iso}, cyc^{01}, and per^{01}) to have the ability to correlate pure sleep without rhythm cues. With these methods, the lifespan can be more directly related to sleep since the genotype and environment can be compared to see how sleep is affected and in return how those factors affect lifespan.

Results showed that as wild-type *CS* flies aged, their sleep significantly increased and they tended to lose their circadian rhythm, which was evident when looking at actual sleep data over an averaged 24 hour period of three days. Changes in sleep parameters also showed that as the flies aged, sleep consolidation increased based on their total night sleep and the number of night bouts the individuals had. Both concepts are widely associated with aging in *Drosophila* and humans (Huang et al., 2002; Koh et al., 2006). Although the circadian rhythm mutants don't have rhythms to start with, sleep significantly increased with age and sleep parameters showed more fragmented sleep in per^{iso} and cyc^{01} males and females. In per^{01} males, sleep did become more

fragmented with age, but in the females, there didn't seem to be any significant changes with age.

5.2. PREDICTING LIFESPAN IN *DROSOPHILA* USING A MATHEMATICAL MODEL AND USING THIS PREDICTION TO CORRELATE A BIOLOGICAL MARKER TO SLEEP AND AGING

Once survival data was completed for all genotypes and genders, predictive models could correlate the predicted lifespan to the actual lifespan. Specifically *Canton S* from Paul Shaw's lab as well as the cyc^{01} flies were selected to do further testing. The *Canton S* are one strain of the wild-type, and the R^2 for the predictive model was high at 75%, so these were selected. The cyc^{01} circadian rhythm mutants were selected since previous antioxidant testing in the laboratory had shown a promising outcome, and the R^2 for the predictive model was also relatively high at 60%. Monitoring in an LD environment was selected for use for both genotypes since that is the most natural environment for the flies. Males of each genotype were also used since it they were the gender used in previous experiments relating to antioxidant levels (Klichko et al., 2015), with the goal to complete more experimentation in females in the future.

The first goal was to measure differences in chronological age, then see if changes in biological age could be found with the same biological methods. to do this, cyc^{01} flies were chronologically aged to 7 days and 21 days old. Glutathoine (GSH), cysteine, and an aminothiol unknown peak, were all detectable in the samples. Glutathione significantly decreased in the heads of older aged flies. Previous literature has shown that glutathione did not significantly correlate with

aging in *Drosophila*, however in the past, only heads have been evaluated (Klichko et al., 2015). Studies in other organisms, such as rodents, have successfully correlated glutathione with aging when measured in the kidneys, livers, and other tissues (Hazelton & Lang, 1980; Pouget et al., 2015). This could be because biological age occurs in tissues and organs in the main portion of the body, such as the liver and kidneys where you commonly find disease leading to death, whereas brain failure is not as common.

Since antioxidant levels were successfully measured for chronological sleep with significant results, biological age was the next step. Once sleep parameters were calculated, the data was input into the model to predict lifespan at various days for each genotype to assess optimal time to estimate future lifespan. It was found that day 30 for *Canton S* and day 20 for cyc^{01} flies yielded the best results. New flies of these genotypes were monitored until these days, and the new data was used to predict lifespan as either long or short lived flies. Heads and bodies were used for each group of long-lived and short-lived flies, and antioxidant levels were measured using the same methods as chronological age. Unexpected results showed that there was no significance between the two groups for the cyc^{01} flies which could be due to the two groups of flies not being significantly separate from each other resulting from a small sample size. However, in *Canton S* flies, there was a significant difference between the two groups for glutathione and cysteine. In both instances, glutathione and cysteine levels were increased in the longer-lived flies. With these data, antioxidant levels show there could be a correlation between aging and the predictive model.

Previous studies in rats correlated a decrease of glutathione with age (Pouget et al., 2015), which would explain why the shorter-lived flies had lower glutathione levels since biologically speaking they were older than the longer-lived flies. Not only can lifespan be predicted, but GSH could potentially be the mechanism that determines how quickly tissues and the body biologically age. This could be helpful in finding a biological marker which could potentially be supplemented or used to intervene with aging to increase lifespan. Although other biomarkers have successfully been found to associate with age, most are factors which cannot be supplemented such as certain components only expressed in selective cells (Krishnamurthy et al., 2004; Ma et al., 2004; Ressler et al., 2006).

The same protocol to measure glutathione and cysteine as a biological marker of age was then replicated with the wild-type *CS* flies since a significant difference between short and long-lived flies in that genotype was shown. With increased biological replications, the replication of the experiment showed that glutathione consistently decreased in the shorter-lived flies. With these results, it can be suggested that glutathione is a good candidate as a biological marker for aging and sleep as well as a testable mechanism for why shorter lived flies are shorter lived.

5.3. ALZHEIMER'S MODEL DOSE CURVES RELATED TO INDUCING THE DISEASE

Alzheimer's can be induced in DaGSw>UAS-APP^{42}, UAS-BACE mutants when dosed with Mifepristone, a system which is carefully explained in the methods. This model is widely used in *Drosophila* to better understand and study the

disease (Greeve et al., 2004), however the goal of the experiment was to see if a dose curve could be created with different dosages of Mifepristone (0 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL). This could determine if the strength of the dose would be correlated with the speed of the induction of the disease and how sleep correlated with the severity of the disease. When log-rank tests were completed, it showed that in both genders, all four groups were significantly different from each other when it came to lifespan (all p-values < 0.001). It showed that in both genders, the 25 mg/mL and 50 mg/ml were not significant from each other, so further testing will be completed with higher dosages of Mifepristone to determine whether different doses do actually matter, but the results showed that higher doses of Mifepristone induced the disease at a quicker rate. Being able to control induction of Alzheimer's in flies could potentially allow more in depth understanding of when sleep starts to change and when to expect onset of the disease.

Measuring sleep changes with age between flies in groups of different dosages could help determine if certain dosages affected sleep more than the others, a factor which is very sensitive to the Alzheimer's disease. By looking at actual sleep data averaged over four days early in life (day 1-4) versus late in life (day 42-45), these data showed that as they age, flies in all the groups seemed to lose their circadian rhythm. This is associated with age in general, that as humans age the circadian rhythm starts to decline (Huang et al., 2002). The sleep parameters measured, total sleep time as well as the night bout duration and the number of sleep bouts, shows that all four groups have increased total

sleep as they age and yet their sleep has a lot more bouts which are all shorter than when they were younger. This shows that the sleep becomes a lot more fragmented (less consolidated with shorter sleep bout duration and more sleep bouts) as the flies age and becomes a well-known significant marker of Alzheimer's disease (Lim et al., 2013). The experiment shows consistent data with the induction of the disease as well as characteristics of sleep changes that occur after the onset of Alzheimer's.

5.4. GLYCOGEN LEVELS ARE AFFECTED AFTER *DROSOPHILA* IS PLACED UNDER STARVATION CONDITIONS

When flies are starved overnight and sleep during starvation is compared to baseline sleep, three groups can be determined based on how that sleep changed. The initial goal of testing the metabolic differences between these groups was to find how glucose, glycogen, ketone bodies, and triglycerides changed dependent on sleep change due to starvation; however some unexpected results were shown.

Glycogen is a widely measured metabolic component in *Drosophila* which the body relies on for energy. Changes in levels of glycogen can contribute to the circadian rhythm, sleep deprivation, and age, to name a few (Petit et al., 2002; Sharma & Sharma, 1980; Zimmerman et al., 2004). Results from comparing the three groups dependent on change in sleep showed no difference in concentration of ketone bodies and triglycerides for either gender, and even in the females, glucose and glycogen showed no differences in concentration between the groups. This could be due to the fact that sleep in the females didn't

alter as vastly between baseline and starvation as it did in the males, which could account for no significant changes in sleep and the way the metabolic stores were used. What was significant, however, was glycogen in the male *Canton S* flies. Glucose concentration was different in the *Canton S* males showing that the different sleep groups used glucose as an energy source, which is expected, but the glycogen levels were not only significantly different for these flies, but also showed a regression which was linearly dependent on how much they slept the night of starvation. The flies that slept less during starvation than the night of baseline used more glycogen, showing that these flies either had to use more energy sources than the other flies or they started with a lower level of energy stores. The group that slept more during starvation had significantly higher levels of glycogen ($p\text{-value}<0.05$) showing that those flies conserved more energy with more sleep.

5.5. CONTINUOUS EXPERIMENTATION TO CORRELATE SLEEP AND AGING

In an attempt to find an initial biomarker relating sleep to lifespan, two experiments with previous significant data were replicated in the lab, both of which gave unexpected results. Both experiments, which included a climbing assay and a change in the amount of yeast in the flies' diet, showed increase lifespan previously with the methods given, but made no correlation with sleep (Balasubramani et al., 2014; Feany & Bender, 2000; Min et al., 2007). The goal was to use these methods to correlate the longer lifespan with the sleep of the

flies since past results had already shown an increase in lifespan, but this did not turn out as hypothesized.

A climbing assay was completed with an initial small sample size in attempt to find any sleep and lifespan correlation. Previous literature showed that when given a chance to climb for 30 seconds, flies which chose to climb lived on average longer than flies which chose not to climb (Balasubramani et al., 2014). When the experiment was replicated, results were initially promising because sleep between the two groups on average was significantly different for both genders (p-value<0.05 by ANOVA). In both males and females, the group which initially climbed during the assay actually slept less than the flies which didn't climb. Unfortunately the conclusions of the experiment showed that although the survival curve for both groups seemed different in both genders, the two groups were not statistically different. The females in the group that didn't climb actually lived on average seven days longer than the group that did climb; a result which was unexpected compared to previous data (Le Bourg & Lints, 1992). In the males, both groups lived until the same day, but for the group that didn't climb during the assay, some flies in the group died quickly before the rest began to die at a linear rate. With a replicated experiment and a larger sample size per each group, this could possibly change the significance between the two groups, which could possibly correlate the change in sleep to the lifespan dependent on the climbing assay, but initial results showed that these methods would not give a successful biomarker when associating sleep with aging.

Caloric restriction was also attempted in which the flies were fed a diet with a higher yeast content compared to a group of flies fed lower yeast content. Previous literature supports that flies which would be fed lower yeast content would actually live longer than flies fed higher yeast content (Min et al., 2007; Piper et al., 2005; Sohal & Weindruch, 1996). With previous research showing these conclusions, the goal was to then take this data and correlate it to a change in sleep between the two fed groups. Initial sleep results showed that the sleep between the two groups did not significantly change for either gender, which showed that a correlation with sleep would be nulled. However, in the females, sleep in the low yeast flies seemed to be more fragmented as shown by increased number of bouts, which is usually associated with aging and unhealthy flies (Lim et al., 2013), the opposite of the hypothesis. The survival curves showed some expected results with the females and males lifespan being significantly different (females $p\text{-value}<0.001$ and males $p\text{-value}<0.05$). However, the female survival curve showed that the flies on the low yeast diet initially started dying quicker, but yet both groups of high yeast and low yeast both concluded on the same day. The inconsistent data makes any correlation between sleep and lifespan impossible when associated with these diet changes for females. The males' survival curve showed that flies in the group fed lower yeast content lived about 10% longer on average than flies which were fed high yeast content, the expected result. These data show that a low yeast diet can significantly increase lifespan in male wild-type flies and allow more experimentation to correlate it as a biomarker of sleep and aging.

5.6. FUTURE DIRECTIONS FOR CURRENT EXPERIMENTATION

The research completed thus far is just the beginning for finding a specific biomarker correlated with aging. With sleep survival data collected for all four genotypes, both genders per each, lifespan predictions can be completed for all sets of data and more biological experimentation can be completed.

In order to further support the claim that antioxidants decrease with age, therefore decreasing support against reactive oxygen species, the statistical model could be used to split wild-type *CS* Shaw flies into shorter and longer lived flies, as before. Once split, the flies' food can be dosed with paraquat, a known reactive oxygen species. If death occurs in the predicted shorter lived flies before the longer lived flies, this would support the idea that antioxidants, specifically glutathione, are decreased in shorter lived flies due to them already being biologically older (Bonilla et al., 2006; Zou et al., 2000).

Although antioxidant levels prove to be a promising track in correlating a biological marker to aging, further experimentation with Western blots could possibly offer a more specific biomarker. *Drosophila* can be aged to a certain time point, lifespan can be predicted with the statistical regression model, and the flies can be split between a long and short lived group, just as the current methods being used. With these groups, homogenate could be used to detect specific protein differences found between the two known groups. A good start would be p16, a tumor suppressor protein which has been previously correlated with age (Krishnamurthy et al., 2004; Ressler et al., 2006). A specific biomarker

correlating sleep and age could be any number of proteins, most likely previously found to correlate with either sleep or aging previously.

Statistical data also has a promising future direction in a sense that there can always be new model conducted and created to find correlations within the data. A model which could split the flies into more specific groups, as well as a model which could offer an increased R^2 at an earlier time point in life, would both be directions in which the project could benefit.

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