Spring 2019

The correlation between sleep and lifespan in Drosophila melanogaster

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THE CORRELATION BETWEEN SLEEP AND LIFESPAN IN *DROSOPHILA MELANOGASTER*

by

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A THESIS

Presented to the Faculty of the Graduate School of the

MISSOURI UNIVERSITY OF SCIENCE AND TECHNOLOGY

In Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE IN APPLIED AND ENVIRONMENTAL BIOLOGY

2019

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ABSTRACT

Adequate sleep is associated with an individual's health. Too little sleep is associated with many health problems, including cardiovascular disease, obesity, and a general increase in all-cause mortality. Yet the molecular changes that link poor sleep and changes in health are still not well understood. Individuals have a unique daily need for sleep, and deviations from the animal's regular sleeping patterns can be indicative of, or result in, underlying changes in its health. Therefore, we hypothesize that changes in the sleep architecture in *Drosophila melanogaster* reflect changes in the fly's health.

We determined sleep architecture in wild-type male flies over their entire lifespan. We converted activity into sleep and wake-bout parameters and determined the best multiple linear regression model that described lifespan. Variables describing sleep stability can predict the actual lifespan with an adjusted R^2 of 0.42. We then re-calculated the model using sleep data to predict lifespan by approximately midlife. The animals were separated into cohorts consisting of short-lived and long-lived flies, giving us the opportunity to study their underlying molecular differences.

Short-lived flies have significantly increased Amylase mRNA expression in the heads, a biomarker for sleepiness. Moreover, long-lived flies had significantly increased levels of the endogenous antioxidant glutathione (GSH) in their bodies when compared to their short-lived counterparts. There were increased levels of polyubiquitinated proteins in our short-lived samples, which is often observed in older animals. Our results indicate that sleep architecture can be used to separate biological aging in flies in a non-invasive manner to study the molecular changes that occur with an individual's sleep patterns.

ACKNOWLEDGMENTS

Thank you to all who have supported me through the graduate program at the Missouri University of Science and Technology. I would especially like to thank my mentor Matthew Thimgan, who has enabled me to become a driver in my experiments. Through his teachings, he has challenged me to become an independent scientist, and a critical thinker. I would also like to thank NIH for funding this project.

Finally, I would like to thank my loving mother, father, and sister for supporting me through my undergraduate and graduate careers.

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1. INTRODUCTION

Regular sleep is imperative to the health of all animals (Luyster, Strollo et al. 2012). Insufficient or erratic sleep has been linked to a myriad of problems including weight gain (Markwald, Melanson et al. 2013) (Taheri, Lin et al. 2004), metabolic stress(Shukla and Basheer 2016), oxidative stress(Gopalakrishnan, Ji et al. 2004) (Villafuerte, Miguel-Puga et al. 2015), cardiovascular disease (Gottlieb, Redline et al. 2006) (Redline, Yenokyan et al. 2010) (Punjabi, Shahar et al. 2004), inflammation(Dumaine and Ashley 2018) and more. Yet for all that is known of the effects of poor sleep, there is much to be learned about why sleep is necessary and so conserved throughout the animal kingdom.

Previous experiments have attempted to address the necessity of sleep by manipulating the animal at the genetic level or by manipulating an animal's regular sleeping pattern via sleep deprivation or fragmentation (Villafuerte, Miguel-Puga et al. 2015) (Everson 1995). Invasive experiments like these often require that the animal is sacrificed to measure the health of the animal through biochemical experimentation. These experiments are great for discerning what goes wrong when an animal is denied sleep, but they do little to elucidate the restorative functions that sleep serves (Brown, Basheer et al. 2012) (Atkinson and Davenne 2007).

The relationship between sleep and health is further complicated by the fact that each individual animal has a unique daily need for sleep, and there is substantial variation between individuals even within the same species (Cirelli , Cirelli and Bushey 2008) (Koudounas, Green et al. 2012). Therefore, it is difficult to qualify what good sleep looks like for any individual organism.

Recent advances in mathematical modeling have opened up opportunities to gain a better understanding as to the restorative function that sleep serves (Swindell, Harper et al. 2008). This may provide a more complete picture as to what healthy sleep looks like, and how it changes with age. This thesis addresses the link between sleep and health in Drosophila melanogaster by modeling the relationship between sleep architecture and lifespan for each individual animal. Our model offers a novel method to better understand how this relationship affects and is affected by the underlying biology of the animal.

1.1. THE RELATIONSHIP BETWEEN HEALTH AND SLEEP

Epidemiological studies in humans have revealed an association between sleep duration and an increase in all-cause mortality for both short- and long-duration sleepers. Deviation from the mean sleep duration in humans is correlated with an increased BMI, cardiovascular and respiratory problems, insulin resistance and other long-term issues (Ohayon, Carskadon et al. 2004). However, studies on humans are often based on subjective surveys and are therefore difficult to interpret. They also cannot assess specific biological differences between good sleepers and bad sleepers which remain largely unknown. Further, these studies are not able to identify subjects with poor sleeping behaviors and thus lack predictive ability.

Studies focusing on insomnia and other sleep disorders have also yielded important information on the consequences of inadequate sleep. Insomnia in humans is associated with health problems later in life, including an increased risk for disease, decreased telomere length, and even an increased risk of death (Carroll, Esquivel et al. 2016). The sleep disorder is also correlated with an increase in inflammation and

disruption of immune and endocrine pathways (Okun 2011). Diseases like insomnia give scientists clues as to what the consequences of poor sleep actually are and highlight the need for additional research.

Animal models allow researchers to evaluate specific changes in biology due to poor sleep and have indicated that sleep duration and health are related. Reduced sleep duration is correlated with a shorter lifespan in *Drosophila*, as well as *C. elegans*.

1.2. *DROSOPHILA MELANOGASTER* AS A MODEL ORGANISM

Drosophila melanogaster is an ideal animal model to examine the relationship between sleep and longevity. The animal's short lifespan of about two months, along with its fast reproduction rate, allows researchers to study many generations within a short period of time. Additionally, the small size of the fly makes storage and feeding cheap and easy - reducing lab storage requirements as well as costs when compared to other animals. This has led to the accumulation of a vast catalog of genetic information from which additional research may be done (Donelson and Sanyal 2015).

1.3. SLEEP IN *DROSOPHILA MELANOGASTER*

Sleep in *Drosophila* has been extensively evaluated (Hendricks, Finn et al. 2000, Shaw, Cirelli et al. 2000) (Cirelli and Bushey 2008). The fruit fly displays the same sleep characteristics as not only other invertebrates, but even that of "higher" forms of life. Like in mammals, sleep characteristics in the fly include extended periods of reversible immobility, also known as quiescence, along with a heightened arousal threshold (Shaw, Cirelli et al. 2000) (Hendricks, Finn et al. 2000). Fruit flies are also negatively impacted

by sleep deprivation and will respond with a sleep rebound when deprived of sleep (Shaw, Cirelli et al. 2000, Ganguly-Fitzgerald, Donlea et al. 2006). Additionally, experiments have shown that flies are responsive to some of the same sleep- and wake-inducing drugs that affect humans, including adenosine receptor antagonists (e.g. caffeine) and GABA agonists (e.g. gaboxadol) (Dissel, Angadi et al. 2015).

Sleep in the fly is diurnal and is governed by the same 24-hour circadian mechanisms as mammals, suggesting that sleep is highly conserved throughout the animal kingdom. In fact, the universal circadian molecules *timeless* and *period* were originally discovered through forward genetic screening of flies bred to lack a circadian rhythm (Dowse, Hall et al. 1987) (Young 1996). This 24-hour process, known as Process C, is regulated by the presence and absence of blue light. The presence of light works to align the circadian "clock" within the animal's brain, which then influences molecular processes like gene expression and regulation, driving broader animal behaviors (Borbély 1982). Fly mutants lacking the any of the essential components of the circadian clock (*cycle, period, clock)* have an irregular sleeping pattern, and tend to sleep randomly throughout the 24 hour day (Sehgal, Price et al. 1994) (Konopka and Benzer 1971).

The second process, known as Process S, describes how waking affects the accumulation of sleep debt and how sleep works to restore the animal to a sleep-satiated state. Process S can be evaluated through the animal's need for a sleep rebound after sleep deprivation. This process has further been studied in *Drosophila* mutants that lack the fundamental circadian molecules which govern cycling, resulting in a fly that is unbound by light cycles. Loss-of-function circadian mutations cause the fly's sleep to "drift" and have an irregular sleep cycle. Still, little is known about Process S, but the

interaction between it and process C is thought to account for the timing and strength of the animal's sleep drive (Borbely et al. 1982).

Like in mammals, there is substantial variability of sleeping characteristics between individual fruit flies of the same genotype. Total sleep time in wild-type male flies follows a normal distribution and can range from 600 to 1200 minutes per day. Numerous studies have attempted to use this variability to breed short- and long-sleeping flies to isolate specific genes responsible for regular sleep behavior.

Selective breeding of short-sleeping flies has also been used to assess the consequences of sleep disruption. Short-sleeping flies selected to model human insomnia exhibited increased sleep latency, decreased sleep consolidation during the dark period, and increased locomotor activity. These characteristics resulted in learning and balance impairment when compared to controls. Biomolecular differences between short-sleeping flies and controls were also evaluated. Short-sleeping flies were found to have elevated mRNA transcripts of the sleep-debt marker *Amylase* in the head, increased dopamine, and increased levels of cholesterol and fatty acids. Additionally, the insomnia model of *Drosophila* had a significantly reduced lifespan when compared to controls, providing more evidence that sleeping behavior is intimately linked with lifespan (Seugnet, Suzuki et al. 2009).

1.4. SLEEP ARCHITECHTURE AND AGE

The link between aging and the deterioration of sleep quality and quantity has been well established in multiple organisms including humans, flies, mice and *C. elegans* (Pincus and Slack 2010). Both humans and fruit flies, for example, tend to sleep less as

they age by total sleep duration, with increasing fragmentation. This decrease in consolidation leads to increased daytime sleep and decreased nighttime sleep as the animal ages (Koh, Evans et al. 2006). Older flies also have a diminished ability to recover after sleep deprivation, suggesting a reduced effectiveness of sleep (Vienne, Spann et al. 2016).

Studies in humans have shown that total sleep time, sleep efficiency, slow wave sleep and wake after sleep onset all change with age (Skeldon, Derks et al. 2016). These same studies, however, show that there is substantial variability as to when in life these changes occur. In other words, chronological age is less important than physiologic age. Additionally, there have been conflicting results when looking at other factors as they change with age including sleep latency, percentage of time spent in sleep stages 1 and 2, and percentage of time spent in REM (Yin, Jin et al. 2017) (Atkinson and Davenne 2007). This could be the result of differences in methodologies including sample size, differences in ages studied, cultural differences, and other confounding variables. Finally, it is notoriously difficult to measure the quality of sleep given the subjective nature of sleep surveys. The differences found between studies highlights the need for a better way to study the effect of age on sleep behavior using animal models under controlled conditions.

1.5. MATHEMATICAL MODELING

There has been a push in recent years to describe the world using advanced mathematical models. Statistical evaluation of large datasets has revolutionized our understanding of the world from economics to ecology to climate and now, biological

processes. The ability to develop models that are based on pattern recognition have proven indispensable when dealing with the massive amounts of data that may be generated in a given experiment. The field of bioinformatics is currently being used in a wide range of studies in genomics and proteomics, as well as population dynamics and pharmacology.

There have been various attempts to predict lifespan based on simple sleep metrics, but with limited success (Wallace, Stone et al. 2018). Previous experiments used basic sleep metrics, like total daily sleep duration, to predict how long an animal will live. This approach is problematic, however, because each individual organism has a unique need for sleep, and so it's hard to simplify the ideal amount of daily sleep to just one number derived from a single cohort (Koudounas, Green et al. 2012). Further, there may also be tradeoffs and compensations that complicate the sleeping behavior of an organism. For example, an organism with a low total sleep time may still exhibit a healthy sleep due to increased consolidation. Therefore, the average time spent asleep is simply not enough information to predict lifespan.

There is evidence that models developed using a multivariable approach have a higher success rate of predicting lifespan in animals than models generated using a single variable, like sleep duration, alone. Linear regression allows for many variables to be incorporated into a single model, while generating lifespan predictions for each individual organism.

1.6. BIOLOGICAL MARKERS FOR INADEQUATE SLEEP

A cross-species biomarker for sleepiness has long been sought after. Due to the complexities of sleep-wake systems, it has been difficult to identify a single molecule which is correlated with rising sleep debt and can be measured endogenously in humans as well as other animals. Amylase was the first cross-species biomarker discovered to be correlated with sleep debt. Amylase mRNA transcripts are upregulated as an animal becomes more sleep deprived and is thus a potent biomarker that can be used to objectively measure sleepiness (Seugnet, Boero et al. 2006).

Produced in the salivary glands and pancreas, amylase is an enzyme which breaks down complex polysaccharides (starch) into simple sugars. The enzyme is upregulated in the presence or anticipation of feeding. It is unknown why this gene is upregulated during sleep debt accumulation, but one may speculate that there is an increased energy need that is associated with waking that drives animals to find more food (Schneyer 1956).

Poor sleep has also been associated with an increase in transcripts related to immune function. Sleep deprivation in rats and humans has resulted in a homeostatic response that is consistent with an elevated immune response and is accompanied by inflammatory signaling markers. Total sleep deprivation caused rats to have increased levels of interleukin-6 when compared to both yoked and non-sleep-deprived controls. Sleep deprivation was also shown to increase salivary IL-6 in humans(Thimgan, Gottschalk et al. 2013). The same study showed remarkably increased levels of a variety of immune-related biomarkers in CS flies after sleep deprivation. These included *Drosocin, Attacin-B, Drosomycin* and *Metchnikowin.* It's worth noting that transcript levels were a function of sleep deprivation duration for many of the aforementioned

genes. These data provide evidence that there are indeed objective ways to quantify sleep debt.

1.7. MARKERS FOR AGING

1.7.1. Oxidative Stress. There is strong evidence that oxidative damage plays a key role in aging (Jung et al. 2013). Free radicals and oxidative species (eg. Superoxide generated by the mitochondria) lead to chemical changes in many parts of the cell. This includes damage to the cell's protein systems as well as its DNA, resulting in either repair, apoptosis, or the passing on of damaged DNA to the next generation of cells. This is thought to be partly responsible for the general deterioration of an organism throughout its lifetime.

The cell has built in defenses to protect itself; one of which, the antioxidant glutathione. Glutathione is a small tripeptide with a reducing thiol group used to neutralize ROS's. This action converts the reduced glutathione (GSH) to its oxidized form, glutathione disulfide (GSSG), and must then be reconverted by the enzyme glutathione reductase (Figure 1.1). The GSH/GSSG ratio is thought to be an indicator of oxidative stress within an organism (Jahngen-Hodge et al. 1997).

Glutathione is endogenously generated in a two-step process. Glutamate-cysteine ligase (GCL) facilitates the creation of a peptide bond between the amino acids glutamate and cysteine and is the rate limiting step in the synthesis of glutathione. GCL is made up of two subunits, GCLC and GCLM, the expression of which have been found to be correlated with increasing oxidative stress (Franklin et al. 2009). Additionally, expression of both subunits can be induced by the transcription factors nrf2, ap-1, and NF-kB.

Glutathione synthesis is down-regulated with an increase in the concentration of GSH and can be limited by both substrate and GCL component availabilities (Lu 2009).

Sleep plays an important role in the regulation of cellular redox state. Sleep deprivation in animal models results in the accumulation of reactive oxygen species, like superoxide (Gopalakrishnan, Ji et al. 2004). This suggests that sleep serves to protect the organism from oxidative stress. Further, short-sleeping *Drosophila* mutants have a significantly reduced lifespan when exposed to H_2O_2 or paraquat compared to their normal sleeping controls (Hill, O'Connor et al. 2018). This phenotype is reversed when the flies are forced to sleep with drugs like the GABA receptor agonist, Gaboxadol.

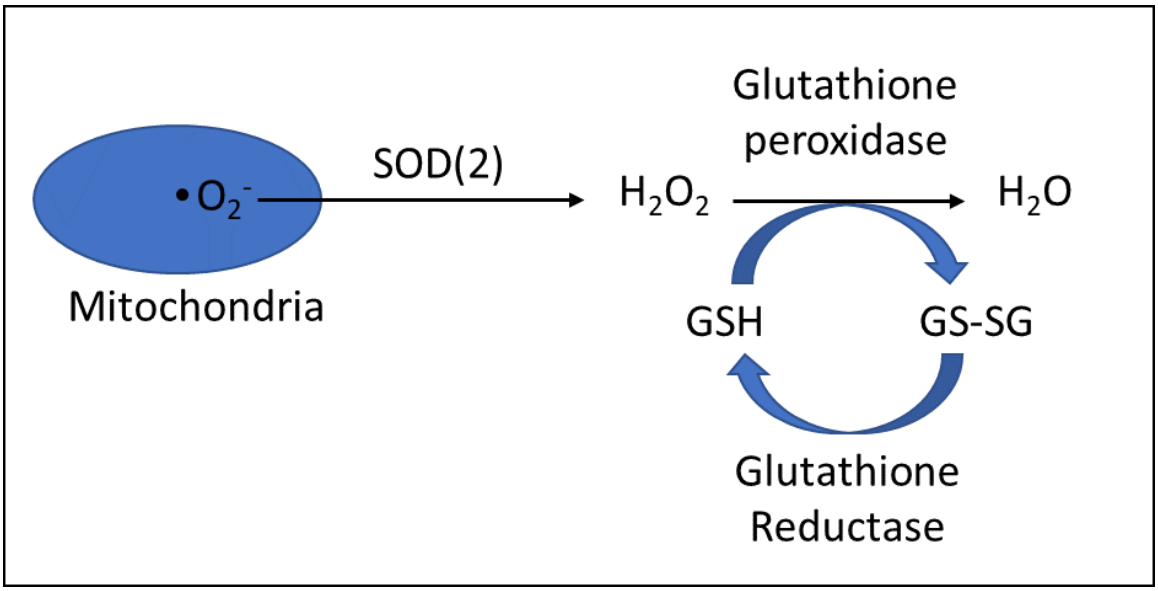


Figure 1.1. Cellular Redox: superoxide free radicals are reduced to hydrogen peroxide by superoxide dismutase. The product is then broken down into water and oxygen by catalase or reduced by glutathione (Li, Yan et al. 2000).

1.7.2. Ubiquitin Proteasome Pathway. Ubiquitin is a small, 76 amino acid protein which serves many purposes from protein degradation to chromatin remodeling. The importance of the ubiquitin-proteasome system is underscored by its almost completely conserved molecular mechanisms from which it acts(Lee, Simon et al. 1988). Additionally, changes within this system are thought to be one of the key causes of cellular and molecular aging, making this system an ideal candidate to study the complex effects of sleep and aging.

All organisms have one or several genes coding for polyubiquitin. The gene contains a homomeric fusion of ubiquitin molecules which are inert until cleaved into individual ubiquitin proteins. Different species have very similar polyubiquitin genes but with one caveat, the number of repeats of ubiquitin within the gene can vary widely from species to species, suggesting rapid evolution of the gene. In humans, the Ubiquitin-B gene encodes 3 repeats of ubiquitin. The polyubiquitin product is then processed into 8.5 kd monomers which can be activated and used when needed. These monomers appear to be difficult to detect, leading researchers to believe that they rapidly bind to proteins once synthesized, and that the process is highly regulated (Hoe, Huang et al. 2011).

Target proteins can be ubiquitinated in several ways and the protein's fate is determined by where ubiquitin is attached. Multiple ubiquitin monomers can attach to a single protein due to the presence of seven lysine residues on ubiquitin itself, creating a thioester bond between the two ubiquitin monomers. K48 linked ubiquitin chains tag the protein for proteolysis and it is then broken down into its constituent amino acids by a 26S proteasome. Inhibition of the 26S proteasome can cause a buildup of polyubiquitinated proteins that can be detrimental to the cell in which this is occurring.

Ubiquitination of a target protein is mediated by a three-enzyme cascade consisting of E1, E2, and E3 components. E1, also known as Ubiquitin activating enzyme, covalently binds to ubiquitin in an ATP dependent process. Once bound and activated, ubiquitin is then transferred to E2, the ubiquitin conjugating enzyme. There have been about 40 discovered E2 enzymes in humans, all of which are nearly identical, with small changes in the active site leading to specificity to E3, also known as Ubiquitin Ligase. The E3 then binds to both the E2 (still holding ubiquitin) and the target protein and mediates the transfer of one or more ubiquitin molecules to the target. There are two categories of E3 proteins with specific active sites. The first contains a RING motif (Really Interesting New Gene) which directly catalyzes the transfer of ubiquitin from E2 to the target (Deshaies et al, 2009). There have been over 600 E3 enzymes discovered, each with specific binding properties, this makes E3 one of the most diverse family of proteins ever described. Mutations in some of these E3s have been associated with various diseases like Alzheimer's and Huntington's (Marblestone et al., 2013).

Cellular redox state has been proposed to be one of the key regulators for the enzymes within the ubiquitin conjugation pathway. The ability for E1 and E2 enzymes to accept ubiquitin depends on the maintenance of the active site, which must be in the reduced state to receive the ubiquitin monomer. Glutathione is thought to be a key regulator of this process, and the ability for E1 and E2 to form thiol esters is correlated with the GSH:GSSG ratio. Thus, lower concentrations of reduced glutathione can suppress ubiquitin conjugation/ Further, exposure to oxidants has been shown to inhibit ubiquitin conjugation leading to a buildup of oxidatively damaged proteins (Jahngen-Hodge, Obin et al. 1997).

After poly-ubiquitination, damaged or misfolded proteins are recognized by a proteasome, starting the degradation process. Proteasomes are multi-subunit protein complexes responsible for the degradation of both misfolded and oxidized proteins into their component amino acids (Jung and Grune 2013). Proteasomes are made up of the 20S cylindrical "core" bound by up to two regulatory adapters capping either end of the core. The 20S core has the ability to degrade a wide range of damaged proteins, but it does not have the ability to unfold them by itself. For this function, it must associate with a 19S subunit which allows the proteasome to unfold and digest ubiquitinated proteins in an ATP dependent manner. When one or more 19s subunits are associated with the 20S core, the complex is known as the 26S proteasome.

The association and dissociation of the 19S subunit is dependent on the cellular redox state (Hohn and Grune 2014). In an oxidative environment, the 19S subunits will disassociate from the 20S proteasome, enabling the core subunit to better process oxidatively damaged proteins. It is thought that increasing oxidative stress gradually causes proteins to expose hydrophobic structures normally located near the protein's core. The 20S core has the ability to recognize these exposed amino acid sequences, leading to the digestion of the protein. A buildup of oxidatively damaged and polyubiquitinated proteins is thought to be a key indication of aging.

1.8. RESEARCH OBJECTIVES

The purpose of this thesis is to evaluate the relationship between an organism's sleep stability and its overall health. An organism's sleeping behavior, health, and biology are intimately linked, and a breakdown in any one will ultimately lead to the

breakdown of the others. Weakness in any one component will lead to an accelerated aging profile that can be detected in both organism behavior as well as the underlying biology. Here, we focus on the effects of poor sleep stability on several biological systems to uncover novel mechanisms in which aging may occur.

The foundation for this thesis was laid by experiments completed in Dr. Thimgan's lab conducted by a previous graduate student. The results suggest that *Drosophila* lifespan can be successfully modeled by linear regression of sleep-related variables. Further, the model can be applied to a new set of 30-day old flies, to generate lifespan predictions. Binning the flies into short- and long-lived groups then gives us the ability to assess the biological differences that may cause an animal to be short- or long-lived as a result of its sleeping behavior (Figure 1.2). We plan to use this method to better understand the biological causes and consequences of poor sleep.

1. To build upon and validate the work done previously in Dr. Thimgan's laboratory by a previous graduate student. This includes expanding the full lifespan model to include a larger sample size so that a broader range of fly sleep behavior may be evaluated and studied.
2. Apply the model to a new set of flies after monitoring them for approximately 30-days.
3. Bin the flies into short- and long- lived groups and confirm the model by testing for known biological aging markers (Figure 1.2).

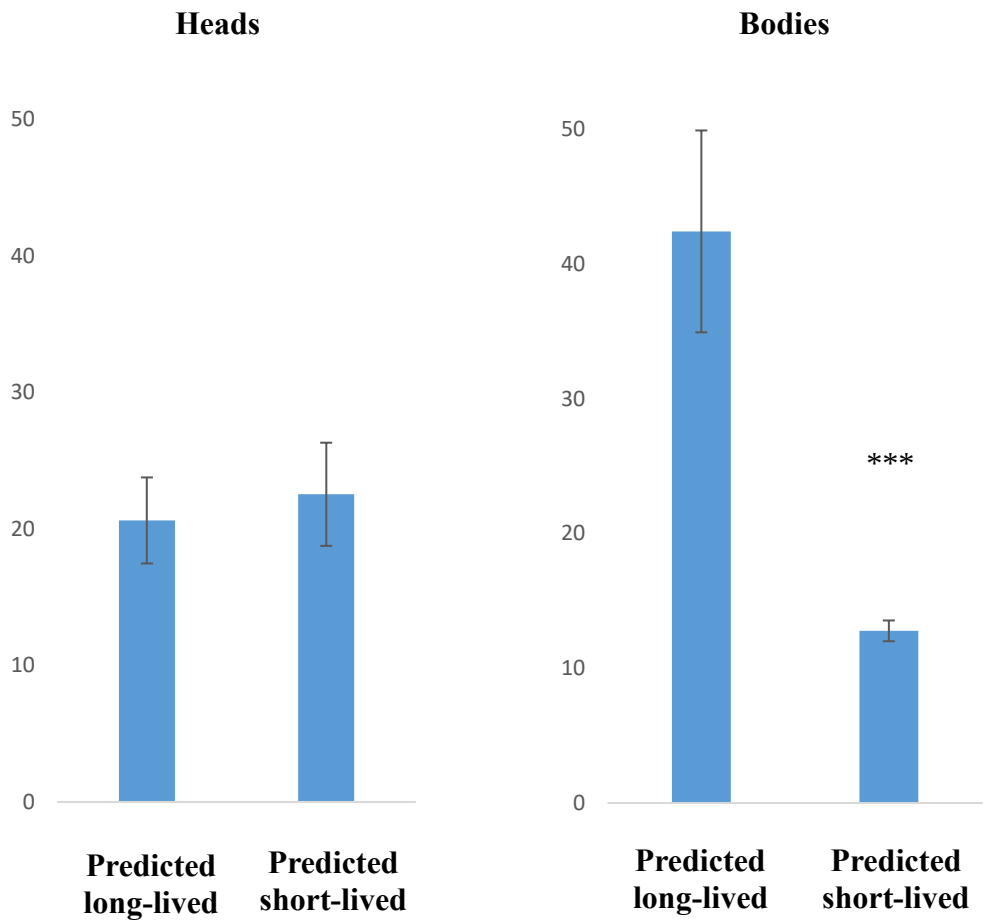


Figure 1.2. Preliminary Results Indicate Lower Levels of Glutathione in the Bodies of Predicted Short-Lived Flies.

2. MATERIALS AND METHODS

2.1. ANIMAL HUSBANDRY

All live animals were stored in polystyrene vials (75mm tall x 25mm diameter) and housed in an incubator set at 25°C and on a 12:12 light:dark (LD) lighting schedule until mating. The flies were "flipped" into new vials and allowed to mate for three days under the same conditions. The parents were then removed from the vials, allowing a new generation of flies to emerge. Flies were reared on a standard diet consisting of agar, corn syrup, molasses, sucrose and yeast. Adult male *Canton S.* flies were used for all experiments.

2.2. FLY ACTIVITY AND SLEEP MONITORING

One- to three-day old *Canton S.* flies were anesthetized under CO_2 and separated by sex. Males were collected and stored in vials for up to three days, allowing them to become socialized. Four- to seven-day old flies were then loaded into individual glass tubes (3mm diameter, 65mm length) containing food with a wax cap on one end, and an air-permeable plug on the other. The flies were loaded into tubes by aspiration to avoid unnecessary CO_2 exposure, and 32 tubes containing flies were placed into *Drosophila* Activity Monitors (DAM, Trikinetics, Waltham, MA, USA) in an incubator with identical conditions as stated above.

Fly activity was recorded and converted into sleep data with an in-house, Excel (Microsoft, Richmond) based program as described previously. Beam breaks were segmented into 1-minute bins, generating a text file of each individual fly's activity during a 24-hour day. The in-house program was then used to populate an Excel file with

raw activity data for every fly in the experiment for each 24-hour period. These data are then moved to another in-house program that converts the raw data into sleep-wake information. The program then generates hourly sleep data for each fly, and calculates the following sleep metrics: latency, total sleep, average day bout duration, average night bout duration, maximum day bout duration, maximum night bout duration, number of day bouts, number of night bouts, and maximum rest time.

Activity was monitored for either the fly's entire life, or until the flies reached an age of 30 days, depending on the experiment. The flies were transferred into new tubes containing fresh food twice a week to prevent the food from drying out. Any deaths were recorded at this time. Activity data were collected and converted into sleep data on a daily basis, and a survival curve was generated after the conclusion of each experiment. Flies obtained from the 30-day experiments were placed in a -80°C freezer until later use.

2.3. MODELING AND PREDICTIONS

Lifespan predictions were generated from a multiple linear regression model developed by Dr. Gayla R. Olbricht from the Department of Mathematics and Statistics at the Missouri University of Science and Technology. Raw activity data was converted into a binary representation of sleep status where "1" represents a fly in its awake state, and "0" represents a sleeping fly. A sliding five-minute window was utilized to determine fly sleep status. Fly activity, as counted by beam breaks, inside any of the one-minute bins within the five-minute window is indicative of a fly in its awake state. The absence of activity for the duration of this period indicates a sleeping fly (Figure 2.1).

Fly sleep and wake bout data were then used to calculate 36 unique variables plus a user term for contribution to the model. Markov chains were used to generate transition probabilities. The following variables were calculated daily for both the light and dark periods.

1. Transition probability of the fly staying asleep from one minute to the next (p00).
2. Transition probability of the fly staying awake from one minute to the next (p11).
3. The percent of time asleep over the first 30 days (pi_0).

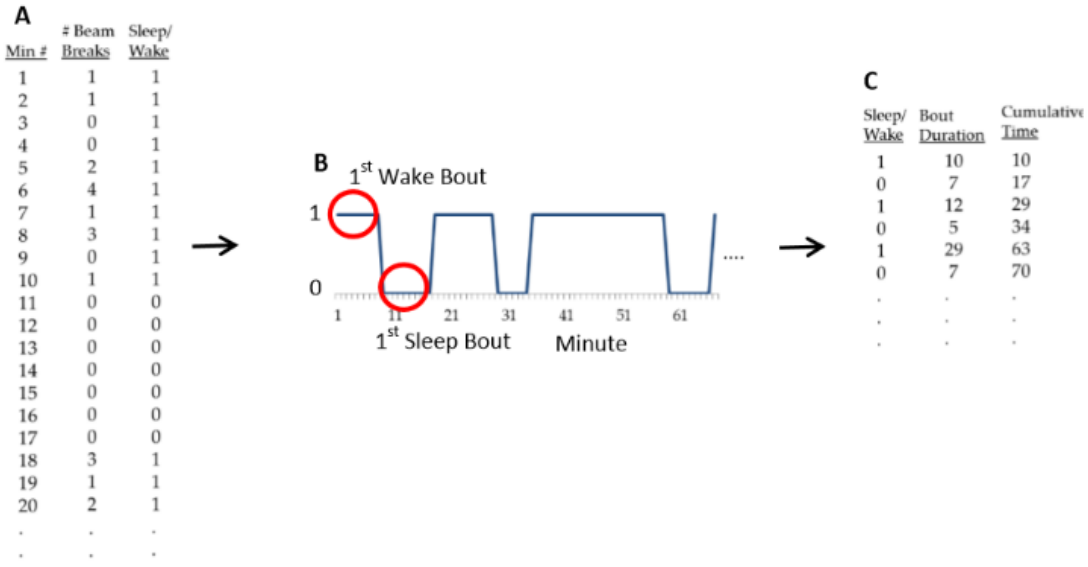


Figure 2.1. Sleep Bout Information can be Derived from Fly Activity Data. A fly is determined to be asleep after a 5-minute period of inactivity. (a): Raw beam break counts are converted to a binary representation of sleep and wake states for each minute, represented visually in (b). Length of each sleep and wake bout is then quantified as shown in (c) (Olbricht, Samaranayake et al. 2014).

The average first differences and the standard deviation of the first differences of the above variables were also calculated, along with a squared term for each of the

aforementioned variables. The resulting 37 variables also included a term for the experimenter. Out of those variables, only 16 were incorporated into the final model. These variables were selected via a model selection process which was utilized to filter out unimportant variables.

2.4. BIOLOGICAL EXPERIMENTS

Frozen flies still inside their monitoring tubes were separated into short- and long-lived groups on dry ice to prevent the flies from thawing. Groups of up to five at a time were then decapitated on a frozen glass plate over dry ice with a clean and sterile razor blade. Groups of heads or bodies were stored in 0.5 mL Eppendorf tubes and placed in the -20°C freezer for immediate use. This ensured that both the fly bodies and their heads remained frozen throughout the process.

2.4.1. Gene Expression. Gene expression was assessed by real time PCR. 20 heads or 5 bodies were homogenized 100ul Trizol (Invitrogen, Carlsbad, CA) using glass Micro Tissue Grinders (Wheaton, Millville, NJ). The samples were then transferred to 1.5mL Eppendorf tubes filled with 900ul Trizol for a final volume of 1mL. RNA was then isolated and pelleted according to the manufacturer's instructions and then dissolved into DEPC-treated water. RNA quantity and quality was then determined with a Nanodrop spectrophotometer (Thermo Fisher, Waltham, MA). 1ug of RNA was DNAse I digested with Optizyme DNAse (Fisher Bioreagents, Hampton, NH) and converted into cDNA with iScript cDNA Synthesis Kit (Bio-rad, Hercules, CA), according to the manufacturer's protocol. Relative cDNA quantity was then assessed by loading equivalent volumes of each sample into iTaq Universal SYBR Green Supermix (Bio-rad,

Hercules, CA) solution as per the manufacturer's instructions along with primers specific to the fly gene Ub-63E. Approximately equivalent amounts of cDNA, determined by Ub-63E concentration, were then loaded into the master mix for gene quantification using a Bio-rad CFX Connect Real-Time PCR Detection System. All genes were normalized to Ub-63E which was used as a reference gene. Gene expression for each cohort was determined by ΔΔCT as described previously (Livak and Schmittgen 2001).

2.4.2. Protein Quantification. Samples were homogenized in lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% Triton X-100, 1% HALT protease Inhibitor, 1mM EDTA) and then centrifuged at 12,000g for 5 minutes. Supernatants were extracted, and protein concentration determined by BCA assay (Pierce Biotechnology, Rockford, IL) with a BMG Labtech FLUOstar Omega plate reader. 100ug of protein was added to 4x Laemmli Sample Buffer (Bio-Rad, Hercules, CA) and 40ug was loaded into each well of a mini-protean stain-free gel (Bio-Rad, Hercules, CA). Protein was then transferred to PVDF membranes using Trans-Blot Turbo mini-size Transfer Stacks and a Trans-Blot Turbo (Bio-Rad, Hercules, Ca). Blots were probed with anti-ubiquitin (1:1000, R&D Systems, Minneapolis) or anti-sod2 (1:1000, Novus Biologicals, Littleton) and were lightly agitated on a rotator at 4°C overnight. The blots were then washed with TBST and then incubated in anti-mouse (1:2000, Abcam, Cambridge) and anti-rabbit (1:2000, Invitrogen, Carlsbad, Ca) for three hours at room temperature. Bands or protein smears were detected with ECL+ (Pierce Biotechnology, Rockford, IL) and imaged using a ChemiDoc Imaging System (Bio-Rad, Hercules, Ca). Band or protein smears were quantified using ImageLab (Bio-Rad, Hercules, Ca), and normalized to total protein images obtained using the stain-free protocol.

3. RESULTS

3.1. FULL LIFESPAN EXPERIMENTS

Sleep data for 192 male adult fruit flies was collected over the life of each fly. Over the 67-day period, data was collected for the following variables on a daily basis: total sleep time, day/night bout duration, maximum day/night bout, number of day/night bouts, maximum rest time and latency. Graphs of these metrics were generated and used for comparison to other experiments to check that the flies are similar and normally distributed (Figure 3.2a). Out of 192 flies, 22 were excluded for various reasons including fly death from unnatural causes (e.g. the fly was crushed during transfer) or it flew away during food exchange. 12 flies were also marked as questionable due to less obvious circumstances (e.g. the fly got stuck in its food and died). In total, 158 flies were ultimately used for modeling. Half of the flies were dead by the 55th day (Figure 3.1).

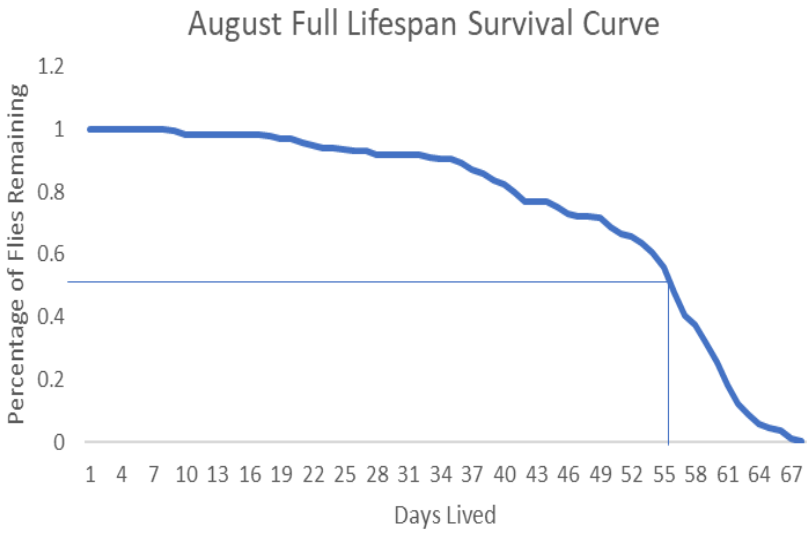


Figure 3.1. Survival Curve for the August Full-Lifespan Experiment. Half the flies were dead by day 55.

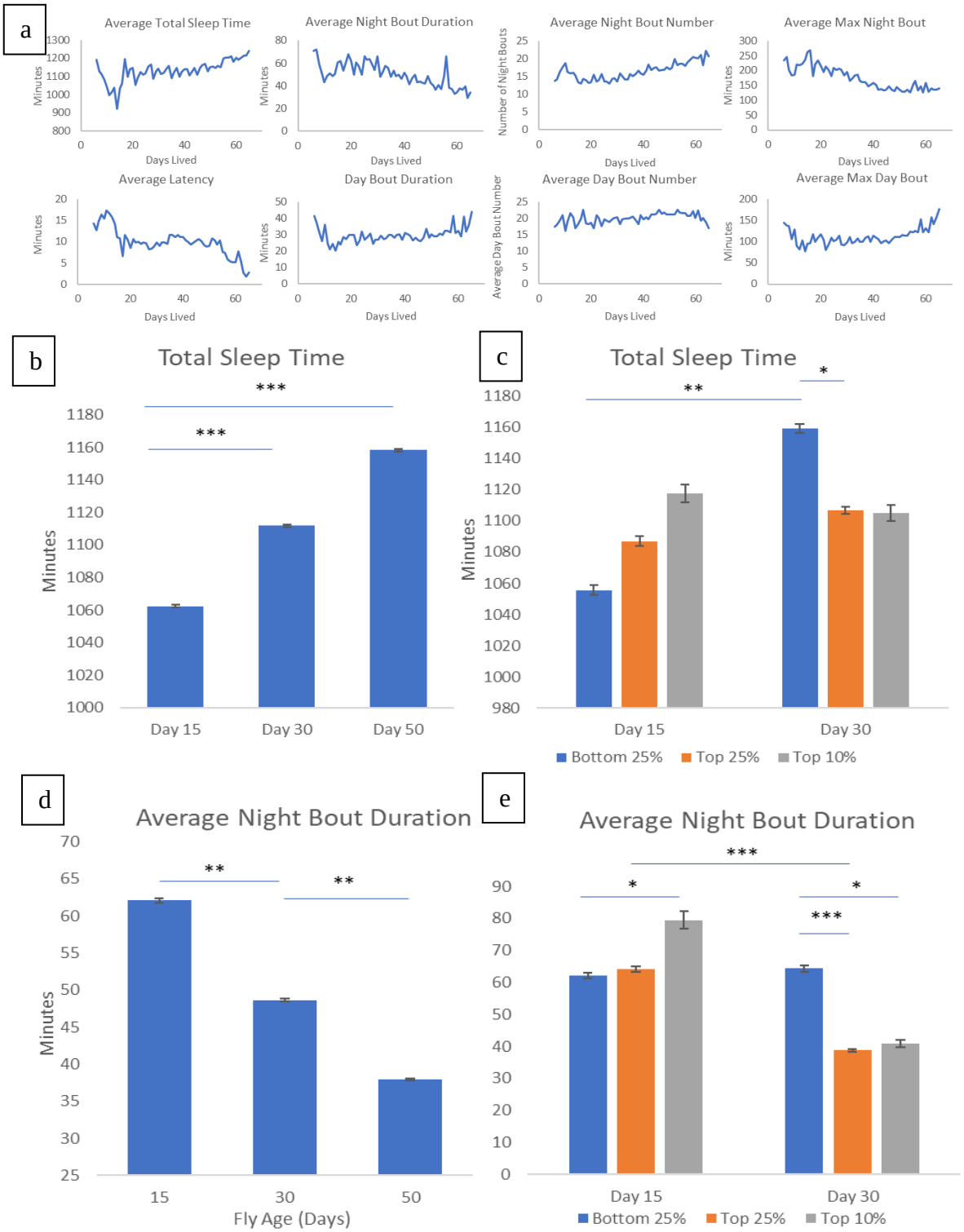


Figure 3.2. Example of Traditional Fly Metrics. (a): Mean daily traditional metrics of the surviving population of flies. (b): Total Sleep Time for 15, 30 and 50 days. (c): Total sleep time for days 15 and 30 by for observed short- and long- lived flies. (d): Average night bout duration for flies at 15, 30 and 50 days. (e): Average night bout duration for observed short- and long- lived flies.

As the flies age, they tend to sleep more by total sleep time (Figure 3.2 b-c). By day 30, this trend was most pronounced in the bottom quartile of flies by days lived. This group of flies slept significantly longer ($p < 0.01$, Student's t-test) at day 30 than day 15. These short-lived flies had a significantly higher total sleep time on day 30 than the top quartile ($p < 0.05$, Student's t-test) but not the top 10% of flies by lifespan, although the trend suggests a similar difference. There was no significant difference between day 15 and day 30 in terms of total sleep time for flies falling in the top quartile and the top 10% of days lived.

The average night bout duration of flies decreases significantly with age (Figure 3.2 d-e). This is most pronounced in flies observed to live within the top quartile of lifespan ($p < 0.001$, Student's t-test), but not in the bottom quartile. Average night bout duration did decrease as the top 10% by lifespan of flies got older, but not significantly. This is possibly due to higher variation.

3.2. FULL LIFESPAN PREDICTION MODEL.

Lifespan predictions were generated by utilizing a multiple linear regression model. The final model was built using fly data (n=380) from four individual experiments, carried out by three different people (Table 3.1).

Table 3.1. Full-Lifespan Experiments Contributing to the Model.

Experiment Title	Researcher	Date	Number of Flies
Full Lifespan Experiment 1	Courtney Fiebleman	April 2015	52
Full Lifespan Experiment 2	Josh Lisse	August 2016	137
Full Lifespan Experiment 3	Josh Lisse	October 2016	117
Full Lifespan Experiment 4	Elizabeth Park	October 2016	74

Out of 37 potential variables, 16 (+3 Experiment terms) were incorporated into the final model. Variables were selected by a stepwise Akaike Information Criterion (AIC) model selection process, ensuring that only the most important variables to lifespan were used in the final lifespan model.

Lifespan prediction was generated inserting values into the following equation: Predicted Days Lived = $\beta_0 + \beta_1 * Var_1 + \ldots + \beta_{19} * Var_{19}$. The first term, the intercept, is represented by β0 and had a value of 116.95. Subsequent terms are comprised of a coefficient (β1-β19), the value of which was determined during model training, and the variable generated for each individual fly. The variables used, along with their parameter estimates can be found in Table 3.2.

a

Predictions vs. Actual Lifespan

		Predicted		
		Short-Lived	Middle	Long-Lived
Actual	Short-Lived	59	29	1
	Middle	34	127	37
	Long-Lived	2	34	57

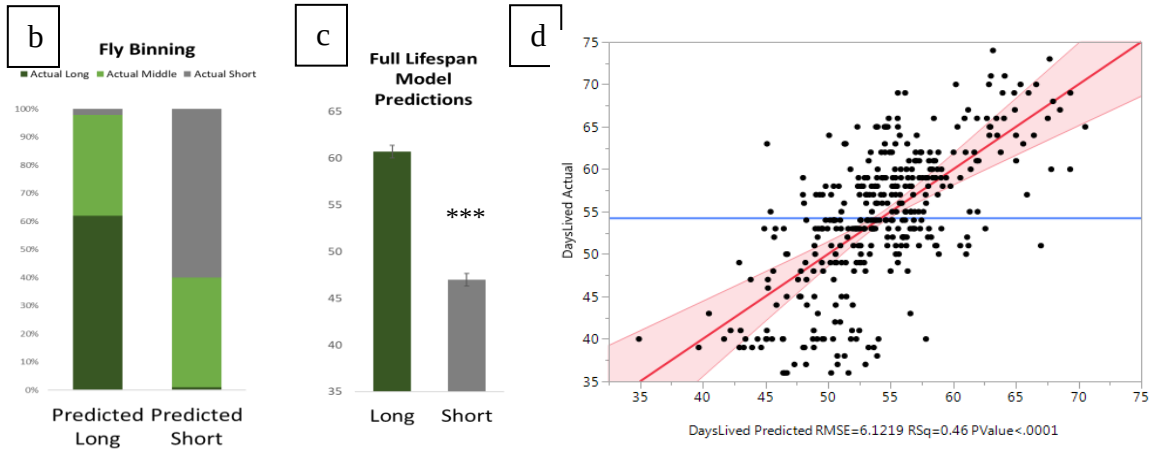


Figure 3.3. Full-Lifespan Model. (a): Fly binning of predicted long- and short-lived flies. (b): Predicted long- and short- lived flies mean actual lifespan. (c): Actual vs Predicted days lived (One-Way ANOVA $p < 0.0001$). (d): Contingency table for actual and predicted lifespan.

Table 3.2. Variables Used for the Full Lifespan Model Sorted by Partial R-Square. FD = First Differences, p11 = wake propabiblity from one minute to the next, p00 = sleep probability from one minute to the next, SD = Standard Deviation.

Variable	Parameter Estimate	P-value	Standardized Estimate	Partial Rsquare
Intercept	116.95	<0.0001	0	.
User Term: Courtney	6.96	<0.0001	0.55173	16.38%
(Percent of Time Asleep – Dark)^2	-299.48	<0.0001	-0.26809	5.31%
Percent of Time Asleep - Dark	-56.91	<0.0001	-0.52449	5.23%
Percent of Time Asleep - Light	34.52	0.0002	0.40063	3.78%
Prob. of Staying Awake - Dark	-45.36	0.0009	-0.32652	3.01%
(Average FD – Light)^2	-43879	0.0011	-0.1403	2.92%
Average FD for p11 - Light	-563.17	0.0012	-0.20191	2.86%
(Average FD for p11 – Light)^2	-63666	0.0023	-0.14791	2.55%
Average FD for p00 - Light	-2549.4	0.0031	-0.19225	2.39%
(Average FD for p11 – Dark)^2	-40675	0.0053	-0.11443	2.14%
User Term: Josh	-1.75	0.0079	-0.17559	1.94%
SD of FD Percent Asleep - Light	60.06	0.0090	0.1699	1.88%
(SD of FD for p11 – Light)^2	957.69	0.0227	0.16879	1.43%
SD of FD for p11 Light	-58.60	0.0555	-0.19299	1.01%
(Prob. of Staying Awake – Dark)^2	-148.34	0.1115	-0.08867	0.7%
Average FD Percent Asleep -	-180.52	0.2749	-0.09205	0.33%
Average FD Percent Asleep -	36.92	0.7100	0.01526	0.038%
User Term: Elizabeth	-0.15	0.8046	-0.01346	0.016%

The percentage of time asleep during the dark period (partial R^2 = 5.23%) and its square term (partial R^2 = 5.31%) were the most influential variables for the prediction of lifespan. This was followed by the percentage of time asleep during the light period (partial R^2 = 3.78%) and the probability of staying awake from one minute to the next, or p11, during the dark period (partial R^2 = 3.00%).

The next five most influential variables were based on first differences, indicating the importance of sleep stability. These included the squared term of the first differences of the mean time asleep during the 30-day light period (partial R^2 = 2.92%), the first differences of the probability that the fly stays asleep from one minute to the next during the light period (partial R^2 = 2.86%), followed by the squared term (partial R^2 = 2.55%).

The resulting model was used to generate lifespan predictions for all 380 flies. When plotted against the actual lifespan of each individual fly, the model was able to successfully predict lifespan with an adjusted R^2 of .435 ($p<.0001$) (Figure 3.3d). The flies were then binned into long-lived (top 25%), middle (middle 50%), and short-lived (bottom 25%) groups by both predicted and actual lifespan (Figure 3.3a). Out of 95 predicted short-lived flies, 62.11% were predicted correctly and were observed to be short-lived, while only 2.11% were predicted incorrectly and were observed long-lived. The remaining 35.79% were observed to be within the middle group. Out of 95 predicted long-lived flies, 60% were observed to be long-lived, while only 1.05% were observed to be short-lived. The remaining 38.95% were observed to fall into the middle group. The predicted short-lived flies had an observed lifespan of 46.95 days, while the predicted long-lived flies had an observed lifespan of 59.37 days. The predicted middle group had an observed lifespan of 54.77 days.

3.3. LIFESPAN PREDICTION AT 30 DAYS

The variables used to generate the full lifespan model were recalculated to be incorporated into a model which predicts lifespan when the fly is 30 days old. 445 flies from 5 different experiments were used when building this model. Out of those 445 flies, 70% (n = 311) were designated as a model training group, while the remaining 30% were withheld from the model for validation. As with the full lifespan model, 36 potential variables were assessed, 14 of which were incorporated into the model (Table 3.3.). Terms accounting for the user were not used for this model.

When the model was applied to the training set (the group of flies used to build the model), it was successfully able to predict lifespan with an R^2 of 0.22 and an adjusted R^2 of 0.183 (Figure 3.4b, One-way ANOVA, $p < 0.0001$). The 311 training set flies were then binned into predicted short- and long- lived groups containing 77 flies each, with 157 predicted to fall into the middle. Out of the 77 predicted short-lived flies, 46.75% were observed to be actually short-lived, with 5.19% having an actual lifespan that would fall into the long-lived category. The remaining 48.05% had an actual lifespan falling into the middle category. Out of the 77 predicted long-lived flies, 46.75% were observed to be long-lived, 11.69% were observed to be short-lived, and the remaining 41.56% of the flies were observed to have a middle lifespan (Figure 3.4a). The mean actual lifespan for the predicted short-lived group was 49.44 days, the predicted middle group was 55.50 days, and the predicted long-lived group was 60.81 days (Figure 3.4d, ANOVA, $p < 0.0001$).

The model was then applied to the withheld group of flies (n = 134), the test set (Figure 3.5). A comparison between the actual days lived and the predicted days lived

yielded an adjusted R^2 of 0.16 (ANOVA, $p < 0.0001$, Student's t-test). The model continued to show strength when binning the flies. Out of the 33 predicted short-lived flies, 45.45% were observed as short-lived, while 6.06% were incorrectly called and were observed as long-lived. The remaining 48.48% had a middle-observed lifespan. Out of the 33 flies that the model predicted to be long-lived, 36.36% were observed to have a long lifespan, while only 9.09% had an observed short lifespan. 54.55% were observed to have a lifespan falling in the middle (Figure 3.5c). The mean actual lifespan for the predicted short-lived flies was 50.45 days, the middle was 56.29 days, and the long-lived was 58.00 days (Figure 3.5d).

3.4. BIOLOGICAL DIFFERENCES BETWEEN SHORT- AND LONG-LIVED FLIES

3.4.1. Amylase. Differences in relative sleep quality between predicted short- and long-lived animals were confirmed by measuring levels of the sleep debt marker, amylase. Heads from three separate 30-day experiments were tested ($n = 20$). Amylase mRNA levels were significantly elevated ($p < 0.05$, Student's t-test) in the heads of short-lived fruit flies when compared to their long-lived counterparts. This is an indication that the predicted short-lived flies have an increase in accumulated sleepiness relative to the predicted long-lived cohort (Figure 3.6).

3.4.2. Drosocin. Drosocin is a marker for inadequate sleep. Short-lived flies from three separate 30-day experiments had significantly more Drosocin expression in the heads ($p < 0.05$, Student's t-test, $n = 3$). Upregulation of this marker in short-lived flies supports our hypothesis that sleep and lifespan have a bi-directional relationship (Figure 3.6.).

a

Predictions vs. Actual Lifespan				
		Predicted		
		Short-Lived	Middle	Long-Lived
Actual	Short-Lived	36	27	9
	Middle	37	95	32
	Long-Lived	4	35	36

30-Day Training Set

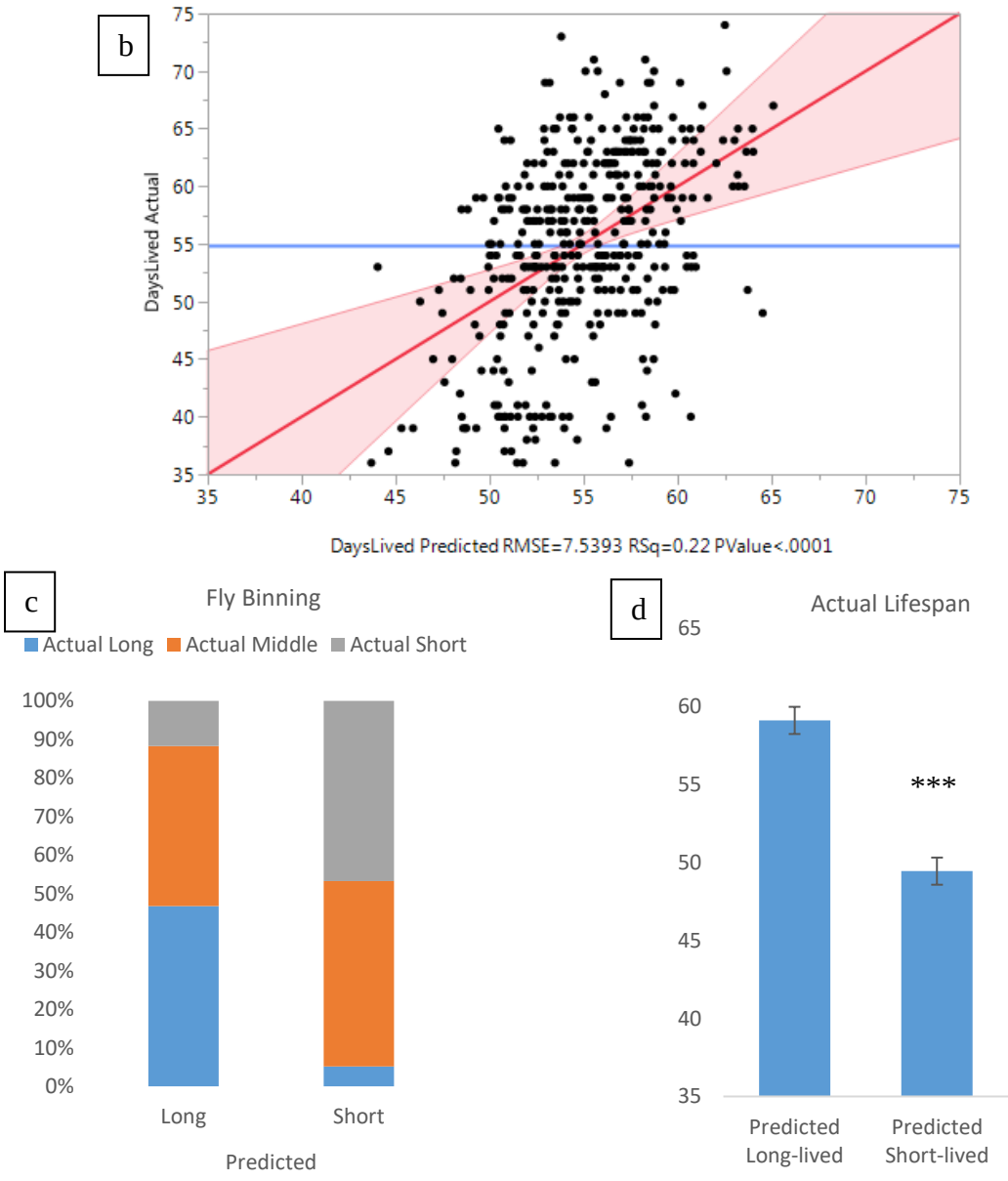
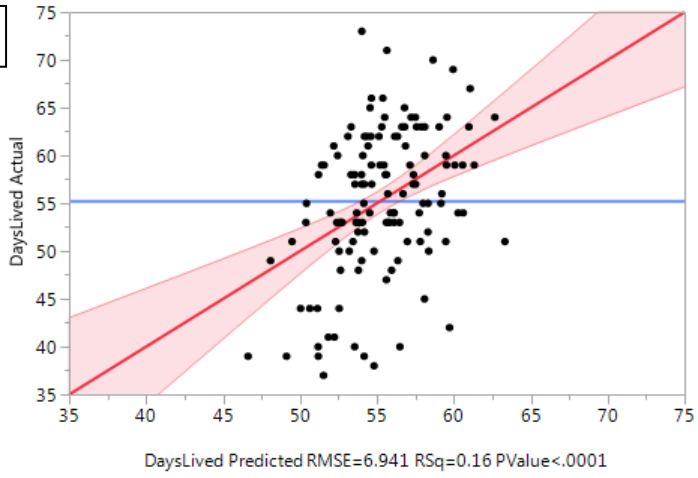


Figure 3.4. 30-Day Training Set. (a): Fly binning by quartile. (b): Predicted days lived vs. actual days lived. (c): Fly binning of predicted long- and short-lived flies. (d): Predicted long- and short- lived flies mean actual lifespan.

a

Predictions vs. Actual Lifespan				
		Predicted		
		Short-Lived	Middle	Long-Lived
Actual	Short-Lived	15	10	3
	Middle	16	40	18
	Long-Lived	2	18	12

b



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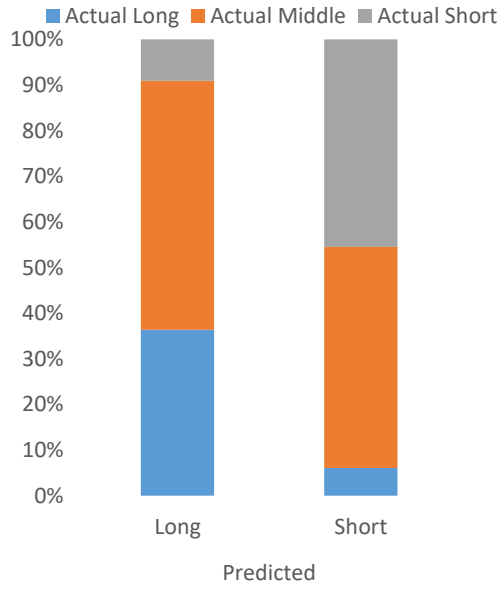

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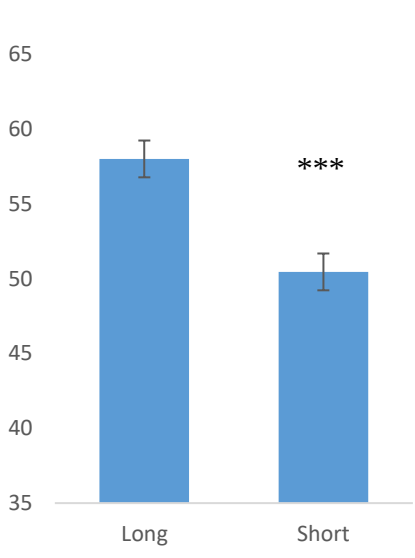

Figure 3.5. 30-Day Test Set: Predictions were generated from flies withheld from the 30-day model and plotted against their actual day lived. (a): Fly binning by quartile. (b): Scatter plot of actual vs. predicted lifespans (n = 134, $p < 0.0001$). (c): A representation of the composition of predicted short- and long-lived flies. (d): The average actual lifespan of predicted short- and long-lived flies (Top/Bottom 25%).

Table 3.3. Variables Used for the Full Lifespan Model Sorted by Partial R-Square. FD = First Differences, p11 = Wake Probability from one minute to the next, p00 = sleep probability from one minute to the next, SD = Standard Deviation.

Variable	Parameter Estimate	p-value	Standardized Estimate	Partial R-square
Intercept	198.78	0.043	0	.
Average FD p11 - Light	-805.06	<0.0001	-0.27	6.81%
Average FD Time Asleep - Dark	-363.41	0.0059	-0.17	2.54%
Percent of Time Asleep - Light	103.42	0.0151	1.19	1.98%
Average FD p00 - Light	-1334.51	0.0186	-0.14	1.86%
Probability of Staying Awake - Light	193.12	0.0224	1.08	1.75%
SD of FD Percent of Time Asleep - Light	51.45	0.044	0.14	1.36%
Percent of Time Asleep - Dark	-14.76	0.0498	-0.18	1.29%
Probability of Staying Asleep - Light	-399.56	0.0524	-0.59	1.27%
(Probability of Staying Awake – Light)^2	525.03	0.0559	0.31	1.23%
(SD of FD Percent of Time Asleep – Dark)^2	-961.13	0.0703	-0.11	1.10%
SD of FD p00 - Dark	327.07	0.0939	0.16	0.95%
SD of FD Percent Asleep - Dark	-42.94	0.2367	-0.14	0.47%
(Average Percent of Time Asleep – Light)^2	11.96	0.8553	0.01	0.01%
(Probability of Staying Asleep)^2	352.84	0.9085	0.01	0.00%

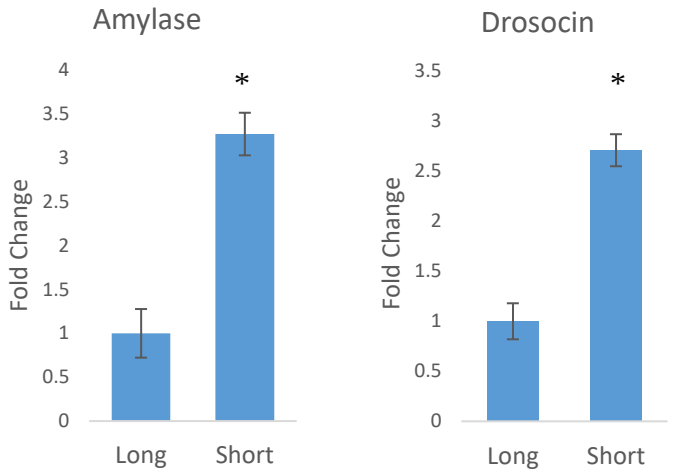

Figure 3.6. Relative Amylase and Drosocin Levels. (a) Relative *Amylase* Levels. Amylase mRNA was significantly higher in the heads of predicted short-lived flies ($p<.05$, Student's t-test). (b) Relative *Drosocin* levels. *Drosocin* mRNA was significantly higher in the heads of short-lived flies ($p<.05$, Student's t-test).

3.4.3. SOD-2. Biological differences between predicted short- and long-lived flies were further assessed by measuring levels of the antioxidant SOD-2. SOD-2 levels were found to be significantly higher in the bodies of flies that were predicted to be short-lived (Figure 3.7). Relative SOD-2 protein concentration as determined by a semi-quantitative western blot, was found to be an average of 25% higher in predicted short-lived flies than in predicted long-lived flies ($n=3$, $p <0.05$ Student's t-test). These results were confirmed by qPCR assay across three different 30-day experiments. The bodies of short-lived flies had an average of 45% more SOD-2 transcripts than their long-lived counterparts ($n = 3$, $p < 0.001$ by Student's t-test).

3.4.4. Ubiquitin. Accumulated ubiquitinated proteins are a known indicator of aging. Western Blots were used to determine the relative concentration of poly-ubiquitinated proteins in both cohorts ($n = 3$). Total ubiquitinated proteins were elevated in the bodies of short-lived samples, suggesting that the predicted short-lived flies are

biologically older than the predicted long-lived flies. Short lived flies had an observed 22% increase in polyubiquitinated proteins (Figure 3.8). This result was confirmed in two separate 30-day experiments.

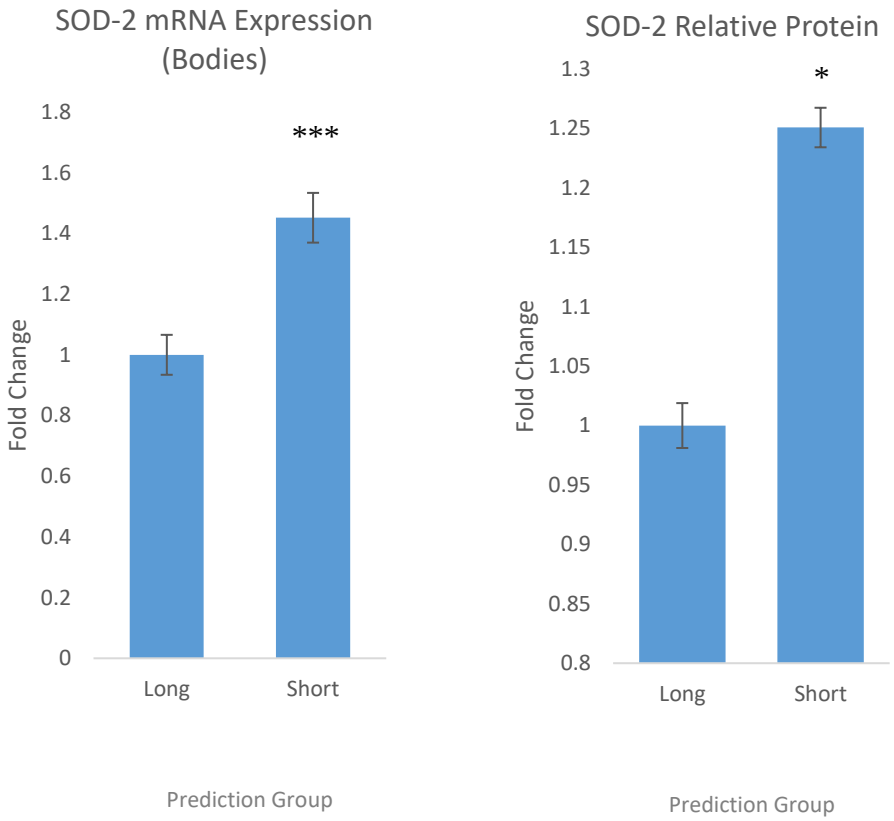

Figure 3.7. Relative SOD-2 Levels. SOD-2 mRNA (n=8, p <.001 Student's t-test) and protein concentrations (n=3, p<0.05 Student's t-test) were found to be significantly elevated in the bodies of short-lived flies .

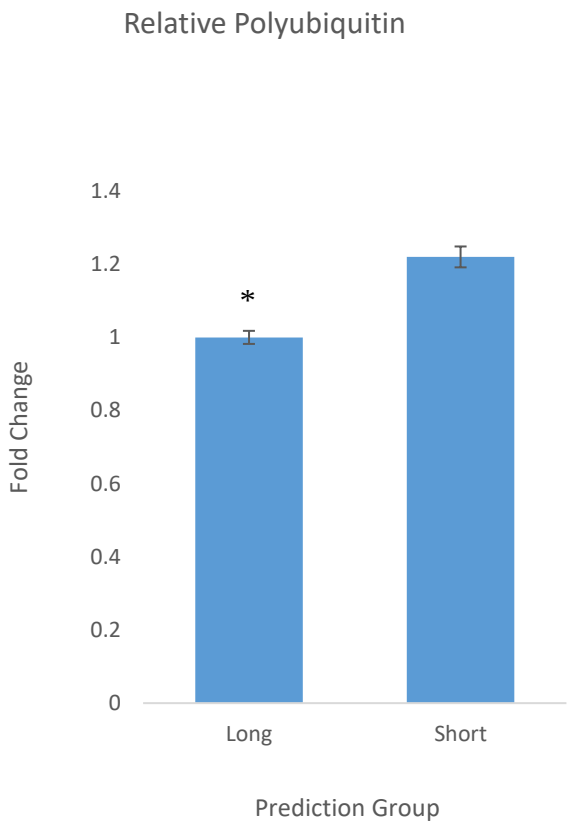

Figure 3.8. Relative Polyubiquitinated Proteins. Polyubiquitin was significantly elevated in the bodies of short-lived flies (n=3, $p<0.01$ Student's t-test).

4. DISCUSSION

Building a model to describe lifespan by sleep architecture has proven to be a monumental task. These data show that wild type *Drosophila melanogaster's* lifespan can be successfully modeled and binned into short- and long-lived groups using a multi-variate approach. The incorporation of variables representing sleep stability was integral when describing lifespan and was much more powerful than measuring basic sleeping metrics alone.

As *Drosophila* age, they spend more time asleep throughout the day, but in a way that is less consolidated. This is especially pronounced in short-lived flies. Long-lived flies, on the other hand, exhibit a more consolidated and stable sleep architecture, even at 30 days old. Unsurprisingly, however, neither total sleep time nor average night bout duration has a strong correlation with lifespan, and both are insufficient when predicting lifespan.

The full lifespan model most accurately predicted lifespan. This model was trained with sleep data from 380 flies which were monitored from about six days after emerging from the pupa stage until death. When the model was reapplied to the same group of flies from which it was built, the generated predictions were highly correlated with actual lifespan (R^2 =.46, $p<0.0001$). The success of the model is even more apparent when the flies are binned into quartiles. 66.29% of observed short-lived flies were correctly predicted to be short-lived, while 61.29% of observed long-lived flies were correctly predicted to be long-lived. Missed calls (i.e. an observed short-lived fly was predicted to be long-lived) accounted for fewer than 1% of all predictions.

Expectedly, the accuracy of the model decreased when only 30 days of sleep data was used in model training. This model was trained using 311 flies and was able to achieve an adjusted R^2 value of 0.183 when the model was applied to the flies used in the training set. When the flies were binned, the model correctly predicted long-lived flies 48% of the time, and short-lived flies 50% of the time. Missed calls accounted for fewer than 4% of all predictions.

The 30-day model performed similarly when it was applied to the 134 withheld flies, with an adjusted R^2 of 0.16. When the flies were binned, the model correctly predicted short-lived flies 53.57% of the time, and long-lived flies 37.50% of the time. Missed calls accounted for a total of less than 3% of flies.

Although each of the 37 variables were calculated for both the full-lifespan and 30-day models, only 15 variables were selected for use in each model. The two models differ because each variable was independently calculated, and model selection was carried out independently for each model. With that said, there is significant overlap between the two models in terms of variables selected.

Sorting by Standardized Estimate revealed which variables have the largest effect on lifespan. The percentage of time spent asleep during both the day and night were strongly correlated to lifespan in both models. Interestingly, and in contrast to available literature, there was a negative correlation between percentage of time spent asleep at night and lifespan. Time asleep during the day, however, was positively correlated with lifespan. In other words, longer-living flies tend to spend less time sleeping during the night and more time asleep during the day. The first differences of the mean transition

probability for wake and sleep during the daytime both had a strong negative correlation with lifespan in both models.

The transition probabilities for both wake and sleep during the day occupied positions two and three in the 30-day model, respectively. Neither variable was selected in the full-lifespan model. This could suggest that transition probabilities during the day play an outsized role during the younger stages of life but lose influence on lifespan as the animal gets older. It's worth noting that the first differences of the mean transition probability of wake during the day was calculated to be extremely similar for both models.

Verification of the model was conducted by comparing known markers of sleepiness and aging for both short- and long-lived flies. Preliminary data collected from a previous graduate student indicated that predicted short-lived flies are less equipped to deal with oxidative stress due to lower levels of the antioxidant glutathione. This evidence suggested a relationship between sleep stability and oxidative stress and is consistent with published research. Subsequent experiments were designed with this evidence in mind, concentrating on the downstream effects of oxidative stress.

Several methods were chosen to carry out the goal of assessing oxidative stress differences between predicted short- and long-lived animals. Gene expression differences between prediction groups were measured via qPCR for genes known to change due to oxidative stress and age. Western blots were used to confirm significant differences in gene expression between the two cohorts for select genes. Many genes associated with cellular redox state are also associated with aging, so it was expected that short- and long-lived flies would exhibit differences in expression of these genes.

A qPCR screen for genes affected by oxidative stress revealed gene expression differences between the two prediction groups. Notably, SOD-2 was significantly elevated in the bodies of predicted short-lived flies. This result was confirmed by a western blot. SOD-2 expression is controlled by the Antioxidant Response Element (ARE) which is responsible for the transcription of several antioxidant genes when activated, including both the catalytic (GCLc) and modifier (GCLm) subunits of γ-Glutamyl cysteine synthase, the rate limiting enzyme in the synthesis of glutathione. The ARE is activated by Relish, the fly analog to Nrf2 in mammals. Upregulation of SOD-2 is consistent with research indicating that organisms become more oxidatively stressed with age, and thus must compensate by increasing antioxidants. This evidence supports our hypothesis that predicted short-lived flies have a more biologically advanced age compared to predicted long-lived flies.

Predicted short-lived flies are biologically different than their long-lived counterparts. Biological experiments resulted in striking differences in both transcript as well as protein levels for several indicators of sleepiness and aging, all of which are consistent with the literature. Predicted short-lived flies have significantly elevated levels of amylase compared to predicted long-lived flies, indicating that they may live in a chronic state of increased sleep debt. Predicted short-lived flies have over three times the expression of amylase in their heads, while nine hours of sleep deprivation resulted in a five-fold change in a previous experiment.

The aforementioned markers of aging are just a few of many that should be tested. These markers are all known to be correlated with age, and now – sleep behavior. From here, lesser understood pathways may be tested for changes between short- and long-

lived organisms. Cell cycle regulation, metabolite clearance in the brain, and kinase function all come to mind. Further, other behavioral consequences of chronically poor sleep can also be tested, like learning differences between predicted short- and long-lived flies.

The biological differences between short- and long-lived flies are an indication that our hypothesis is correct; sleep stability can be modeled to assess organism health. Poor sleep stability is an indication that the organism's biological age is further advanced, and that intervention may be required. From here, the next question that must be asked is whether or not poor sleeping short-lived organisms can be rescued through the stabilization of their sleeping behavior.

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