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THE VALIDATION OF PREDICTED BIOLOGICAL AGE OF DROSOPHILA MELANOGASTER FROM COMBINED STATISTICAL MODELING

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THE VALIDATION OF PREDICTED BIOLOGICAL AGE OF *DROSOPHILA MELANOGASTER* FROM COMBINED STATISTICAL MODELING

by

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ABSTRACT

Sleep is essential for maintaining a healthy body and mind and is associated with aging and aging related diseases. There are individual differences in fly as well as human sleep behavior and lifespan. Between and within individuals, sleep varies in characteristics including consolidation, rhythmicity, continuity, duration, and more. Various evidence in the literature suggests there are many molecular pathways involved with aging and they may be different for individuals. Our research is interested in a possible restorative mechanism of sleep and the ramifications of that mechanism to aging. We have developed two predictive models of aging using the fruit fly *Drosophila*. These models allow us predict if a fly will be 'long-lived' or 'short-lived' based on their first 30 days of sleep data. We hypothesize that sleep characteristics are related to age. *Our hypothesis is that poor sleep qualities lead to a shorter lifespan, and conversely good sleep characteristics lead to a longer lifespan*. This work describes research done verifying the biological validity of these models. Specifically, we find that the models are able to separate out unique subsets of flies in different aging groups, including one group affected by a disruption in proteostasis.

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TABLE OF CONTENTS

Page

LIST OF ILLUSTRATIONS

Page

NOMENCLATURE

Symbol	Description
ROS	Reactive oxygen species
FRT	Free Radical Theory of aging
AP	Antagonistic Pleiotropy theory of aging
DS	Disposable Soma
4-HNE	4-Hydroxynonenal; lipid peroxidation byproduct
SOD2	Mitochondrial Superoxide Dismutase (manganese dependent)
IEC	Intestinal epithelial cell
TOR	Target of Rapamycin
CR	Caloric Restriction
DR	Dietary Restriction
LR	Multiple Linear Regression statistical model
FPCA	Functional Principal Component Analysis statistical model
NOX	NADPH oxidase
DUOX	Dual oxidase
GST	Glutathione S-transferase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GPx	Glutathione peroxidase
HSF	Heat shock factor
DAM	Drosophila Activity Monitoring system

ETC	Electron transport chain
IIS	Insulin/Insulin-like signaling pathway
RING	Rapid Iterative Negative Geotaxis assay

1. INTRODUCTION

1.1. THEORIES OF AGING

1.1.1. Classical Theories of Aging. Aging is universal across species and is a complex process whose mechanisms and molecular characteristics are not well-understood. This thesis will discuss the molecular mechanisms of biological aging that are attributed to sleep characteristics. Rather than comprehensively review every aging theory, a few theories will be chosen based on popularity, current relevance, and focus on molecular mechanism. The first notable theory was proposed in 1882 by August Weismann, commonly referred to as the 'wear and tear' theory. The wear and tear theory proposes that as we live, cells are exposed to the 'wear and tear' of being used and accumulate damage over time. This theory isn't accepted today because it doesn't explain why stem cells and protein maintenance systems fail to do their job, since this was before their discovery. The next mechanistic theory was the free radical theory of aging (FRT) proposed in 1956 by Denham Harman (Harman, 1956). Free radicals are molecules that have an unpaired electron which causes them to react with and disrupt various biological molecules, generating more free radicals and causing widespread damage. The FRT states that cellular macromolecules accumulate free radical damage from oxygen free radicals over time which leads to aging and death. The evolutionary theories Disposable Soma (DS) and Antagonistic Pleiotropy (AP) propose aging is driven by genetic tradeoffs. The DS theory says that with limited energy to use, energy usage is prioritized for reproduction fitness and not cell maintenance. AP says something similar: genes that provide beneficial traits early on are responsible for later harmful phenotypes, and

because of the early benefit they are selected for evolutionarily. The AP theory doesn't explain what causes the traits to be harmful later in life. Because DS and AP are evolutionary theories, they prioritize explaining the 'why' in aging, but don't explain the 'how'.

Currently, there is no single theory of aging that is popularly believed to be the true explanation for aging, but many researchers agree that aging involves an accumulation of damage and a growing inability for cells to repair damage, proliferate, and survive (Barja, 2019; Golubev, Hanson, & Gladyshev, 2018; López-Otín, Blasco, Partridge, Serrano, & Kroemer, 2013; Schieber & Chandel, 2014; Shaposhnikov, Proshkina, Shilova, Zhavoronkov, & Moskalev, 2015). Beyond that, there are competing ideas about the central mechanism although damage accumulation via free radicals and genetic aging programs are commonly reoccurring concepts.

1.1.2. Modern Theories of Aging. Modern researchers realize that aging is not due to a single cause and so researchers are no longer limiting themselves to a single classical theory of aging but are rather creating updated theories that incorporate aspects of multiple classical theories (Barja, 2019; Golubev et al., 2018; Jin, 2010). One recent paper by Golubev, Hanson, and Gladyshev propose that the FRT and the AP theory can be merged (Golubev et al., 2018). They propose that the AP theory by itself isn't sufficient to explain aging because it doesn't explain what the switch for the harmful gene phenotypes seen later in life is, and the FRT by itself is too exclusive, pinning the blame for a variety of free radical damage on oxygen. They argue that reactive oxygen species (ROS) are theorized to be there from the beginning of life so it was potentially the initial driver of damage accumulation and therefore aging. With renewable cells and

complex structures that emerged later, however, ROS was no longer the sole driver of aging. Instead, genes that have largely beneficial products for early reproductive life also produce secondary damaging molecules, and these damaging molecules including ROS accumulate over time eventually presenting phenotypically as late-acting pleiotropic effects. They argue that aging seems to be cumulative and there's no 'switch' or defined point of aging. Aside from ROS, their 'secondary damaging products' responsible for aging are not well defined or described. Despite their insistence that ROS isn't the main culprit for aging, it still plays the most defined and direct role for aging in their hypothesis.

Another more modern theory of aging also proposes combining preexisting theories into one, called the Cell Aging Regulation System (CARS) model (Barja, 2019). In his model, Barja proposes that there exists a genetic program within species that modulate aging in response to environmental signals. This model proposes cellular modulation of aging has 3 main components:

1) Cytoplasmic signaling: second messenger proteins that respond to an environmental signal. One example would be dietary restriction which causes a decrease in TOR signaling, ultimately leading to extended lifespan (Igarashi & Guarente, 2016).

2) Genetic aging program: various transcription factors, genes, and epigenetic components that respond to the cytoplasmic signaling in 1). An extension of the above example would be downstream genes of TOR that are affected by dietary restriction, like antioxidant genes.

3) And post nuclear aging effectors, which work together to modulate aging. The main causes of aging are described as mitochondrial DNA fragment insertion into nuclear DNA (derived from ROS attack on mtDNA), lipid peroxidation, and insufficient autophagy (insufficient disposal of damaged cells and proteins). Post nuclear effectors would work to lower or relieve aging effects, for example through antioxidants that lower mitochondrial-produced ROS.

At its most basic level this proposal designates mitochondrial-produced ROS as the main cause of irreversible damage in aging, given that the reason for decreased autophagy is also very likely due to disrupted ROS homeostasis (Filomeni, De Zio, & Cecconi, 2015). The author didn't discuss ROS as more than a merely contributing factor even though it's the central component for aging his proposal and despite admitting that "…ROS seem of paramount importance for aging…".

Even as modern theories of aging have begun to move away from single-cause theories, aspects of the free radicle theory remain the most consistently included. Despite its popularity, the theory has in recent years come under heavy criticism (Buffenstein, Edrey, Yang, & Mele, 2008; Gladyshev, 2014; Lewis, Andziak, Yang, & Buffenstein, 2013; Salmon, Richardson, & Pérez, 2010). Some researchers have attacked the exclusive nature of the original theory posited in 1956 and point out the major contradictory evidence is organisms that have unusually high baseline levels of oxidative stress, such as the naked mole rat. At a young age this organism has high levels of markers indicting high oxidative stress and relative low levels of major antioxidants, yet they have a longer lifespan than other comparable organisms (Lewis et al., 2013). First, these criticizing papers are misleading in calling it 'oxidative stress'; although the naked mole rats have

higher levels of markers, they aren't necessarily experiencing *damage* which results from being out of ROS homeostatic balance. If the naked mole rat didn't have its ROS levels under control, it wouldn't live very long. Additionally, there's growing evidence for the importance of ROS as a beneficial signaling molecule and that more baseline ROS is not inherently bad (Khan, Abidi, Skinner, Tian, & Smith-Bolton, 2017; Schieber & Chandel, 2014) as long as they're in homeostatic balance with antioxidant systems. Lastly, the exclusivity of the original theory doesn't prevent it from being partially true in that it contributes to the overall aging mechanism rather than being the sole cause. Even those firmly against free radicals being the sole cause of aging acknowledge that free radical damage does largely contribute to aging and aging-related diseases (Buffenstein et al., 2008; Liguori et al., 2018; Salmon et al., 2010). Others highlight the importance of ROS homeostasis which extends beyond prevention via antioxidants to include repair mechanisms (Lewis et al., 2013). The biggest debate currently isn't whether ROS contributes to aging but how it's involved, and there's a growing acknowledgment of the complex temporal and spatial dynamics of ROS homeostasis.

1.2. THE DYNAMICS OF REDOX HOMEOSTASIS

1.2.1. ROS Generation and Control. Under normal conditions ROS is generated endogenously by NADPH oxidases (NOX), dual oxidases (DUOX), and by cell respiration through mitochondrial electron transport. As electrons are moving down the electron transport chain (ETC) in the mitochondria, some fail to make it to complex IV and turn oxygen into a superoxide free radical, whereas if electrons make it through the ETC to complex IV, they combine with oxygen to create water and protons (proton

gradients drive ATP production). Superoxides are high reactive molecules capable of damaging lipid membranes, proteins, and genetic material (Tower, 2015). Highly efficient ATP production exacerbates free radical creation because of a higher proton pressure which causes more electrons to 'escape' (Korshunov, Skulachev, & Starkov, 1997). Mitochondrial ROS levels are controlled through the antioxidant superoxide dismutase 2 (SOD2) and mitochondrial uncoupling. SODs neutralize superoxide radicals into hydrogen peroxide, which can be further broken down into water and oxygen by catalase. Mitochondrial uncoupling leaks protons across the membrane thereby relieving some of the proton pressure and decreasing the amount of 'escaping' electrons. High rates of mitochondrial metabolism combined with high rates of proton leak/uncoupling seem to promote long life (Salin et al., 2015; Speakman et al., 2004). Antioxidants like glutathione peroxidase (GPx) neutralize a variety of radical species whereas other enzymes are more specific: catalase breaks down hydrogen peroxide and the superoxide dismutases neutralize superoxide radicals. Free reduced glutathione (GSH) is an important scavenger for ROS and other free radicals (Haenen & Bast, 2014). The ratio of GSH to oxidized glutathione (GSSG) is commonly used as a marker for the redox state of an organism (Zitka et al., 2012). The glutathione metabolic pathway is fairly complex and includes cysteine as a rate-limiting step for the *de novo* synthesis of glutathione (Figure 1.1). The brain is thought to be particularly susceptible to oxidative damage given it's an environment of high oxygen and high lipid content. Alpha glutathione S transferases neutralize major lipid peroxidation byproduct 4-HNE (Y. Yang, Sharma, Sharma,

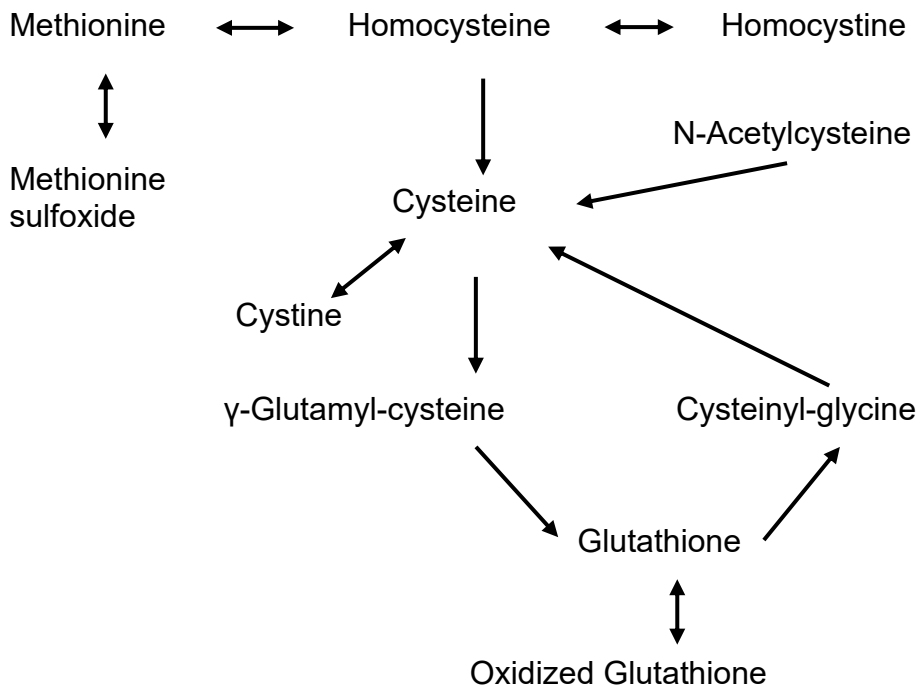

Figure 1.1 Thiol redox metabolic pathways. Curtesy of Jiandong Wu.

Awasthi, & Awasthi, 2003). As a last line of defense, free radical species that have already caused damage may be fixed via repair enzymes or degradation systems like autophagy. Repair and cleanup is considered equally important for ROS control in healthy aging rather than just an alternative backup to antioxidants (Garschall et al., 2017; D. H. Lim, Han, Kim, Lee, & Kim, 2012).

If ROS is the main cause of aging and if antioxidants and repair systems are vital for healthy aging, then gross overexpression of antioxidants and repair factors should always lead to better outcomes. This, however, is not always the case. Ubiquitous over-expression of various repair proteins and antioxidants frequently end in lethality. Garschall et al. found that DNA repair factor Prp19 overexpression extended female

Drosophila lifespan, but only if restricted to the nervous system. Furthermore, no life extension was found in males, hinting at an underdiscussed importance of sexual differences in aging (Garschall et al., 2017). Shaposhnikov et al. also found that overexpressing repair genes had either extending or restricting effects on lifespan depending on the specific repair gene, sex, genetic driver, and the spatial and temporal parameters (ubiquitous vs nervous only and constitutive vs facultative) of the overexpression (Shaposhnikov et al., 2015). Thus, redox homeostasis is more than just antioxidant prevention.

1.2.2. Redox Signaling. Excessive ROS may be potentially damaging, but some is necessary for healthy cell signaling (Jones, 2008; L. Zhang et al., 2019). ROS generated through NOX signals for intestinal cell proliferation following various stressors, especially bacterial infection (Patel et al., 2019) and it's also required for regenerating ablated *Drosophila* wing imaginal discs (Khan et al., 2017). The lipid peroxidation byproduct 4-HNE can directly activate SAPK/JNK, a major regenerative pathway, and is also said to be largely contributive to cell cycle signaling along with other lipid peroxidation byproducts. SOD upregulation extends lifespan, but not if catalase is coincidently upregulated, suggesting an important signaling role for hydrogen peroxide (Sun, Folk, Bradley, & Tower, 2002; Tower, 2015). Complete elimination of ROS is clearly not wanted, and this is seen not just through ROS generators but negative feedback mechanisms. For example, neutralized HNE in the form of 4-HNE-GS conjugates inhibit GSTs (Y. Yang et al., 2003). This means the more lipid peroxidation that's being neutralized by glutathione, the less glutathione is allowed to recycle, preventing a complete elimination of lipid peroxidation. If ROS plays a contributive role

in aging but is simultaneously necessary for healthy signaling, then aging isn't affected by total ROS levels and is instead due to a disruption in ROS homeostasis whether at the level of ROS generation, control, or signaling.

1.3. OTHER COMMON MOLECULAR PATHWAYS IMPLICATED IN AGING

1.3.1. P-p38 MAP Kinase: A Convergence Point. Throughout the literature, three commonly reoccurring pathways that are very involved in aging are Erk, Jnk, and p38, where p38 seems the best described. P38 is a stress-activated transcription factor that has a variety of upstream activators, like the ROS-activated Ask-signalosome, TOR, and sestrins (which are able to coordinate p38, Jnk, and Erk pthways). Downstream, p38 activation has a variety of effects including immune responses through NFkB, circadian function through PER phosphorylation, antioxidant involvement through Nrf2, and proliferation and apoptotic effects through TOR and p53 respectively (Dusik et al., 2014; Han et al., 1998; He et al., 2020). Lastly, though Erk, Jnk, and p38 may operate independently, they may also be coordinated into one complex by anti-aging, pro-immunity sestrins (Lanna et al., 2017)

Clearly p38 is a major convergence point for multiple pathways including aging, oxidative response, immune response, and response to external stressors. In the intestines, Nox-mediated ROS generation leads to increased pp38 and increased intestinal epithelial cell (IEC) proliferation together with Jnk and Jak-Stat (Patel et al., 2019). On the other hand, the age-related decrease in IEC proliferation is due to excessive TOR-p38-p53 signaling (He et al., 2020). P38 is also able to activate Nrf2 (Ewald et al., 2017), a major transcription factor involved in antioxidant transcription. Ask1-signalosome-p38 and

p46-Jnk signaling is inhibited by Klotho which called a ‘suppressor of aging’ and greatly promotes Nrf2 nuclear localization. Long-lived Klotho-overexpressing mutants are said to live longer because of the protective effects of increased base-line antioxidants (Hsieh, Kuro-o, Rosenblatt, Brobey, & Papaconstantinou, 2010). In summary: under some circumstances, more pp38 signaling leads to better regeneration and antioxidant response. In other circumstances, repressing pp38 is good for longevity and high pp38 signaling can be a sign of stress. Since it’s so involved in a variety of pathways, context and the status of upstream and downstream signaling molecules are important in determining pp38’s relationship to lifespan.

1.3.2. Target of Rapamycin (TOR). The most universal method of life extension across different species is dietary restriction. Dietary restriction (DR) involves the reduction of specific nutrients in the diet, like changing the ratios of protein to sugar without changing the overall calorie content. Similarly, caloric restriction (CR) involves a limitation of calorie intake such that lifespan is extended but health isn’t harmed by way of starvation. Although CR is sometimes considered interchangeable with DR (Apte, 2014; Kaeberlein, 2013), it has been suggested that calories are less important for extending life, and instead the reduction of specific nutrients is what’s important (Mair, Piper, & Partridge, 2005). CR is thought to work as least in part through the Target of Rapamycin (TOR) pathway (Igarashi & Guarente, 2016; Kapahi et al., 2004), which is another common pathway reoccurring throughout aging literature, especially in regards to gut aging (Barja, 2019; Fan et al., 2015; He et al., 2020). Specifically, increased TOR-pp38-p53 signaling is found in aging mouse intestinal stem cells, and decreasing this signaling pathway improves stem cell proliferation and villi nutrient absorption ability

(He et al., 2020). Additionally, long-lived TOR mutants which result in decreased protein synthesis are long-lived because of compensatory mechanisms involved in energy allocation (L. Yang et al., 2019). Thus, TOR signaling is strongly implicated in aging, especially in the gut.

1.3.3. Protein Homeostasis. An often overlooked aspect of aging is the wide world of protein handling which encompasses diverse networks responsible for protein synthesis, maintenance, and degradation. In *C. elegans,* protein maintenance in particular is shown to be one of the first systems that breaks down with age—as early as 10 days into adulthood (Ben-Zvi, Miller, & Morimoto, 2009). If a misfolded protein is unable to be rectified via chaperones, then it is either tagged for proteasomal degradation with ubiquitin or ushered to a lysosome. In some cases, whole sections of a cell may be engulfed into an autosome, fused with a lysosome, and the contents are degraded. Some proteins, called metastable proteins, are mostly stable until exposed to a slight challenge like a 'contagious' misfolded protein. These metastable proteins depend heavily on chaperones and are liable to compromise shortly into *C. elegans'* adulthood but are restored upon HSF or DAF16 upregulation (Ben-Zvi et al., 2009).

Protein synthesis by itself isn't a significant contributor to lifespan when viewed independently from maintenance and clearance. A paper by Syntichaki, Troulinaki, and Tavernarakis in 2007 showed that by reducing global somatic cell protein synthesis via a deletion mutation of IFE-2, an isoform of mRNA translation initiation factor 4E, they could extend *C. elegans* lifespan. The reduction in protein synthesis itself likely wasn't the immediate cause of the altered lifespan; they hypothesized that this lifespan extension works by shunting energy normally used by protein synthesis towards maintenance and

repair, as seen by an increased resistance to various stressors (Syntichaki, Troulinaki, & Tavernarakis, 2007). Loss of IFE-2 on top of dietary restriction or TOR mutation extends lifespan more than by itself, indicating differing mechanisms by which loss of IFE-2 extends life. A newer study found that proteostasis machinery and mitochondrial-related proteins generally decrease with normal aging (L. Yang et al., 2019). This group also ultimately concludes that protein maintenance is the most commonly affected pathway during aging.

Protein clearance also plays a role in lifespan. Vincow et al. found that proteostasis disruption in aging flies is due equally to changes in synthesis and decreased degradation (Vincow, Thomas, Merrihew, MacCoss, & Pallanck, 2020). Autophagy, a large contributor to protein degradation, is modulated in part by ROS (Filomeni et al., 2015) and negatively regulated by mTOR. It's also at least partially regulated by sleep (Bedont et al., 2021), making it an good target for model validation.

1.4. PHYSIOLOGICAL MARKERS OF AGING

Aside from molecular markers of biological age, there are also a variety of physiological indicators. One common physiological indicator of biological age is measuring locomotor and geotaxis ability, commonly through the Rapid Iterative Negative Geotaxis (RING) assay (Julia Warner Gargano, Ian Martin, Poonam Bhandari, 2005). As the name implies, flies are repeatedly tapped to the bottom of a container, after which they climb upwards due to an intrinsic behavior called negative geotaxis. Younger flies climb up faster than older flies (Julia Warner Gargano, Ian Martin, Poonam

Bhandari, 2005; Simon, Liang, & Krantz, 2006), thereby providing a simple and effective physiological measurement for aging.

Another physiological assay used in *Drosophila* aging studies is the cardiac pacing assay (Ocorr, Vogler, & Bodmer, 2014; Paternostro et al., 2001). Unlike mammals, *Drosophila* have an open circulatory system, and their heart can be seen on

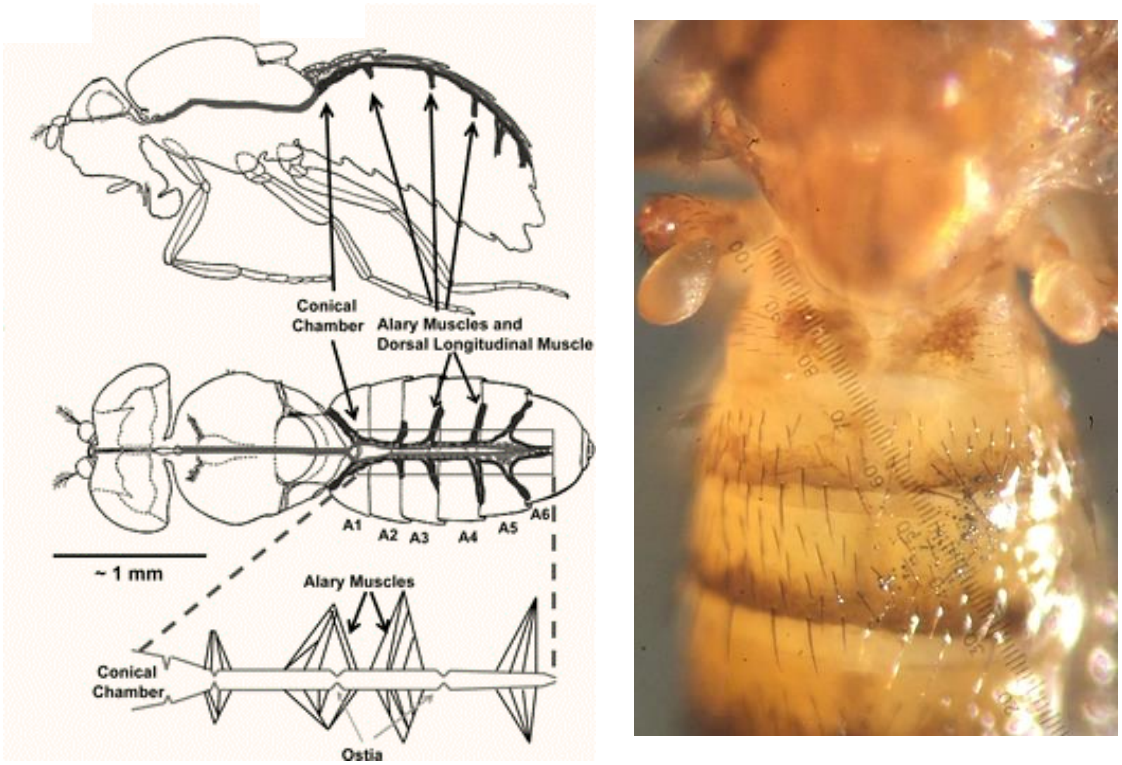

Figure 1.2 Internal tubular structure of the adult *Drosophila* heart. Left diagram adapted from figure 4 in "Drosophila, Genetic Screens, and Cardiac Function" Wolf and Rockman, 2011.

their dorsal side through their semi-transparent cuticle (Figure 1.2). This makes visual observation and counting of heart beats possible. In the heart pacing assay, flies are shocked by a square wave stimulator such that their heartrate is forced to beat two times

faster than a healthy heart (Wessells & Bodmer, 2004). Younger flies are able to recover from the electrical stress faster than older flies and have fewer heart irregularities (Paternostro et al., 2001). In humans, heart health and cardiovascular disease is strongly related to sleep (Cappuccio, Cooper, Delia, Strazzullo, & Miller, 2011; Grandner et al., 2016; Suzuki et al., 2009).

1.5. AGING AND SLEEP

Sleep is essential for both health (Cappuccio, D'Elia, Strazzullo, & Miller, 2010a; A. S. P. Lim, Kowgier, Yu, Buchman, & Bennett, 2013; Suzuki et al., 2009) and survival (Cappuccio, D'Elia, Strazzullo, & Miller, 2010b) and is highly conserved throughout the animal kingdom. It's also necessary for proper learning, memory, immune function (Besedovsky, Lange, & Born, 2012; Gais, Lucas, & Born, 2006). Sleep is defined physiologically as a period of inactivity alongside an increased arousal threshold. Good sleep characteristics include appropriate duration, consolidation, and consistent timing. Sleep duration is associated with cardiovascular disease, stroke, and type 2 diabetes, where less than 5 hours of sleep and greater than 9 hours of sleep were both associated with negative outcomes (Cappuccio et al., 2011, 2010a). Consolidation refers to the consolidation of sleep episodes, as opposed to sleep that is highly fragmented, which is associated with Alzheimer's Disease and cognitive decline (A. S. P. Lim et al., 2013). Consistent timing, called sleep regularity, is about entraining a strong circadian rhythm by going to sleep and waking up at around the same times every day. The recent formulation of the Sleep Regularity Index (SRI) created a reliable and widely applicable method of measuring regularity (Phillips et al., 2017). For one example of the impact that

regularity has on health, high irregularity is emerging as a risk factor for high blood pressure (Makarem, Zuraikat, Aggarwal, Jelic, & St-Onge, 2020).

Fruit flies have long been used as models in aging research (Hyde, 1913), but in the year 2000 it was shown for the first time that fruit flies truly sleep (Shaw, Cirelli, Greenspan, & Tononi, 2000). Using a system called the Drosophila Activity Monitoring system (DAM), flies are placed in a narrow glass tube with food, and the tube is inserted into the monitor. A beam of infrared light shines through the middle of the tube, and every time the beam is broken by a fly crossing, a count is taken. Shaw et al. showed in their research that flies that don't cross the beam for five minutes or more are truly asleep as opposed to performing still waking activities like grooming and eating. Another sleep characteristic, sleep depth, has been difficult to characterize in flies. In humans, it's typically measured by brain wave patterns, but in flies the primary technique for measurement involves lightly probing them. One system in particular, called the Drosophila Arousal Tracking system (DART), attempts to measure sleep depth by gently probing the fly while it's sleeping with increasing stimulation intensities (Faville, Kottler, Goodhill, Shaw, & Van Swinderen, 2015). They were able to separate out fly sleep duration from intensity and show that different genetic backgrounds had different sleep intensity profiles. Although this is the best option for measuring fly sleep depth intensity to date, the very act of taking a measurement is by definition fragmenting the flies sleep, which could be applying a subtle negative stressor. In other words, some of the differences seen in sleep intensity profiles could actually be differences in fly response to mild sleep fragmentation.

Although sleep is so essential for multicellular life, the function of sleep is not currently well understood. Several ideas have been proposed, like sleep is for restoring brain energy metabolism (Joel H. Benington, 1995) and sleep is for processing new memories into long-term memories (J. Zhang, 2004). Another theory for the function of sleep is that it's restorative. Under this theory, energy is used during the night for biosynthetic activities to restore cellular damage accumulated during the day. This damage can't be addressed during the day as it occurs because that energy is instead being devoted to wake-time activities (Adam, 1980). Much like with aging, the true function of sleep likely isn't limited to a single theory but instead includes multiple aspects of various theories, however, there is compelling evidence that sleep restoration is one such aspect, especially regarding oxidative restoration. Sleep deprivation causes an initial antioxidant upregulation as a first response, then later an increase in oxidative stress as defenses become overwhelmed (Villafuerte et al., 2015). Moreover, a recently-published study found that sleep and oxidative stress share a bidirectional relationship: when fruit flies were made to sleep, oxidative stress resistance improved, and when oxidative stress was decreased in neurons, sleep was also decreased (Hill et al., 2018). Additionally, acute sleep deprivation leads to death through ROS accumulation in the guts of *Drosophila* (Vaccaro et al., 2020).

There is a distinction between biological age and chronological age. Chronological age refers to the numerical amount of time an organism has been alive and does not indicate how much time the organism has left to live. Biological age, however, refers to what state of the aging process the organism is in and directly relates to how long the organism has left to live. For example, in some flies intestinal barrier integrity

can indicate biological age, and it's independent from the number of days the fly has been alive (Rera, Clark, & Walker, 2012).

If sleep is a restorative process, and different sleep characteristics are strongly associated with age and age-related diseases, then biological aging status should be inferable from sleep characteristics. Under this idea, our lab has developed several statistical models interpreting sleep characteristics as predictors of remaining lifespan in male Canton-S *Drosophila melanogaster*. For my research, I used two of them: multiple linear regression (LR) model developed by Dr. Gayla Olbricht and functional principal component analysis (FPCA) model developed by Luyang Wang. The models require approximately 30 days of sleep data and are based off of wake and sleep activity data measured using DAM system. The LR model provides more of an average snapshot into fly sleep in that it averages the values for different sleep variables across the first 30 days of a fly's life into one number. The FPCA model provides slightly more dynamic insight into fly sleep and may be more strongly influenced by sudden changes in sleep near the end of the 30-day data collection. It has been my priority to biologically verify the validity of these predictions and to reach a clearer understanding of fly aging in individuals. *We hypothesize that poor sleep qualities lead to a shorter lifespan, and conversely good sleep characteristics lead to a longer lifespan.*

2. RESULTS

2.1. LR-PREDICTED LONG-LIVED FLIES HAVE PHYSIOLOGICALLY YOUNGER HEARTS

Since heart physiology changes with age and younger flies are reported as having faster baseline heartrates than older flies (Paternostro et al., 2001; Wessells & Bodmer, 2004), we wondered if predicted long-lived flies would have younger-looking hearts than

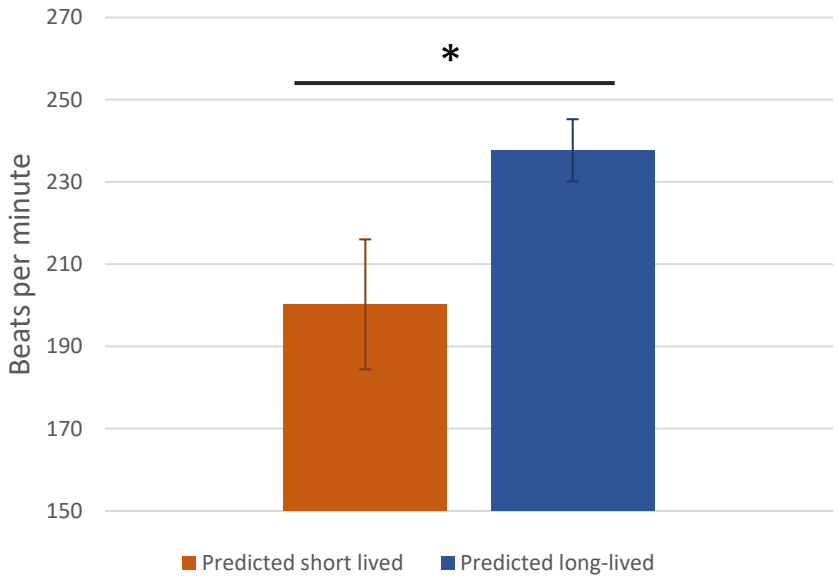

Figure 2.1 Predicted long-lived flies have higher basesline heartrates than predicted short-lived flies. N = 10 for each group. Error bars are SEM. Student's t test: p = 0.0233

our predicted short-lived (see Methods Section 4.2 for definitions of long and short-lived). Counting baseline fly heart rate at multiple time points revealed that our flies predicted to be long lived, i.e. predicted to be biologically younger, do in fact have higher baseline heartrates than our predicted short-lived flies from the LR model (Figure 2.1,

data collected by undergraduate Natalie Leach). Fly chronological age was matched between predicted long and short-lived. Additionally, the exact numerical value of fly age prediction correlates positively and significantly with baseline heartrate (Figure 2.2).

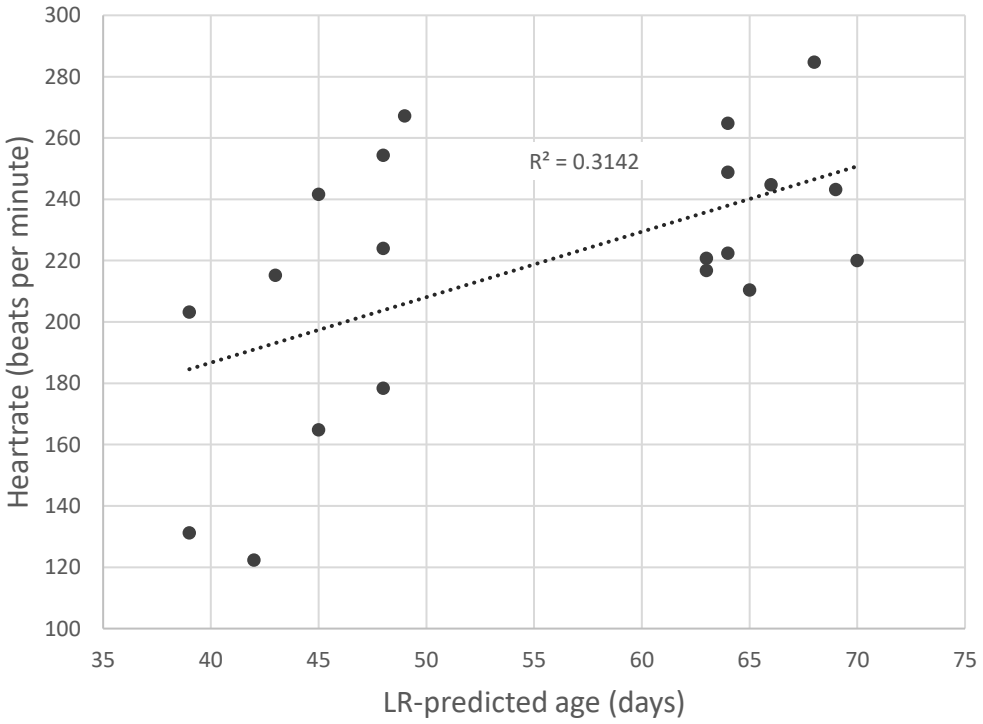

Figure 2.2 Predicted age correlates positively and significantly with heartrate. N = 20. Pearson's correlation: p = 0.010141.

2.2. USING BOTH MODEL PREDICTIONS IN TANDEM IDENTIFIES BIOLOGICALLY UNIQUE SUBSETS OF FLIES

When looking for potential molecular markers of aging, various literature describes a correlation between longevity, oxidative stress, and PP38, a stress-activated transcription factor (Ewald et al., 2017; He et al., 2020; Hsieh et al., 2010; Pérez et al.,

2018). Unlike with heart physiology, when only the LR model was used for age predictions, there didn't appear at first to be a correlation between PP38 and fly predicted age, nor was there a correlation when just FPCA was used either.

Since the models generate predictions based from different combinations of sleep variables, we wondered if the models were detecting different underlying pathways affected by aging. This would suggest that taking both model age predictions for one fly into consideration and combining the two model predictions in specific ways might be more revealing of fly aging. So instead of using one model at a time when choosing flies to use for experiments, flies that were predicted to be long-lived for LR were chosen

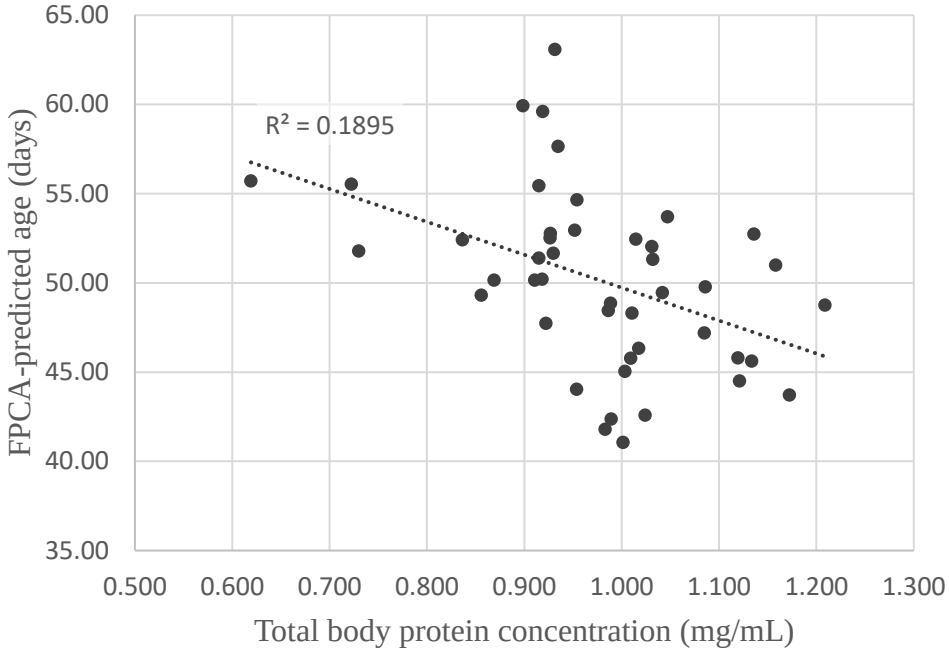

Figure 2.3 Total Body Protein Concentration Correlates Significantly with FPCA-Predicted Age

alongside of varying FPCA predictions (see Figure 4.2 in methods for visual reference). When this specific combination of models is used, fly FPCA-predicted age correlates significantly with total body (Figure 2.3), Pearson's p = 0.0028) and total head (Figure 2.4, Pearson's p = 0.0248) protein concentration. It should be noted that removing the three far left points on Figure 2.3 still results in a significant negative relationship (new $R^2 = 0.15$, Pearson's p = 0.011) and thus the relationship isn't being driven by a few flies. When model predictions aren't being used in tandem, the relationship between total protein concentration and fly predicted age isn't seen (Figure 2.5).

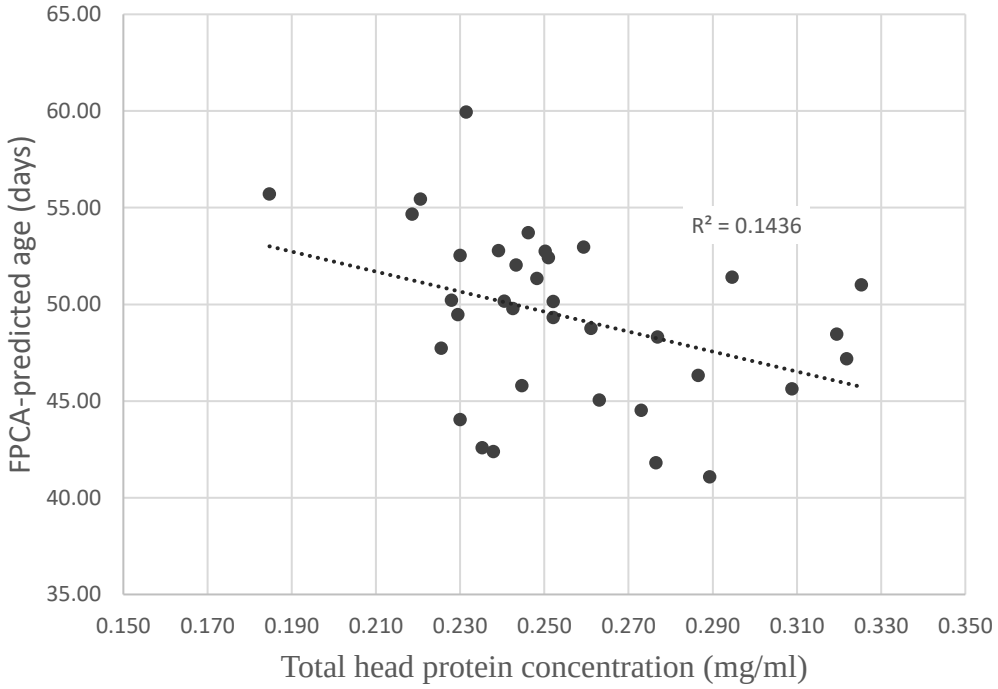

Figure 2.4 Total head protein concentration correlates negatively and significantly with FPCA-predicted age in flies with a LR prediction of 'long-lived'

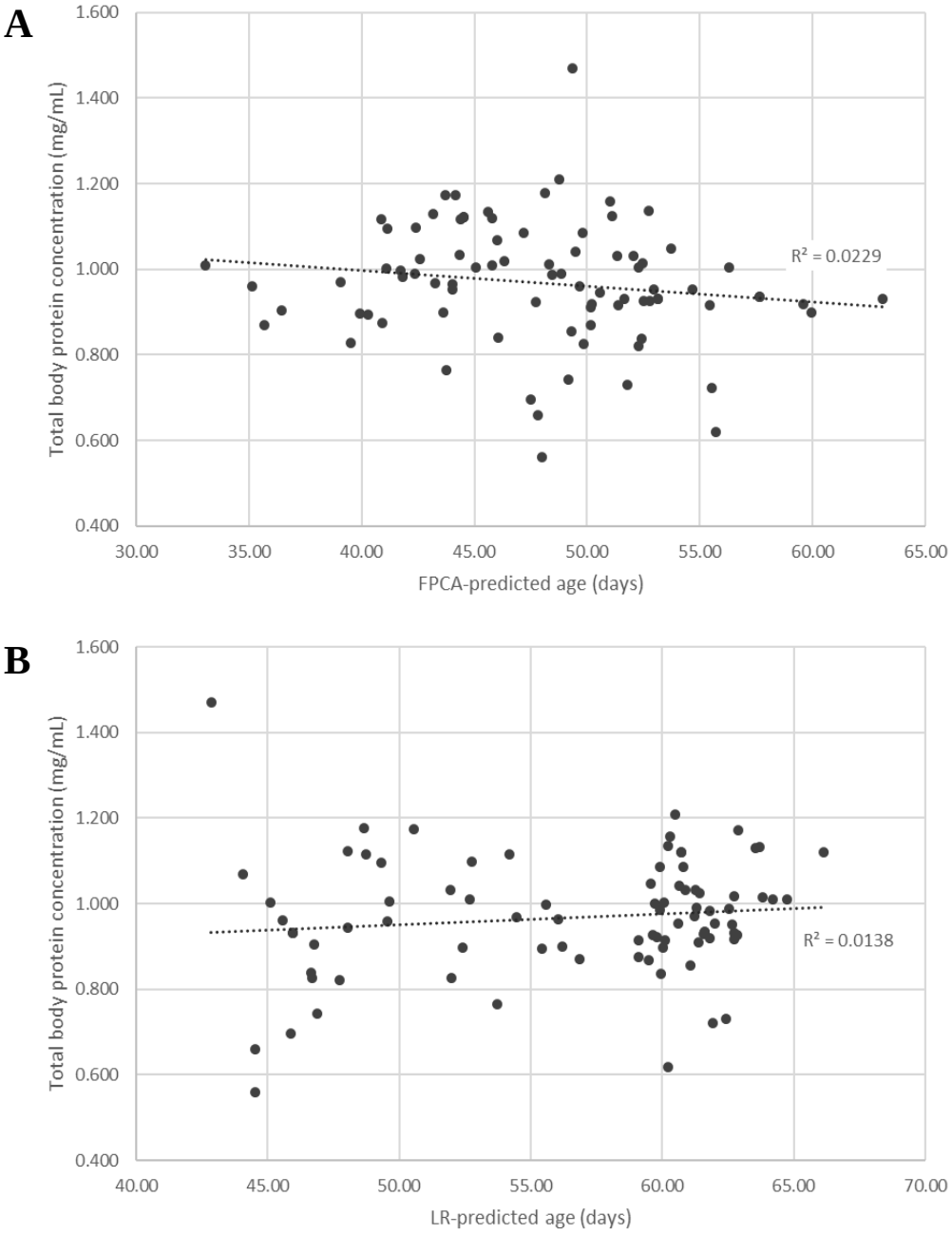

Figure 2.5 Without Further Subdividing Flies, FPCA (A) or LR (B) Models Aren't Sufficient on Their Own to Separate Out the Flies That Are Measurably Affected by a Change in Proteostasis. A & B are not significant ($p > 0.15$)

2.3. FLY PREDICTED AGE CORRELATES NEGATIVELY WITH PP38

Since the fly age predictions seen above are negatively correlated with total protein concentration, normalizing PP38 levels to protein negates any potential relationship between predicted age and PP38. Thus, protein was not used to normalize PP38 levels in these flies and a very significant (Pearson's $p < 0.00001$) and negative relationship was found between predicted fly age and PP38 (Figure 2.6).

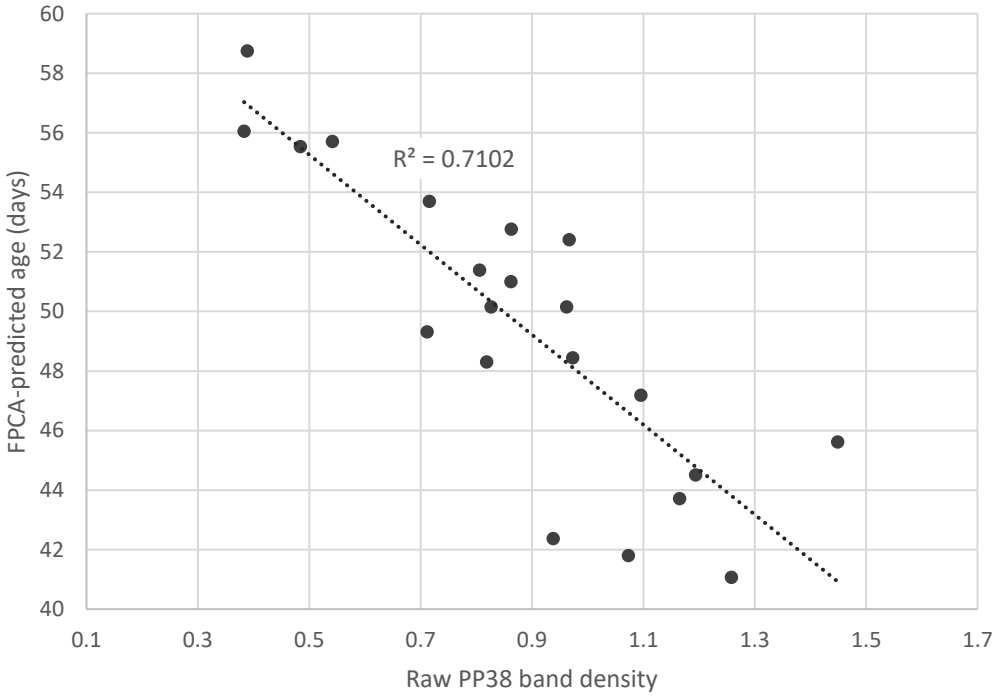

Figure 2.6 PP38 protein levels in the body correlate negatively and significantly (Pearson's $p < 0.00001$) with FPCA-predicted age. N = 23

Instead of using total protein concentration for normalization, reprobing the blots for Tubulin or Actin would normally be the next step, except the small amount of starting protein makes blots difficult to reprobe. Additionally, I've found that TUB varies in

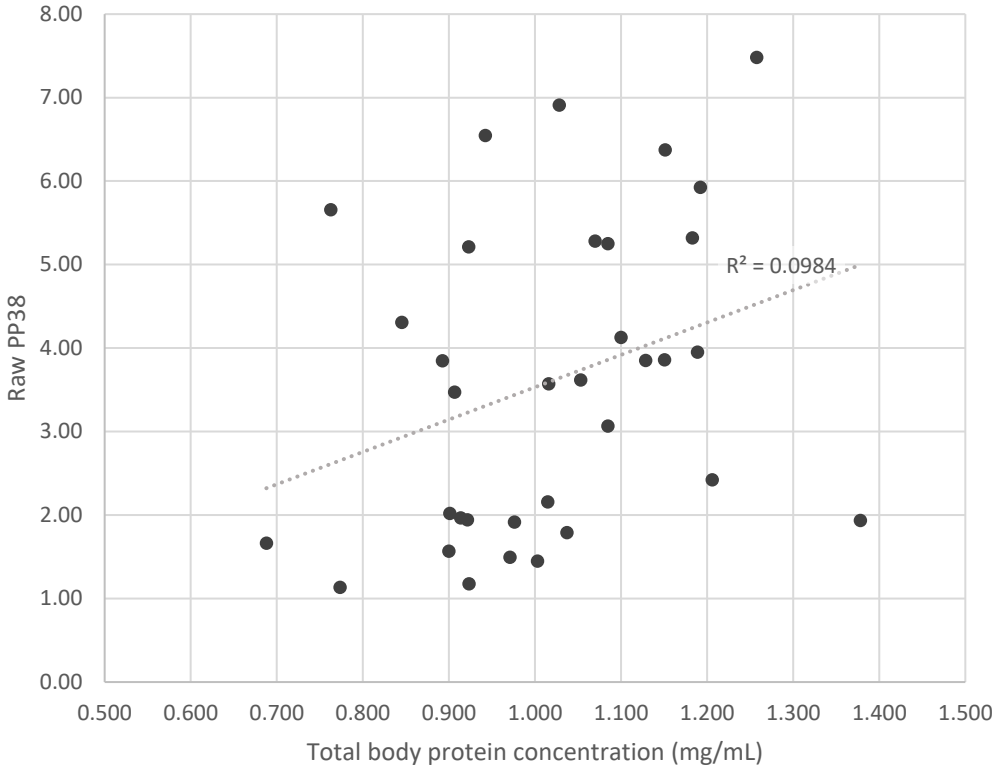

Figure 2.7 Body PP38 Levels Do Not Strongly or Significantly Correlate with Total Body Protein Concentration in Typical Flies

individual flies up to 1.8-fold (Figure A.1). The relationship seen in Figure 2.6 is not likely due to a technical error, however, since total protein concentration does not correlate significantly or strongly with PP38 levels in other flies (Figure 2.7).

In short summary, a subset of flies identified by specific combination of LR and FPCA predictions display higher total protein and elevated PP38 with shorter predicted age.

Looking at these same proteostasis-disrupted flies from a different perspective reveals another piece of evidence showing our models are saying different things: when FPCA predictions are subtracted from LR age predictions, the resulting disparity between

the two model's age predictions is very significantly ($p < 0.00001$, Pearson's) and positively correlated with body PP38 levels (Figure 2.8).

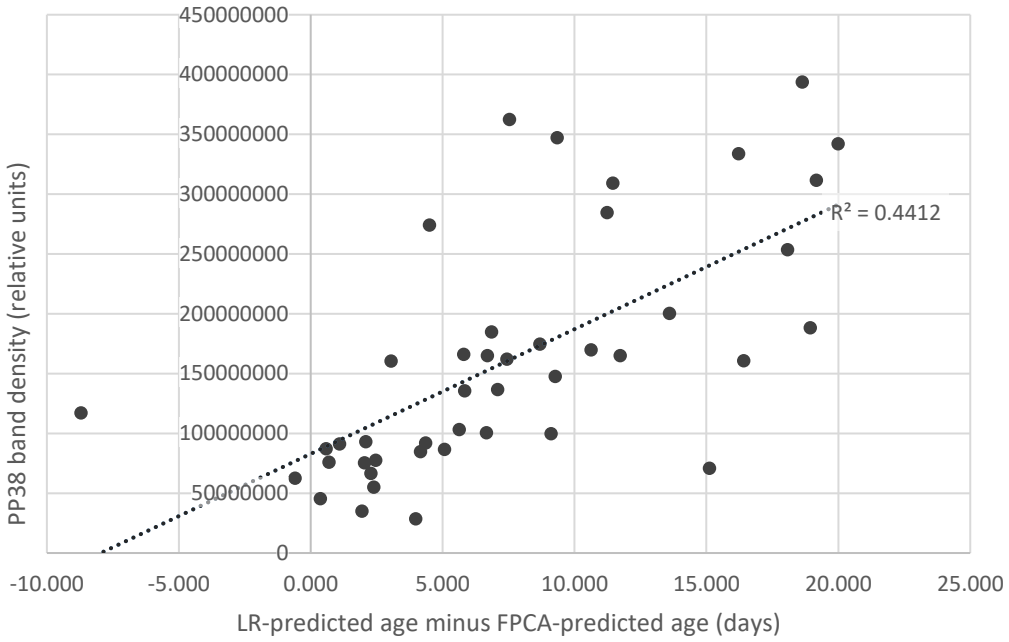

Figure 2.8 The difference between LR and FPCA predicted ages is positively correlated with PP38 protein levels in the fly body. N = 44 and Pearson's correlation P value < 0.00001

2.4. INDIVIDUAL FLIES SHOW HIGH DEGREES OF VARIATION IN HEAD SOD2

If disrupted ROS homeostasis may be largely contributive to aging, then markers of oxidative stress might still be an important indicator of biological age. With the intention of confirming a previous graduate student's results of finding higher SOD2 levels in pooled predicted-short-lived flies, we next aimed to evaluate SOD2 in individual

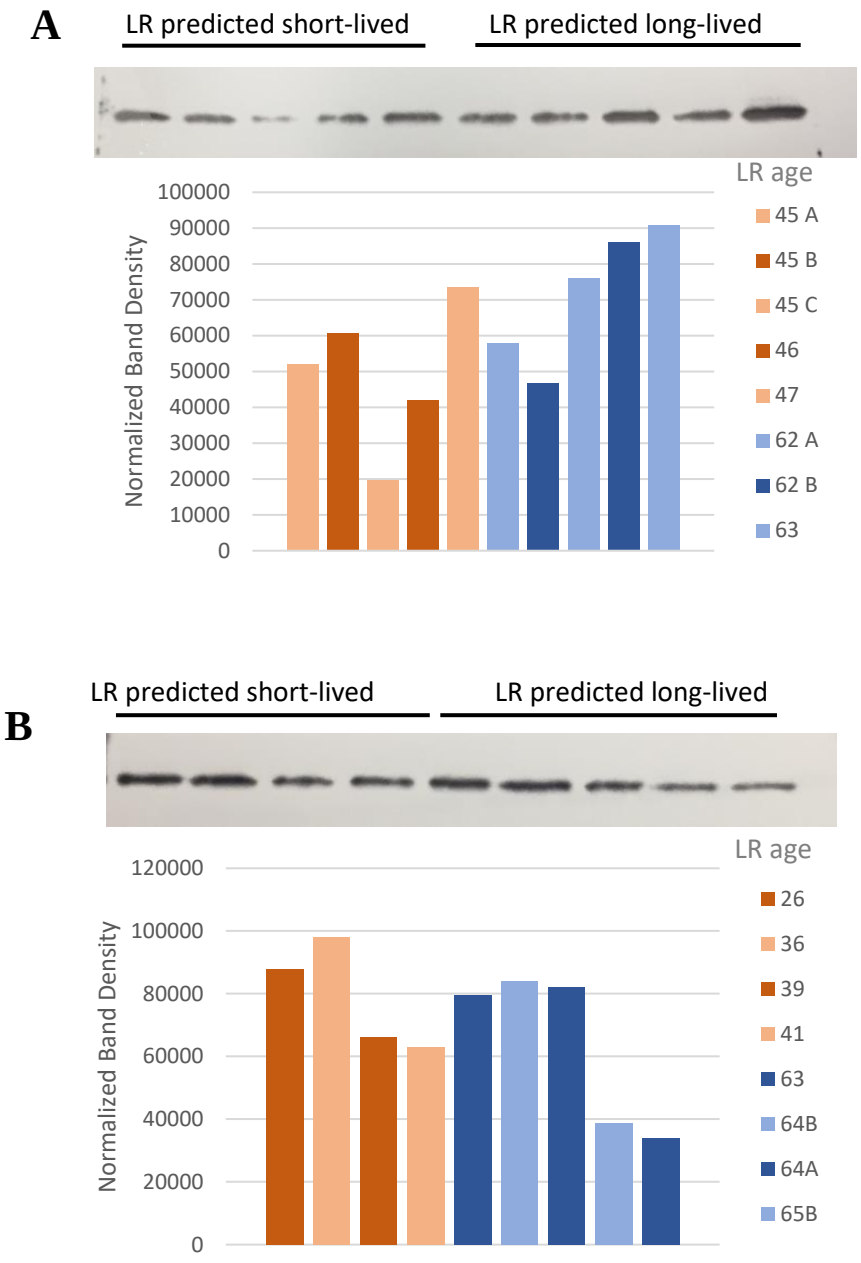

Figure 2.9 Two representative examples of SOD2 western blots in individual fly heads. Orange colors represent predicted short-lived flies and blue represents predicted long-lived flies (LR only).

flies. The results were mixed with a great degree of individual variation between flies (Figure 2.9). Reasons and implications are discussed in detail in the Discussion Section.

3. DISCUSSION AND CONCLUSIONS

3.1. INDIVIDUAL DIFFERENCES

Variation between wildtype individuals of a given species is enough to produce surprisingly large molecular and behavioral differences (Ahadi et al., 2020; Augier et al., 2018; Speakman et al., 2004; Zhao et al., 2017). Naturally, this is exemplified in our own data as well: flies under the same experimental conditions, same sub-strain, same sex, same genetic background, frozen at the same time of day, and even the same chronological age are capable of exhibiting 3.7-fold different levels of PP38 (Figure 2.6) and 4.6-fold difference in SOD2 (Figure 2.9A).

This variability in individual levels is obscured when samples are pooled. A previous graduate student Josh Lisse found 0.2-fold significantly more SOD2 in predicted short-lived flies compared to predicted long-lived flies, while I did not. Josh used pooled data (3 flies per sample, 8 samples) while I used individual data. Considering the magnitude of variation between individuals that I've seen (Figure 2.9), likely what was happening was 1 or 2 flies in Josh's pooled samples were pulling the values for the whole group up. Another group saw a similar phenomenon in humans; Ahadi et al. found that the majority of individuals in their cohort had a negative correlation for age and creatinine even though populational creatinine is positively correlated with age (Ahadi et al., 2020). Researchers should continue to be aware of the variability that individuals are capable of and that pooling samples together may be hiding important biological information.

3.2. SLEEP CHARACTERISTICS CAN BE USED TO PREDICT BIOLOGICAL AGE

Aging is a dynamic process that occurs at different rates and through different molecular pathways for members of a given species (Zhao et al., 2017). It can also vary in characteristics between species and between different tissues of the same organism. Thus, it's unlikely that there's a single common molecular aging marker for all individuals even of the same strain and sex. Having previously used only one model for predictions, this clarifies why we've had difficulty identifying a strong molecular marker for just LR predicted 'long-lived' or 'short-lived' flies; not enough models were used to separate out subsets of flies sharing the same pathways affected by aging. A correlation with heartrate was found in one model (Figure 2.1), but one physiological outcome might reflect multiple sets of pathways. Two models better separate flies out into aging subgroups, although even a second model doesn't perfectly separate out proteostasis flies, seeing as only ~19% of the differences in total body protein concentration is explained by the differences in age predictions (Figure 2.3).

Rather than being absolute predictors of the exact day on which a fly will die, our results suggest that the models are working by identifying different sets of sleep characteristics that reflect different aging pathways. When combined, the models separate out different aging subgroups of flies. Specifically, when a fly's predicted age is anchored as predicted long-lived (~59-63 days) under the LR model and a corresponding range of FPCA predicted ages are chosen, this combination of models separates out the subset of flies for which proteostasis disruption is the strongest predictor of lifespan (Figure 2.3), given that excess proteins and elevated stress markers are indicators of

disrupted proteostasis (Y. Zhang et al., 2018). I hypothesize that combining the model predictions in different ways will reveal other subsets of aging.

I didn't find a correlation with SOD2 and predicted lifespan, and this likely due to a multitude of reasons. First, there's the problem of baseline SOD2 vs elevated SOD2 in response to oxidative stress. Because of natural individual variation, some flies are born with higher baseline SOD2 levels which would confer a protective ability from the antioxidants in preventing ROS damage. This concept is seen with heat shock proteins; heat shock proteins are considered only partially predictive of lifespan because longer lived strains were found to have higher baseline levels of heat shock proteins which act protectively and preventatively for the organism (J. Yang & Tower, 2009). In our flies, if at 30 days of age before the end of data collection there's a stressor that leads to an increase in ROS, SOD2 would elevate in response to keep ROS levels in balance. In this case, the fly's normal baseline variation would be indistinguishable from an elevation in response to oxidative stress in our experiments. Second, because we're taking measurements at only ~30 days of age, some flies may not be far enough along in their aging pathways to be at the point of significantly different levels of elevated antioxidants. Even not knowing baseline SOD2, we may be able to find differences in moderately short vs extreme short predictions (e.g. age prediction of 46 vs 32). I would hypothesize that in the case with the extreme low predictions, the models are indicating the fly is very near death and so would have a higher chance of having elevated SOD2, much in the same way *hsp22* has been reported to spike in expression hours before death (Grover, Yang, Ford, Tavaré, & Tower, 2009). Lastly, the relationship that SODs have with lifespan is complex. SOD overexpression can lead to increased or decreased lifespan depending on

diet and genetics, although it's unclear which combinations of diet and genetic backgrounds allow SOD to have what effects on lifespan (Tower, 2015).

Protein homeostasis and abundance change with age (Ben-Zvi et al., 2009; Koga, Kaushik, & Cuervo, 2011; Vincow et al., 2020). One recent paper found that disrupted proteostasis in aging flies is due to both changes in protein synthesis and decreased autophagy, the later resulting in proteins with an increased abundance with age (Vincow et al., 2020). We found a negative correlation between total body protein concentration and predicted age, where predicted short-lived flies have higher body protein abundance. This same subset of flies has a coincident negative correlation between predicted age and PP38, a mitogen-activated kinase. These results suggest this subset of flies have age-related disrupted proteostasis, and it's at the point where it's causing the fly stress. These flies are identified when the LR model predictions are held at about 59-63 days, and the biomarker correlations are found with the corresponding range of FPCA predictions. In other words, after sub-grouping the flies with the LR model, the FPCA model finds sleep characteristics that indicate that the flies are biologically older in a way that directly relates to decreased autophagy. The question is, are these poor sleep characteristics causing the decreased autophagy or are they simply associated with the disruption as a parallel consequence of another cause? Conversely, is the decreased autophagy causing poor sleep? A recent study by Bedont et al. reveals a complex relationship between macroautophagy and sleep in fruit flies. They found that autophagosomes normally accumulate during the day and sleep resets their levels. At the same time, genetic manipulation causing an increase in autophagosomes reduces total sleep and decreasing autophagosomes increase sleep (Bedont et al., 2021). While the exact relationship

between autophagy and sleep is still unclear, this study does provide evidence of a direct relationship, rather than poor sleep and decreased autophagy being independent aging effects.

Oxidative stress is thought to be one of the reasons that proteostasis becomes disrupted with age (Santos, Sinha, & Lindner, 2018), and PP38 elevates in response to oxidative stress, especially in relation to aging, so it's unsurprising that we also found higher PP38 in our predicted short-lived flies with disrupted proteostasis.

In short summary, our results show that sleep characteristics can be used to predict biological age. Additionally, our models identify specific pathways affected by aging, like proteostasis. This is shown through predicted short-lived flies that have higher protein concentration levels and higher PP38 levels.

3.3. CONCLUDING REMARKS AND NEXT STEPS

The problem of baseline SOD2 vs elevated SOD2 may be solved by using a luciferase or GFP strain of flies. That way we could measure SOD2 at the beginning of fly lives as a baseline and then track how SOD2 changes alongside their sleep characteristics, however, the altered genetic background would present a new problem. It may be that SOD2 is too convoluted to use in our cohort as a marker, but oxidative stress markers are still a promising avenue to pursue. A more direct measurement of oxidative stress may be more desirable, like measuring protein carbonyls.

Since gut aging pathways are well described (He et al., 2020; Rera et al., 2012; Vaccaro et al., 2020) and a subset of our flies are showing elevated PP38 which is implicated in gut aging (Figure 2.6), then gut aging is one promising area to pursue next.

The first step would be to find the combination of models that identify flies whose sleep characteristics are largely due to gut aging. This could be done by evaluating TOR, PP38, and P53 signaling, completing the major molecular pathway for gut aging (He et al., 2020). I would hypothesize that some combination of models would subset out those flies, and the whole TOR-PP38-P53 pathway would be elevated in the predicted short-lived flies.

Since the aging pathways identified by our models are ultimately reflected through sleep characteristics, markers of bad sleep that are also implicated in aging would be interesting to explore. Jnk and Erk are a start, since they are two pathways involved both in aging and sleep regulation.

Finally, if one model is seeing one set of sleep characteristics reflecting a certain type of aging, and two models begin to separate out fly groups, then more models will provide additional angles of view to more accurately narrow down aging subgroups.

4. MATERIALS AND METHODS

4.1. FLY HUSBANDRY

Canton-S flies were reared in incubators kept at approximately 25°C and no more than 40 flies per vial on standard agar-yeast-molasses media containing 5% penicillin/streptomycin. Flies were flipped onto fresh food every 1-2 weeks.

4.2. 30-DAY MONITORING AND SLEEP ANALYSIS EXPERIMENTS

Figure 4.1 outlines the general workflow for experiments, which goes as follows: newly emerged flies are allowed to develop social skills 2-4 days before being briefly sedated under CO_2, separated by sex, and allowed to recover and for at least a day before the males were loaded into monitors for experiments. Flies were loaded into individual glass tubes with food in one end and a sponge plug in the other, and their activity was

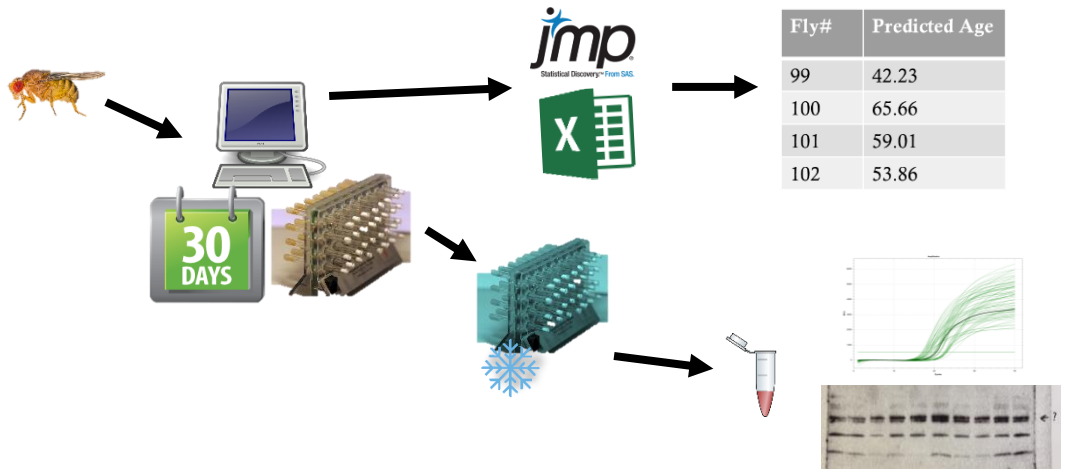

Figure 4.1 Workflow for experiments. Flies are loaded into monitors for 30 days, after which the sleep data is run through a series of Excel, R, and jmp programs to generate predictions. At the ~30 day endpoint, flies are frozen at -80°C until needed for Western Blot or qPCR experiements

tracked for 30 days via the Drosophila Activity Monitoring system. Fresh tubes were changed out every Tuesday and Friday. At the end of ~30 days, live flies were frozen away at -80°C and their activity counts were interpreted into sleep data using Excel,

Blue font: 'disrupted proteostasis' flies

Fly ID	LR Predictions	Fly ID	FPCA predictions	
247	63.726	25	57.198	
22	61.769	34	57.118	Top 15%: predicted long-lived
33	60.785	106	55.694	
14	60.557	124	55.055	
253	60.477	14	53.864	
12	59.402	121	53.308	
32	59.063	12	53.217	
249	58.818	32	52.871	
34	58.598	99	52.773	
102	58.552	247	51.556	
106	58.069	105	51.542	
99	56.189	102	51.435	
25	55.862	13	51.215	
125	55.800	218	51.091	
124	55.231	107	50.750	
121	54.650	248	50.545	
13	54.627	33	49.477	
23	54.478	252	48.979	
105	54.305	125	48.595	
217	53.970	97	48.545	
107	53.144	249	48.537	
126	52.663	217	48.293	
252	51.946	24	48.125	
123	50.955	123	46.585	
97	49.764	126	46.517	
251	48.718	254	45.656	Bottom 15%: predicted short-lived
248	48.398	22	45.642	
218	48.038	251	44.388	
254	47.022	253	40.731	
24	43.007	23	39.435	

Figure 4.2 Example of a list of predictions and flies chosen for 'proteostasis' experiments

where 5 minutes of inactivity (no beam crossings) corresponds to sleep (Shaw et al., 2000). The sleep data was then processed through a multiple linear regression model and a functional principal component analysis model. Each statistical model generates sleep

characteristic variables deemed important for predicting lifespan then predicts the age at each fly will die based on those variables. The numerical value of the predicted age is not exact to the day, instead the numbers provide information about the biological age of the flies. For each 30-day experiment, the top 15% of flies are called predicted long-lived and the bottom 15% are predicted short-lived. See Figure 4.2 for an illustrated example.

4.3. CARDIAC PACING ASSAY

Protocol for the heart pacing assay were followed roughly according to Wessells & Bodmer, 2004. Data shown in Results were performed and collected by undergraduate Natalie Leach.

4.4. SAMPLE PREPARATION FOR WESTERN BLOT AND BCA

Whole flies, bodies, or heads were homogenized in triton-x lysis buffer with a microtissue grinder and kept on ice. After centrifuging for 5 minutes at 13,000 rpm, 10μL was taken out to use for Bicinchoninic acid assay (BCA). Samples or bovine serum albumin (BSA) standards were incubated with BCA working reagent (Pierce BCA Protein Assay kit, Thermo Scientific) at 37°C for an hour, allowed to come to room temperature, then absorbance was measured 462 nm with a plate reader. Absorbance values for BSA standard serial dilution were plotted against concentration values, and simple linear regression was performed in order to determine sample protein concentration.

Remaining sample was incubated with 1.1X SDS loading buffer at 95°C for 5 min., allowed to cool, spun briefly, then returned to ice before loading into gels.

4.5. SDS-PAGE AND WESTERN BLOT

Samples were loaded into 4–20% or 12% Mini-PROTEAN TGX precast gels (Bio Rad). When needed, one lane of the gel was used for 10μL of ProSieve Color Protein Marker (Lonza). One lane was used for loading control. Using cold running buffers, samples were run at 100 Volts or lower until the dye front reached the end of the gel.

Charged nylon or PVDF membranes were wet briefly in 100% methanol then equilibrated in cold Power Blotter 1-Step Transfer buffer (Invitrogen). Gel cassettes were broke open and gels were also equilibrated in transfer buffer. Transfer stacks (WypAll cut to size) were soaked thoroughly in transfer buffer, and the sandwich was assembled as follows: 4 layers of WypAll, PVDF membrane, acrylamide gel, then 4 last layers of WypAll. After transferring using the 'mixed MW' option (constant 1.3 Amps for 7 minutes) in the TransBlot Turbo transfer system (Bio Rad), PVDF membranes were blocked in either 5% (w/v) BSA in tris-buffered saline-tween-20 (TBST) or 5% skim milk-TBST for 1 hour at room temperature.

After washing in TBST for 15 minutes then 5 minutes X 3, blots were incubated in primary antibody overnight at 4°C, rinsed again in TBST like previously and incubated for 1.5 hours in secondary antibody conjugated with horseradish peroxidase at room temperature before once last set of TBST washes. Blots were then set on plastic wrap and incubated evenly with 5mL of ECL working reagent. After 5 minutes, plastic wrap was folded down over top and the excess ECL reagent was pushed out. Blots were developed using the Gel Doc system (Bio Rad) and band density was measured using ImageJ. Except where indicated in proteostasis-disrupted flies, band density was normalized to total protein concentration as a loading control. Concentration was used instead of weight

because total input volumes, such as the lysis buffer, were kept consistent across experiments. Additionally, the loading order for gel lanes and BCA wells were pseudo-randomized to avoid obvious technical artifacts.

APPENDIX

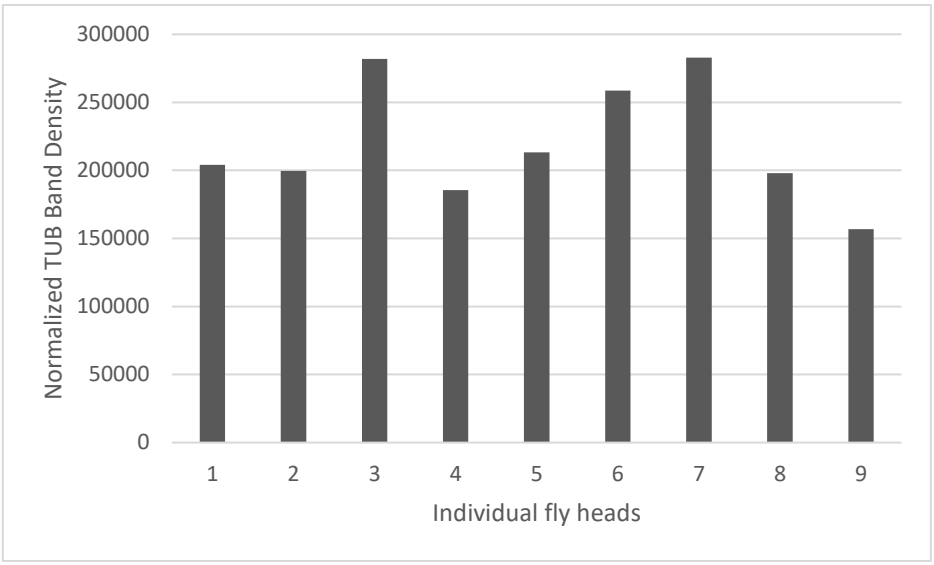

Figure A.1 Western Blot analysis showing individual variation of TUB.

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