Investigating the role of glutathione in *Drosophila* lifespan and neuronal health

By

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Contents

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Abstract

The greatest risk factor for developing Parkinson's disease is aging, and the accumulation of reactive oxygen species is thought to play a significant role in the aging process. Glutathione is a universal antioxidant in animals and plants that helps maintain redox homeostasis within cells, preventing or delaying excessive buildup of reactive oxygen species that may damage or destroy the cell. Using the fruit fly *Drosophila melanogaster* as an animal model, I sought to investigate the extent of glutathione's influence over fly lifespan and neuron viability. I took advantage of the Drosophila Genetic Reference Panel (DGRP) to conduct experiments on flies with longer and shorter lifespans and first determined that peroxide production was negatively correlated with average lifespan. Next, I measured the glutathione levels in the heads of the short- and long-lived flies and found them to be correlated with lifespan in younger flies only. I then designed double-transgenic Drosophila lines containing glutathione overexpression and knockdown constructs under the direction of ubiquitous and neuronal drivers that were crossed into the short- and long-lived DGRP genetic backgrounds. From this, I observed a sex-specific difference in effects, with female flies experiencing lifespan reductions and males experiencing lifespan extensions in a construct- and expression-independent manner. Finally, a pilot experiment involving dihydroethidium staining of fly brains was done to visualize potential redox differences between short- and long-lived flies, where no difference was seen in week-old flies. The work presented in this thesis adds to our understanding of how glutathione affects the aging process and its therapeutic potential in aging-related neurodegenerative diseases like Parkinson's disease.

Abbreviations

PD = Parkinson’s disease

AD = Alzheimer’s disease

ROS = Reactive oxygen species

RNS = Reactive nitrogen species

GSH = Glutathione (reduced)

GSSG = Glutathione disulfide (oxidized)

DGRP = Drosophila Genetic Reference Panel

SL = Short-lived (DGRP)

LL = Long-lived (DGRP)

TH = Tyrosine hydroxylase

LS = Lifespan

BSO = Buthionine sulfoximine

GCL = Glutamate-cysteine ligase

GCLC = GCL catalytic subunit

DHE = Dihydroethidium

Chapter 1: Introduction

Aging and Neurodegenerative Disease

The process of aging is incredibly complex and spans many integral biological pathways and systems within organisms. While the details differ across species, the underlying phenomenon is always the same: a progressive accumulation of molecular and cellular dysfunction that ultimately results in the breakdown of tissues necessary to sustain life. In an attempt to better categorize the different types of aging-related progressive breakdowns, aging researchers have identified distinct groups, or "hallmarks", of aging; these include genomic instability, epigenetic alternations, altered intercellular communications, deregulated nutrient sensing, and loss of proteostasis, to name a few[3,4]. Determining the origins, mechanisms, and consequences of aging-related dysfunctions across these hallmarks is a fundamental goal of the field of aging research principally in order to identify entry points for therapeutic intervention.

Aging is the greatest risk factor for the development of many prominent neurodegenerative diseases including Parkinson's disease[5-7] (PD) and Alzheimer's disease[3,8] (AD). PD is a neurodegenerative disorder biologically characterized by a progressive loss of dopamine neurons in the substantia nigra pars compacta (SNc) region of the brain[9,10]. While symptoms can vary between cases, the loss of these critical neurons most commonly results in some form of locomotive dysfunction that increases in scope and severity over time. In advanced cases, cognitive impairments can also occur[11]. AD neurodegeneration is characterized by the presence and accumulation of intraneuronal fibrillary tangles and extracellular plaques which ultimately result in dementia[12]. While PD and AD have unique pathologies, their shared

risk factor of aging and resultant neurodegeneration puts them into similar focus with the field of aging research. With the world's population of elderly adults set to triple in the next few decades[13], incidence rates of PD, AD, and other aging-related diseases are likely to increase dramatically in that time, putting extraordinary pressure on healthcare systems and societies alike. To combat this coming trend, aging research seeks to better understand the biological mechanisms underlying the aging process in order to better identify early changes that might lead to or signal the development of aging-related diseases like PD.

Mitochondria and the oxidative stress theory of aging

While many theories exist to explain the most fundamental processes underlying aging, one of the oldest and most vetted theories is the "free radical theory of aging", first described by Dr. Denham Harman in 1956[14]. Dr. Harman argued that the intracellular generation of free radical molecules, which are highly reactive biochemically, caused progressive damage to cells in ways that result in the physiological changes we see in aging. From this free radical theory of aging came the oxidative stress theory of aging, based on the notion that reactive oxygen species contribute to an increase in cellular damage known as oxidative stress[15,16].

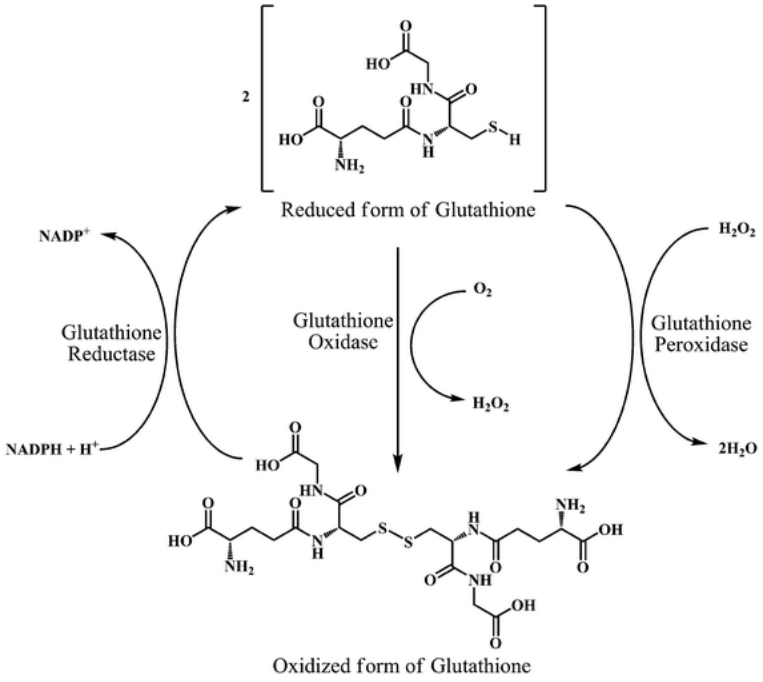
Mitochondria are the source of the majority of reactive oxygen species found in the cell at any given time[17,18]. The use of oxygen creates the opportunity for the formation of superoxide ($O_2^{\cdot -}$) radicals that can give rise to other reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$). While recent research has supported that these ROS or RNS molecules may have essential signaling functions within the cell[19-21], their high

biochemical reactivities can also cause disruption through oxidative or nitrosative stress, respectively[22]. Mitochondria harboring dysfunctional ETC complex component(s) have increased potential for generating $O_2^{\cdot -}$ (produced predominantly by complexes I and III) which can elevate overall oxidative stress in the cell. Since these reactive oxygen species could target the mitochondria themselves, a putative feed-forward cycle of mitochondrial dysfunction and reactive oxygen species generation has been proposed to account for the progressive nature of cellular aging. The widely-held hypothesis that oxidative damage alone is sufficient to account for functional losses associated with aging has recently been challenged[23]. An alternative hypothesis posits that an age-related shift toward a pro-oxidizing cellular redox state leads to a disruption of redox-regulated signaling pathways that in turn promotes cell senescence and death.

Glutathione antioxidation

Regardless of their specific effect on aging or PD, the production of oxidative stressors through routine mitochondrial function is mitigated in large part by the activity of the antioxidant glutathione. Glutathione is a tripeptide composed of L-glutamate, L-cysteine, and glycine, synthesized in two consecutive steps by the enzymes γ-glutamyl-cysteine synthase and glutathione synthase[24]. Glutathione exists in two forms: reduced glutathione (GSH) and oxidized glutathione (GSSG). The primary antioxidative function of glutathione involves the glutathione peroxidase-catalyzed reaction of a GSH thiol group with a negatively charged free radical, wherein the thiol hydrogen is transferred to the free radical and used to reduce and effectively neutralize it[25] (Figure S1). This process also results in the formation of oxidized glutathione as two glutathione molecules lacking their thiol hydrogens form a disulfide bond and become GSSG. This

Figure S1



Supplemental Figure 1: Main antioxidation mechanism of glutathione

Figure from Nimse, S.B., and Pal, D.[2] Glutathione exists in both a reduced, monomeric form (GSH) and an oxidized, disulfide form (GSSG). GSH is able to neutralize reactive oxygen species such as hydrogen peroxide via a peroxidase in order to neutralize it. This results in the formation of GSSG, which can be reverted to GSH using an NADPH-facilitated reductase reaction.

reaction is reversed by glutathione reductase, which utilizes riboflavin and NADPH to break the disulfide bond and provide new hydrogen atoms to the glutathione thiol groups, refreshing them into two GSH molecules[26].

Glutathione can also be utilized to protect other proteins in the cell with exposed thiol groups in a process called glutathionylation[27]. In this process, either GSH forms a disulfide bond with the exposed thiol of the protein or GSSG exchanges its disulfide connection with the protein, in either case protecting it from attack by reactive oxygen species or other free radicals within the cell. Once the cellular environment becomes

less saturated by reactive species, the protein is deglutathionylated and free to resume its normal function.

As research has uncovered more functional implications for reactive oxygen species in the cell, so too has interest and understanding grown about the diversity of roles that glutathione may play. The rate-limiting enzyme γ-glutamyl-cysteine synthase is able to sense the intracellular redox state and adjust GSH synthesis accordingly[28]. Therefore, the functional ability of the cell to neutralize reactive oxygen species is regulated by a threshold of biologically permissible oxidative stress. This may explain how key protein tyrosine kinases such as Lck and Fyn have been shown to be activated by hydrogen peroxide[29,30], and protein kinases B and C have shown similar activation patterns[31,32]. If certain amounts of peroxide or other reactive oxygen and nitrogen species are needed for proper cell signaling, it stands to reason that the synthesis and activity of glutathione safeguards a finely-tuned balance between necessary and excessive oxidative stress. Additional research has also demonstrated how coordinated glutathione efflux from the mitochondria may play an active role in the regulation of apoptosis, or programmed cell death[33,34], further implying that glutathione likely contributes to many important cellular processes in ways the field has yet to fully understand. While such broader implications are fascinating to consider, glutathione's direct antioxidation capabilities will be the main focus of this thesis.

Using *Drosophila* to study PD and aging

Drosophila melanogaster, the common fruit fly, has proven to be an excellent animal model for studying aging and neurodegenerative diseases including PD[35,36]. Drosophila have a complex nervous system that mirrors mammalian systems in key

ways, such as the presence of distinct neuronal subtypes and clusters[37]. Drosophila also contain all the relevant genes for dopamine synthesis and secretion, and utilize dopaminergic signaling in analogous ways to mammals such as for locomotion and circadian rhythm maintenance[38]. There is also a wide range of tools for genetic manipulation of Drosophila, allowing for the temporal and tissue-specific induction or knockdown of genes of interest. Of even more interest for PD studies is the fact that Drosophila share many key aging-related genes with humans[39,40], providing additional translational credence to their use in medical research. There are also well-developed methods for measuring changes in Drosophila memory and locomotion, two phenotypes of interest in aging and neurodegeneration research[41] The average life span of a fruit fly is approximately sixty days[42], and when combined with the fact that hundreds of Drosophila can be raised inexpensively and in a relatively small amount of space, this allows for experiments of strong statistical power to be established and completed within a few months.

There are important caveats when it comes to using Drosophila for PD research that are worth consideration. For one, Drosophila do not synthesize epinephrine and norepinephrine from dopamine like mammals do, instead opting to use octopamine and tyramine[43]. Also, mammals have a highly vascularized central nervous system, while Drosophila utilize a circulating hemolymphatic system to account for their brain's metabolic needs[44]. Differences such as these impede certain avenues of translatable research, such as investigating the vascular assistance roles of non-neuronal cells in the central nervous system. Despite these and other key distinctions between the invertebrate Drosophila model and other vertebrate mammalian models, the utility of

fruit flies for aging and PD research remains strong and so they are the primary one used in our lab.

In order to find clues to the mechanistic links between aging and PD-linked neurodegeneration, our lab has employed the use of the Drosophila Genetic Reference Panel (DGRP), a suite approximately 200 fully-sequenced homozygous fly lines that can be used as a sample population to search for genes that correlate with phenotypes of interest[45]. The DGRP fly lines exhibit a bell curve of average lifespans, with a suite mean of 55 days[46]. However, the fly lines of most interest to us are the ones that live much longer or shorter than that average, lines that we call “short-lived” or “long-lived”. Our thinking is that by studying these short- and long-lived DGRP flies, we can find genetic and metabolic factors that are conferring these extensions and reductions in lifespan. We can then look at these lifespan-related factors in the context of PD and hope to find related treatments or preventative strategies that directly target the aging-related elements involved in disease progression.

Correlation between lifespan and PPL1 dopamine neuron viability in DGRP flies

One of the initial experiments our lab conducted after adoption of the DGRP was to assess neuron viability across the fly lines to determine any correlations with line average lifespan. Female flies from 24 DGRP lines of varying average lifespans were raised until they were five weeks post-eclosion, at which point the brains were dissected and incubated with anti-tyrosine hydroxylase (TH) antibodies for dopaminergic neuron labeling. Confocal imaging was then used to identify and count the number of viable neurons present in each of the six dopaminergic clusters in the fly brains across the

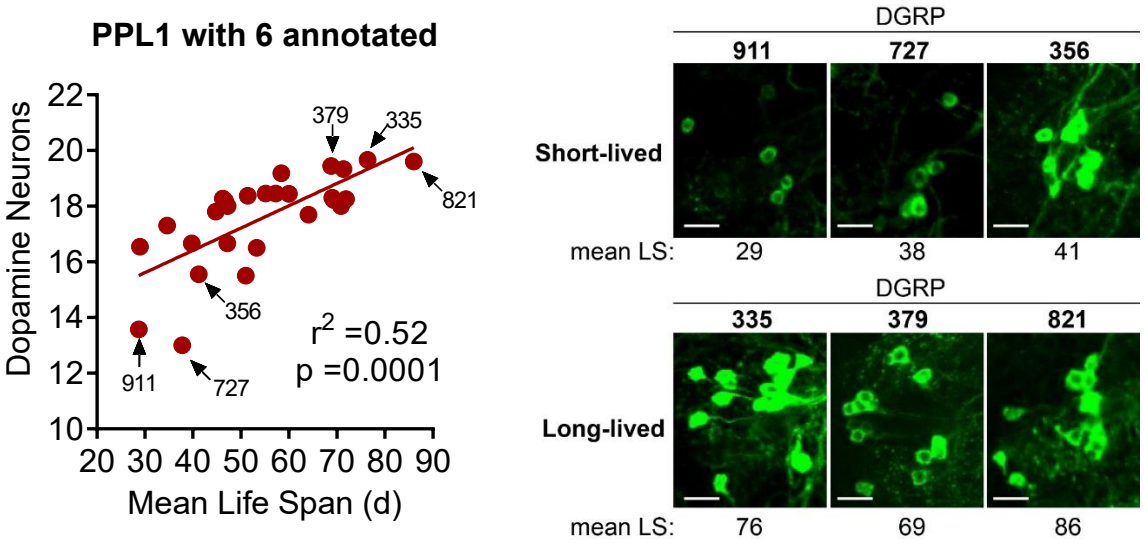
lines, and that data was then plotted against the average lifespan data to assess correlation.

While there was no correlation found across five of the clusters observed, there was a statistically significant correlation between average lifespan of the DGRP line and the number of viable dopamine neurons in just the PPL1 cluster of fly brains five weeks post-eclosion (Figure S2). Put more simply: the short-lived DGRP lines had on average fewer PPL1 dopamine neurons at the aged timepoint than their long-lived counterparts. This PPL1 cluster has been linked to wakefulness, taste memory, and motor function[47-49], although the true functional extent of these neurons is still far from fully understood. To rule out the possibility that these neuron count differences were due to impaired neurodevelopment instead of progressive neurodegeneration, the same experiment was conducted using one-week-old flies; at this younger timepoint, all fly lines had a similar number of dopamine neurons across all clusters, supporting that the correlation seen at five weeks post-eclosion was reflective of neurodegeneration.

Correlation between lifespan and peroxide vulnerability in DGRP flies

Next, our lab decided to further investigate the DGRP's relevancy for PD research by assessing the link between oxidative stress resistance and lifespan of the long-lived and short-lived flies. This was done by exposing four-week-old flies of three long-lived and three short-lived DGRP lines to a high dose of hydrogen peroxide, a potent oxidative stress inducer, and measuring their survival. There was a significant correlation between DGRP line lifespan and survival during peroxide exposure,

Figure S2



Supplemental Figure 2 – Lifespan is correlated with PPL1 neurodegeneration in DGRP flies

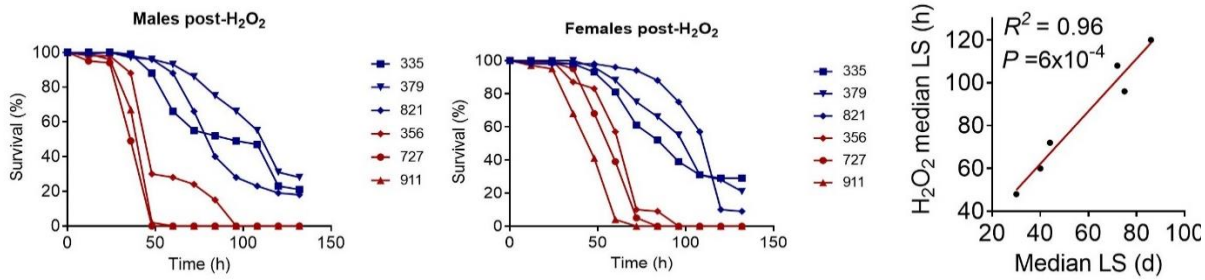
Data and graphs generated by Dr. Ian Martin. 26 female DGRP fly lines of varying average lifespans were aged five weeks before brain extraction. The brains were then stained using anti-TH antibodies to allow confocal microscopy visualization of the dopamine neurons. All dopamine neuron clusters were counted, but only the PPL1 neuron cluster showed neuron counts correlated with line lifespan. Representative microscopy images of the PPL1 neuron clusters for three short-lived and three long-lived lines are shown. The same experiment was repeated with one week old flies and it was observed that all flies had similar dopamine neuron counts (data not shown). This implies that the correlation seen at five weeks is due to neurodegeneration and not impaired neurodevelopment in the shorter-lived DGRP flies. n = 7-12 brains/genotype

meaning that the long-lived flies survived longer while subjected to oxidative stress than the short-lived flies (Figure S3). This trend was consistent across both sexes as well.

Metabolomic analysis of long- and short-lived DGRP flies

Seeking to cast a wider net in our exploration of the differences between the short- and long-lived DGRP lines, we worked with our collaborators in the Promislow Lab at the University of Washington to conduct a broad metabolomics assay. For this

Figure S3



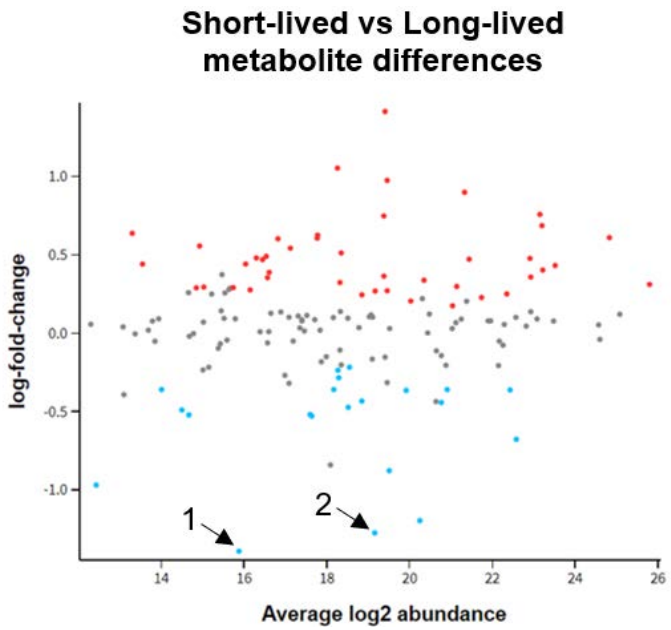
Supplemental Figure 3 – Hydrogen peroxide vulnerability is correlated with lifespan in DGRP flies

Data and graphs generated by Dr. Ian Martin. 100 flies each from three long-lived (blue) and three short-lived (red) DGRP lines were transferred to food containing a high dose of hydrogen peroxide and then their longevity was measured. Long-lived flies lived significantly longer during peroxide exposure than the short-lived flies; this was observed for both males and females. Additionally, longevity during peroxide exposure was correlated with DGRP lifespan.

assay, they processed the heads of four-week-old flies from the same six (three short-lived, three long-lived) DGRP lines that we used in the peroxide survival experiment and analyzed the levels of 147 metabolites, spanning many key biochemical pathways. When checking for correlation between metabolite abundance and lifespan, several interesting candidates were flagged but the one that caught our attention the most was the antioxidant glutathione. We observed a strong correlation between DGRP lifespan and the abundance of both the reduced (GSH) and oxidized (GSSG) forms of glutathione (Figure S4). In conjunction with our and others' studies implicating oxidative stress in aging, these results led to my interest in glutathione and the consequent work presented in this thesis.

The following experiments and results represent my aim to investigate the role that glutathione may play in lifespan determination in Drosophila, leveraging

Figure S4



Supplemental Figure 4 – Results from DGRP metabolomics experiment

Data generated by Drs. Ian Martin and Daniel Promislow; graph generated by Drs. Theo Bammler and Lu Wang. Heads of four week old female flies from three short-lived and three long-lived DGRP lines were sent to the Promislow lab at the University of Washington for metabolomics analysis. Data on 147 different metabolites were collected, and then difference in abundance between the short- and long-lived flies were assessed and plotted as seen here. Each dot represents a single metabolite, with position in the y-plane reflecting the scale of any fold-change difference observed between the short- and long-lived flies tested. Positive y values indicate higher levels were observed in the short-lived flies than long-lived flies tested, and negative y values indicate the inverse. Oxidized glutathione (1) and reduced glutathione (2) were both higher in abundance in long-lived flies than short-lived flies, with log-fold differences of -1.391 and -1.274, respectively. n = 3 biological replicates of 25 flies each/genotype.

biochemical assays to measure the antioxidant's abundance in different DGRP lines across the lifespan of the fly, and utilizing genetic constructs for manipulating its expression in different cell types. I hypothesized that the reduced lifespans seen in the short-lived DGRP flies were due to reduced levels of glutathione compared to the

average, impairing their antioxidation upkeep needs and resulting in accelerated aging phenotypes. Further, I hypothesized that such reduced lifespans could be rescued by increasing glutathione synthesis in those short-lived flies, increasing their lifespans. This work expands our understanding of glutathione's impact on lifespan and contributes to future directions exploring its connections to PD neurodegeneration.

Chapter 2: Materials and Methods

Drosophila Strains

The Drosophila Genetic Reference Panel (DGRP) is made up of 205 fully-inbred, fully-sequenced fly lines[45]. From this panel, I chose to use several lines based on their average lifespan, which was measured previously in our lab. The DGRP lines that I used and their average lifespans (LS) are as follows:

Line ID	Avg. ♂ LS (days)	Avg. ♀ LS (days)
136	43.90	57.35
177	21.52	21.77
335	53.99	61.99
356	40.85	35.19
360	43.62	48.23
373	35.26	48.95
379	46.69	58.59
409	27.55	25.95
727	31.17	28.49
765	19.00	28.97
819	38.32	46.11
821	44.51	55.14
911	40.41	21.62

I also used the lines listed below when generating the double-transgenic flies to be crossed into the DGRP backgrounds; the crossing scheme for that experiment is described in more detail later in this section.

Sp/CyO; TM3, Ser/TM6B, Tb, Hu (double-balancer, previously generated in lab)

Sp/CyO; Dr/TM6, Sb, Tb (double-balancer, previously generated in lab)

elav-GAL4 (pan-neuronal driver, RRID:BDSC_8760)

TH-GAL4 (dopamine neuron driver, RRID:BDSC_8848)

Da-GAL4 (ubiquitous driver, RRID:BDSC_55850)

UAS-GCLc (GCLc overexpressor, generated and courteously provided by the Orr lab[50])

UAS-GCLc$^{RNAi\text{-}67753}$ (GCL dsRNAi, RRID:BDSC_67753)

UAS-GCLc$^{RNAi\text{-}67936}$ (GCL dsRNAi, RRID:BDSC_67936)

Lastly, the following line was used in the DHE experiments, crossed into the DGRP backgrounds:

UAS-GFP-nls; TH-GAL4 (dopamine neuron labeling, generated by Dr. Judit Pallos)

Respirometry

All respirometry experiments were conducted at the University of Washington, utilizing equipment and guidance from the Marcinek lab. 40 flies each from three short-lived (911, 356, 727) and three long-lived (821, 335, 379) DGRP lines were aged to four weeks post-eclosion. For each line, four biological replicate samples were prepared by homogenizing 10 cold-anesthetized flies in mitochondrial isolation buffer (MIB, 0.32 M sucrose, 10 mM EDTA, 10 mM Tris/HCL). These homogenates were then lightly centrifuged (200 g, 90s) and the supernatant was collected into a new tube. The supernatant was then centrifuged at a higher speed and time (10,000 g, 10min), after which the supernatant was removed and the pellet resuspended in MIB (110µl). At this point, the samples were ready for use in the respirometer. A small amount of this sample was used in a Bradford assay to determine protein concentration for normalization.

The respirometer used was an O2k-FluoRespirometer (Oroboros Instruments, Innsbruck, Austria), capable of both high-resolution respirometry and simultaneous

fluorometry. The following protocol was used for each sample, with the respirometer cleaned and recalibrated between uses. 2.2ml of respiration buffer (120 mM KCl, 5 mM KH_2PO_4, 3 mM Hepes, 1 mM EGTA, 1 mM $MgCl_2$, 0.2% BSA) was loaded into each respirometry chamber and allowed to equilibrate to 28˚C. Amplex Red and horseradish peroxidase (HRP) were added to the chamber to allow for hydrogen peroxide (H_2O_2) fluorometry, after which the O_2 flux (rate of O_2 change) and H_2O_2 flux (rate of H_2O_2 change) fluorometry readings were calibrated. 40 µl (~50-100ug) of isolated mitochondrial sample was then added to the chamber. As the respirometer collected both O_2 flux and fluorometry data, a series of cell respiration substrates and inhibitors were added to the chamber, one at a time and allowed to stabilize before the next was introduced. The series is as follows:

- Superoxide dismutase (SOD, 55 µM)
- Pyruvate (5 mM)
- Proline (5 mM)
- ADP (5 mM)
- Glycerol-3-Phosphate (10 µM)
- Cytochrome C (10 µM)
- Rotenone (0.5 µM)
- Antimycin A (3 µM)
- TMPD (200 mM)
- KCN (500 mM)

Glutathione Measurements in Drosophila Heads

All 13 DGRP lines listed above were used in this experiment. The flies were segregated by gender into separate vials within 24 hours post-eclosion. The flies were aged either one, two, or four weeks, and were transferred into fresh standard yellow medium vials twice per week until such time for their use. 2-4 replicates of 25 flies each were used for each line and timepoint combination; the variance in number of replicates is due to sample processing errors or a low number of surviving flies available at the time of experimentation. The tissue processing for each experiment was performed in the morning (between 9-11am) to maintain similar circadian metabolic profiles. Biological replicates were performed on separate days.

For each sample, the flies were transferred to a 15ml conical tube and then submerged in liquid nitrogen for approximately 20 seconds to flash freeze them. The tube was vortexed to separate the heads from the bodies, and the heads collected into a microcentrifuge tube containing 100 mM phosphate buffer. The heads were homogenized and centrifuged (17,000 g, 10 min, 4°C). An aliquot of the supernatant was taken and used in a Pierce BCA Protein Assay (Catalog #: 23225) to measure protein concentration. The remainder of the sample was processed for glutathione measurement using the Invitrogen Glutathione Colorimetric Detection Kit (Catalog #: EIAGSHC). Glutathione concentrations and protein concentrations were read at 405nm and 562nm, respectively, in a SpectraMax i3 Microplate Reader. Wells containing fly head homogenate without assay reagents were used to control for any background absorbance due to eye pigments. Glutathione concentration data was normalized using

the associated sample protein concentration data and expressed in units of $\mu Mol \cdot L^{-1} \cdot \mu g^{-1}$.

All head collections were performed in the morning (between 9-11am) to maintain similar circadian metabolic profiles. Biological replicates were performed on separate days.

BSO Media Preparation

Media containing BSO was prepared by first creating a 1mM BSO solution in previously boiled dH_2O, to which 12.5µl/ml of a 10% benzoic acid stock solution was added as an antifungal agent. 1.125 g of dry Drosophila food powder (Carolina Biological Supply Co, Cat. #: 173200) was added to each Drosophila vial needed, and then 5 ml of the BSO solution was added. The food was allowed to rehydrate for 5 minutes, after which the aging flies were transferred into the newly prepared vials. New BSO media was prepared for each transfer, which occurred twice weekly.

Survival Assays

Flies were kept in a 25°C incubator with an automatic 12-hour light cycle to maintain circadian rhythms. Flies were transferred into new vials containing fresh food twice weekly, at which point deceased flies were counted and logged. Flies that escaped during the experiment or became stuck in the media while alive were culled and not included in the data set. This process continued until all flies had perished.

Generation of Double-transgenic Drosophila Lines for Glutathione Level Manipulation

The double-transgenic flies containing the GAL4-UAS expression constructs were generated using the mating scheme depicted in Supplemental Figure 9. After the

double-transgenic lines were established, males were mated to virgin females from the DGRP lines 335, 356, 821, and 911. Control crosses were created by mating w^{1118} (a standard wild-type line) males to DGRP virgin females, resulting in DGRP heterozygote progeny lacking any transgenic constructs. Offspring from these crosses were collected immediately post-eclosion, separated by sex, and then their lifespan assessed via the method described above.

Imaging of Oxidative Stress Using DHE

For this pilot experiment, male 821 and 911 flies were crossed with female flies containing a nuclear GFP reporter construct for labeling dopaminergic neurons (UAS-GFP-nls; TH-Gal4). The offspring from these crosses were aged for one week on standard yellow medium. After one week, the brains of the flies were dissected in standard Schneider's medium using the method described by Wu et. al.[51] and then treated with DHE following the protocol described by Owusu-Anash et. al.[52] In short, the isolated brains were incubated in a 30μM DHE solution in DMSO for five minutes at room temperature in the dark, washed in Schneider's medium three times for five minutes each, fixed for five minutes in 4% paraformaldehyde in PBS, washed briefly in PBS and mounted with SlowFade Gold (Fisher) for imaging.

The posterior dopaminergic neurons were imaged using a 40x objective in a Zeiss LSM 900 confocal microscope, managed by the OHSU Advanced Light Microscopy Core. 500nm excitation and 582nm emission settings were used to visualize DHE fluorescence. 488nm excitation and 510nm emission settings were used to visualize GFP fluorescence. Acquisition settings were standardized across all images in order to maintain data integrity for fluorescence intensity comparison.

Statistical Analysis

Respirometry and fluorometry data was analyzed using Oroboros DatLab software. O_2 flux and hydrogen peroxide flux measurements were calculated for each respiration state by averaging the flux values across a stabilized representative measurement window, taken between the appropriate substrate or inhibitor additions. The data was initially background corrected, and then the values were normalized by sample protein concentration and citrate synthase activity[53] to account for differences in total protein and total mitochondria used, respectively. Per the push for simplified and standardized units of measurement with regard to respirometry, the normalized flux values are expressed in $J_{O2/CS}$ [pmol·s^{-1}·mg^{-1}] for O_2 flux and $J_{H2O2/CS}$ for hydrogen peroxide flux. These data were then analyzed in GraphPad prism, where simple linear regressions were performed to assess the relationships between DGRP lifespan, O_2 flux, and hydrogen peroxide flux. Associated graphs were plotted, as seen in Figure 1.

Statistical analyses for the head glutathione measurements were conducted in GraphPad Prism. Simple linear regressions were performed to assess the relationships between DGRP lifespan and head glutathione levels at the aging timepoints of interest. Regressions were plotted with the standard error of the mean, as seen in Figure 2.

For the survival assays shown in Figure 3, lifespan values were inputted into SPSS (IBM) and analyzed using the Kaplan-Meier survival curve, and pairwise log rank p-values were generated to assess significance of the changes.

For the confocal microscopy in Figure 5, the images were analyzed using Imaris software (Oxford Instruments). Briefly, using the “surfaces” module and the green fluorescent signal, the PPL1 dopamine neurons were designated as surfaces within the

images, mean DHE fluorescence intensity of each PPL1 dopamine neuron “surface” were calculated, and then an average value for each brain was determined. These whole-brain averages were then used in GraphPad Prism to conduct an unpaired t-test between the short-lived 911 brain values and the long-lived 821 brain values.

Chapter 3: Results

The strength in the DGRP lies in its diversity of genetic backgrounds from which phenotypic correlations can be found. This strength is not without caveat, as without a strong understanding of the mechanisms driving the observed phenotype, any sort of manipulation of those genetic backgrounds may fundamentally alter the phenotype of interest in unexpected ways. For that reason, my initial experiments were purely observational: I measured the inherent mitochondrial respiration profiles and head glutathione levels of long- and short-lived DGRP flies to assess for lifespan-associated differences. Once those experiments yielded data that warranted more causal experimental designs, i.e. the integration of genetic upregulation or knockdown of glutathione synthesis, I took extra care to ensure that any manipulations of the DGRP genetic backgrounds did not compromise the integrity of their long- or short-lived phenotypes and the usefulness of their comparison.

Peroxide production rates are negatively correlated with average lifespan in adult DGRP flies

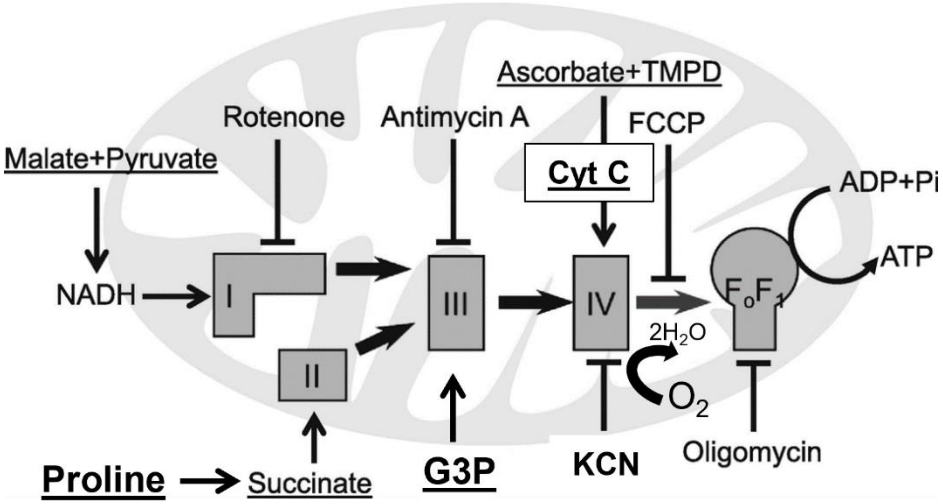
As mentioned earlier, mitochondria are the primary sources of oxidative stress within the cell. This is due to their use of oxygen and free electrons in aerobic respiration to generate ATP. While healthy mitochondria have the means to tightly compartmentalize the respiratory process, impaired mitochondria have the tendency to "leak" electrons, leading to the formation of reduced oxygen species such as superoxide that are highly reactive. Based on our lab's previous data showing that short-lived DGRP flies are more vulnerable to peroxide than long-lived flies, we theorized that this discrepancy may be due to mitochondrial impairments. To assess the mitochondrial

health of these flies, I worked with our collaborators in the Marcinek Lab at the University of Washington to run respirometry analyses of our candidate short- and long-lived lines.

A respirometer is able to measure the rate of oxygen consumption of a mitochondrial sample, which reflects their respiratory activity. By adding substrates and inhibitors of the electron transport chain (ETC) to the respirometry chamber and measuring the change in oxygen consumption, I would be able to identify which specific ETC complexes, if any, were impaired in the mitochondrial preparations from the various DGRP fly lines tested (Figure S5). Not only that, but the addition of Amplex Red, a compound that interacts with peroxide in the presence of horseradish peroxidase to produce a highly fluorescent product, also allowed for the measurement of peroxide production over the course of the entire experiment.

I used two short-lived (911, 356) and three long-lived (821, 335, 379) DGRP lines, aging the flies until four weeks post-eclosion (hereafter referred to as “weeks old”) before their use in this experiment. I isolated mitochondria from four samples containing 10 flies each for each DGRP line. I loaded the isolated mitochondria into the oxygen-saturated respirometry chamber, then I added key respiration substrates and inhibitors into the respirometry chamber in a specific order and measured their impact on oxygen consumption and peroxide production rates. The respirometer was cleaned and reset between each sample in order to ensure quality control and data integrity. The samples were mass-normalized by protein concentration and corrected for mitochondrial content by citrate synthase concentration measurements.

Figure S5



Supplemental Figure 5 – Diagram of electron transport chain activation and inhibition in respirometry

Figure adapted from Long et. al.[1]. Respirometry involves the ordered addition of respiration substrates and inhibitors to isolation functional measurements of specific electron transport chain (ETC) complexes. By measuring and comparing the rate of oxygen consumption or hydrogen peroxide production before and after each addition, mitochondrial health can be assessed on a complex-by-complex basis, uncovering particular impairments or vulnerabilities within the cellular respiration machinery.

Analysis of the collected data showed no significant correlation between mean lifespan and respiration rate via oxygen consumption for any stage of aerobic respiration (Figure 1A-B). This analysis included uncoupled state 2, or “resting state”, respiration as well as complex I state 3 respiration, when ADP is phosphorylated into ATP in the final stage of the process. Despite the lack of respiration rate correlations, there were significant correlations between DGRP line lifespan and peroxide production rates at all respirations states measured (Figure 1C-D). The mitochondria of DGRP flies with longer average lifespans had significantly lower rates of peroxide production when

Figure 1

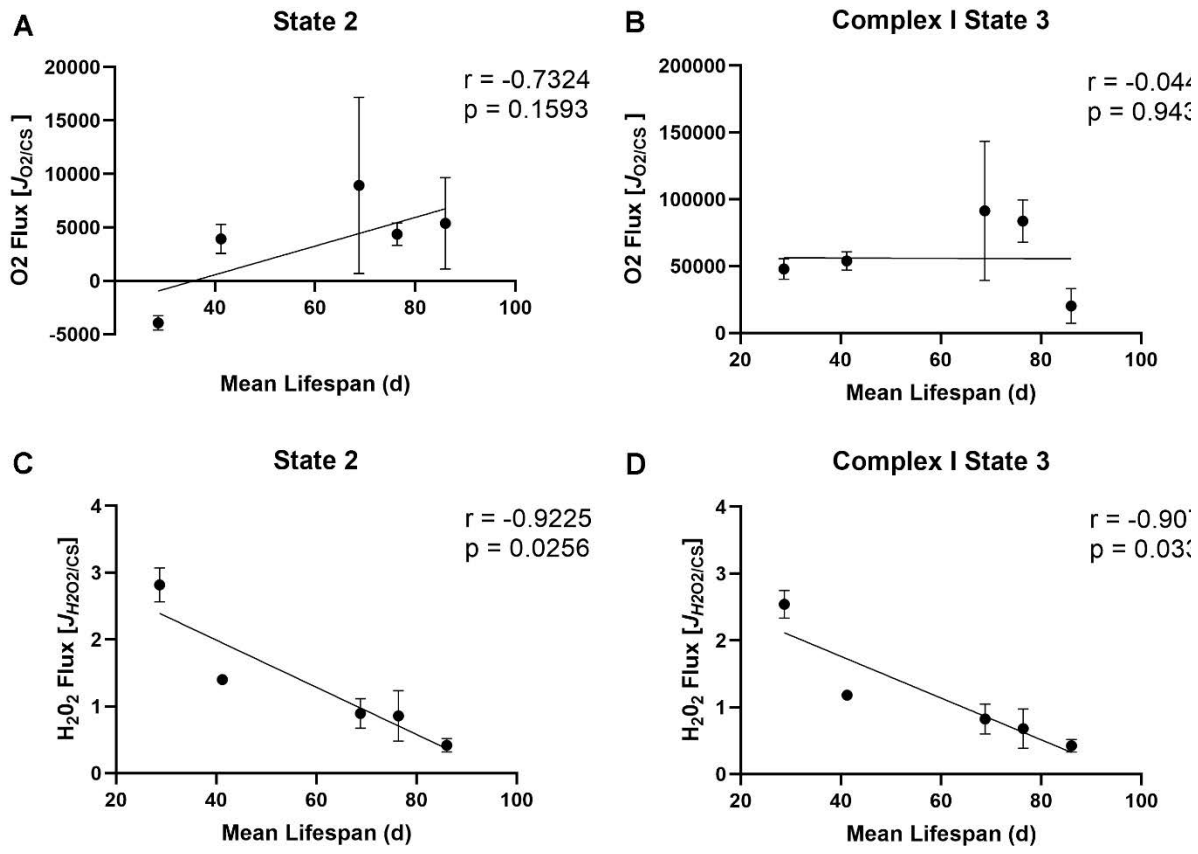


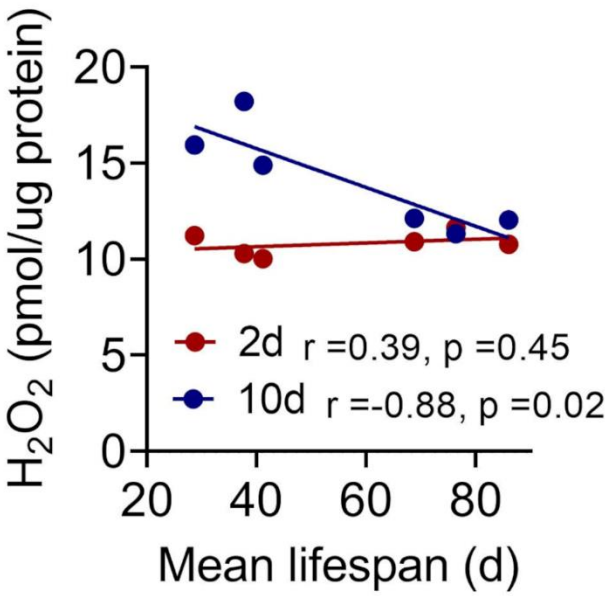
Figure 1: Peroxide production rates are negatively correlated with average lifespan in adult DGRP flies

A,B) No significant correlation was seen between DGRP lifespan and oxygen consumption rates in State 2 (resting, A) and Complex I State 3 (ADP-limited, B) respiration. No significant correlation was seen in any of the other respiration states tested as well (data not shown). Each data point represents the average flux value across four biological replicates of 10 flies each; error bars are SEM. **C,D)** Significant correlations were seen between the DGRP lifespan and hydrogen peroxide production rates in State 2 (C) and Complex I State 3 (D) respiration. Significant correlations were see in all other respiration states tested as well (data not shown). Each data point represents the average flux value across four biological replicates of 10 flies each; error bars are SEM.

compared to the flies with shorter lifespans. To confirm and expand upon these results, our lab later conducted a follow-up experiment wherein Amplex Red was used to

measure the peroxide levels of fly head homogenates from the same DGRP lines. 2-day-old and 10-day-old flies were used in this experiment, and for the 2-day-old flies no significant correlation between lifespan and peroxide abundance was observed (Figure S6). However, in the 10-day-old flies, a similar correlation as the one seen in the respirometry experiments was observed where the short-lived flies had significantly higher levels of peroxide compared to the long-lived flies.

Figure S6



Supplemental Figure 6: Peroxide differences only appear after one week in short- vs. long-lived DGRP flies

Data and graph generated by Dr. Judit Pallos and Alicia Arreola-Bustos. Three short-lived and three long-lived DGRP lines were used to test lifespan correlation with hydrogen peroxide levels at 2 and 10 days post-eclosion. No significant correlation was seen at the two-day timepoint, but a significant correlation was seen in the 10-day-old flies. This data contributes evidence to the hypothesis that early glutathione impairments might lead to later oxidative stress phenotypes, resulting in reduced lifespan.

It is worth noting that this follow-up experiment differed from the respirometry experiment in key ways, including tissue type used (whole body mitochondria vs. head homogenate), aging timepoints measured (1-2 weeks old vs. 4 weeks old), and data collected (rates of peroxide production vs. static level of peroxide observed). However, the conserved trend of increased peroxide observed in short-lived flies compared to long-lived flies was encouraging. These results motivated me to investigate further into the potential oxidative stress differences separating the short- and long-lived flies by focusing on glutathione, the antioxidant which was found to be in much greater abundance in the long-lived DGRP lines compared to the short-lived lines in our metabolomics experiment.

Head glutathione levels correlated with lifespan in younger flies

In order to confirm and expand upon the glutathione data produced by the metabolomics experiment, I decided to conduct my own glutathione measurement assays using the DGRP flies. I used a total of 13 different DGRP lines, which covered a wide range of average lifespans and included the 3 short-lived and 3 long-lived lines which we had used for our previous preliminary experiments. I also chose to use three aging timepoints – 1 week, 2 weeks, and 4 weeks old – to observe how glutathione levels might change over time, and to see how such changes may correlate to lifespan. I also decided to specifically measure glutathione levels in fly heads, focusing on potential antioxidation changes in the central nervous system and tying back to our lab's interest in the short-lived flies' neurodegenerative phenotype.

The results showed that at four weeks post-eclosion, there was no correlation between glutathione levels and mean lifespan values (Figure 2C,F). There was also no

Figure 2

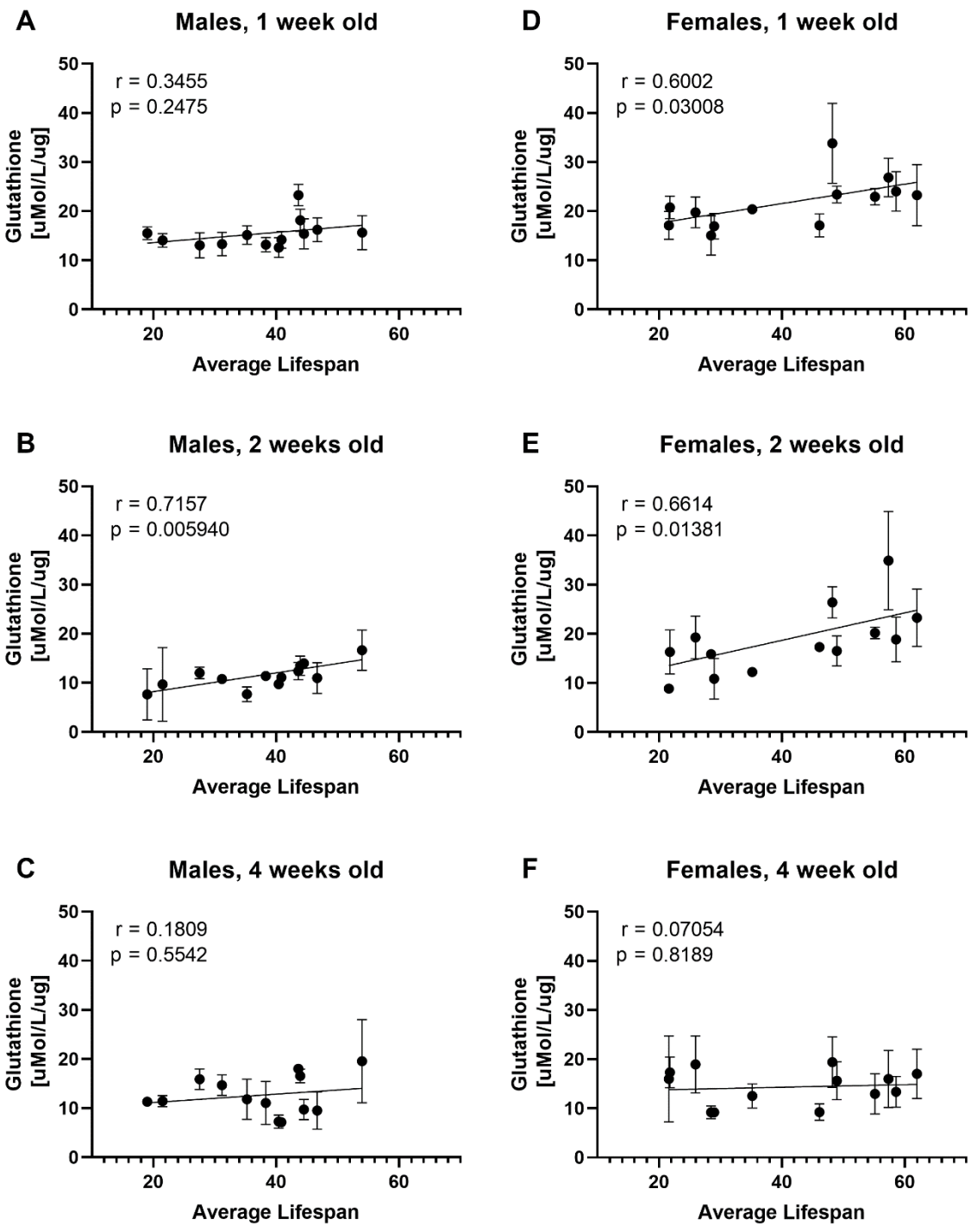


Figure 2: Head glutathione levels correlated with lifespan in younger flies

(continued on next page)

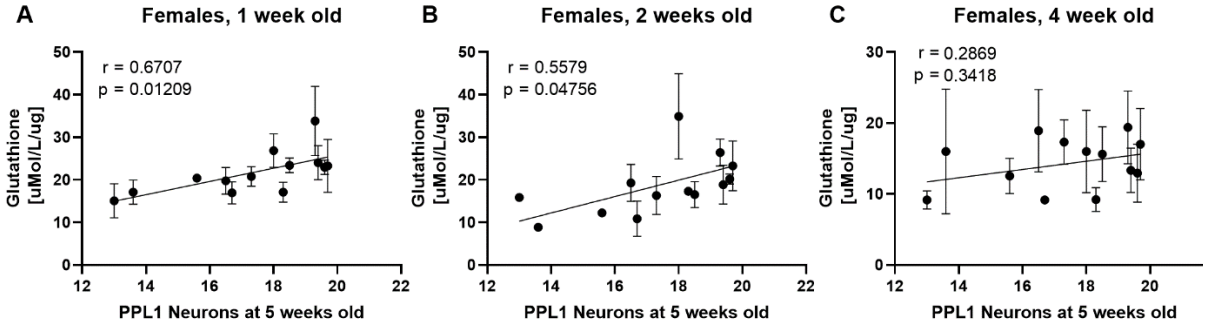
Figure 2: Head glutathione levels correlated with lifespan in younger flies

Mass-normalized glutathione concentrations were calculated for homogenates of 25 fly heads per sample. 13 different DGRP flies spanning a wide range of lifespans were used. **A-C)** With male flies, there was a significant correlation between DGRP lifespan and glutathione concentration in two-week-old flies, but not in one- or four-week-old flies. Each data point represents the average glutathione concentration value across 2-4 biological replicates of 25 flies each; error bars are SEM. **D-F)** With female flies, there was a significant correlation between DGRP lifespan and glutathione concentration in one- and two-week-old flies, but not in four-week-old flies. Each data point represents the average glutathione concentration value across 2-4 biological replicates of 25 flies each; error bars are SEM.

correlation seen at one week post-eclosion for the male flies (Figure 2A). However, for the one-week-old females and two-week-old flies of both sexes, there was a correlation between glutathione levels and lifespan values (Figure 2B,D-E). In other words, for those timepoints the shorter-lived DGRP flies had lower concentrations of glutathione in their heads compared to the longer-lived DGRP flies. An additional observation was that overall, female flies had substantially higher levels of glutathione compared to their male counterparts for each DGRP line tested; this difference will be addressed in greater detail later in this discussion section.

Using our previously collected data on female DGRP PPL1 neuron counts at five weeks post-eclosion, we also determined that there was a significant correlation between such neuron counts and glutathione concentrations in the female flies (Figure S7), meaning that the DGRP flies that had fewer PPL1 neurons at five weeks of age likely also had lower concentrations of glutathione in their heads at one and two weeks post-eclosion. Unfortunately, we did not have the same five-week-old PPL1 neuron count data for male DGRP flies and therefore could not perform the same analysis for that gender. Also worth noting is that in all correlations tested, there was no significant

Figure S7



Supplemental Figure 7: Glutathione levels correlate with neurodegeneration in DGRP flies tested

Glutathione measurements for 1, 2, and 4 weeks old female DGRP flies were plotted against previously collected PPL1 dopamine neuron counts for those same DGRP flies at 5 weeks old. Significant positive correlations between glutathione levels and aged PPL1 neuron viability were seen in the 1 and 2 week old flies, but not with the four week old flies.

differences seen at the four-week-old timepoint. This may be due survival selection bias, where glutathione-deficient flies were unable to survive to that timepoint to be measured, or it might imply that compensatory mechanisms for restoring glutathione levels are engaged later in the fly lifespan.

Assessment of pharmacological inhibition of glutathione synthesis in DGRP flies

While observational studies provided useful insights into glutathione's potential governance over lifespan variability in the DGRP flies, in order to truly test its relevance, I needed to manipulate glutathione levels in the flies and measure the biological response. I devised two complementary but distinct methods to conduct such manipulations: pharmacological inhibition using buthionine sulfoximine (BSO), and genetic upregulation or knockdown using the GAL4-UAS system. The general

hypotheses for each of these experiments were similar: that glutathione reduction or knockdown would reduce the extended lifespan of long-lived DGRP flies by reducing their survivability, and that glutathione upregulation would extend the reduced lifespan of the short-lived DGRP lines by increasing their survivability.

BSO is a potent inhibitor of glutamate-cysteine ligase (GCL), the enzyme that facilitates the first rate-limiting step of glutathione synthesis. This compound has been used in many animal models, including Drosophila, to investigate effects of glutathione deficiency. The delivery mechanism for BSO in Drosophila models is by supplementing their food with the chemical[54]; the plan was compare the lifespans of short- and long-lived DGRP flies on the BSO-containing food and standard medium. Unfortunately, the nature of the media preparation led to major complications in data integrity that meant no data could be reliably used. In short, the media recipe used for this experiment, which was different that the normal food used in the lab for husbandry and other experiments, caused a large number of flies to get stuck in the food and had to be culled from analysis. The control flies raised on food lacking BSO still displayed dramatically reduced lifespans compared to their lifespans on normal media as well. This media recipe likely differed in significant and unexpected ways from the more commonly used core-prepared media, but due to the proprietary nature of the media ingredients used, such differences could not be properly determined.

Genetic manipulation of glutathione synthesis using the GAL4-UAS system

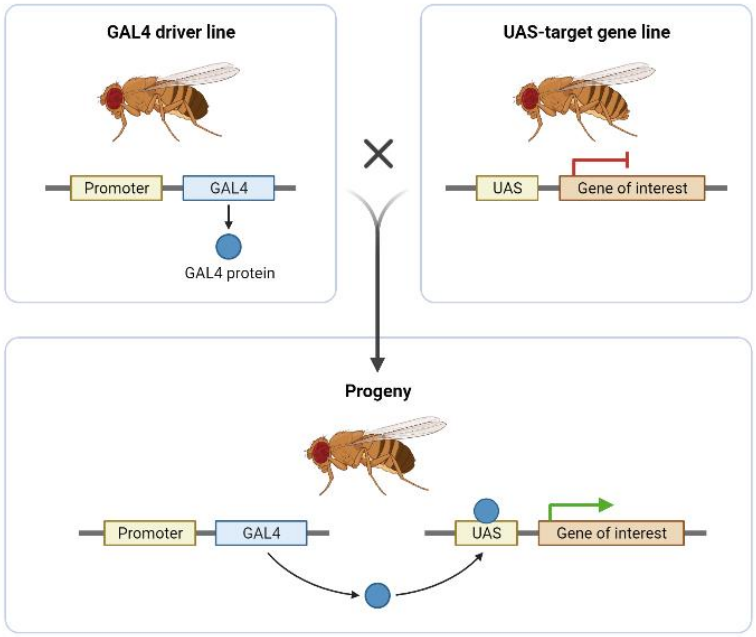
A major strength of the Drosophila model is the depth of genetic manipulation systems that can be used with flies in targeted and compelling ways. One of the key tools that is often used is the GAL4-UAS system, which allows for cell-type specific

gene upregulation or RNAi-mediated knockdown (Figure S8). In this system, the gene of interest is under transcriptional control of an enhancer region called the upstream activation sequence (UAS). Separately, the gene encoding for the yeast Gal4 protein is inserted under the control of a cell-type specific promoter in order to limit its expression. In those specific cells in which the Gal4 is expressed, the protein binds the UAS region and drives expression of downstream sequences such as protein-encoding genes or double-stranded RNAi sequences.

For my project, I wanted to use the GAL4-UAS system to drive glutathione synthesis or inhibition either ubiquitously using daughterless-Gal4 (da-Gal4), or in all neurons using elav-Gal4. These were used to express either the GCL catalytic subunit (Gclc) to promote increased glutathione synthesis, or Gclc RNAi in order to reduce glutathione synthesis. I generated double-transgenic fly stocks that contained the GAL4 and UAS constructs on separate chromosomes which were subsequently crossed with four DGRP lines to assess the effects of altered glutathione synthesis in the context of the short-lived or long-lived genetic backgrounds (Figure S9). Previous experiments done in our lab demonstrated that outcrossed DGRP flies containing only a heterozygous DGRP background maintain their lifespan and peroxide vulnerability profiles, suggesting that the key genetic factors contributing these phenotypes are dominant, and providing rationale for my experiments to evaluate the role of GCL activity on DGRP life span determination (Figure S10).

Three successful double-transgenic lines were created: a ubiquitous Gclc overexpression line (da-GAL4; UAS-Gclc OE), a pan-neuronal Gclc overexpression line (elav-GAL4; UAS-Gclc OE), and a pan-neuronal Gclc knockdown line (elav-GAL4; UAS-

Figure S8

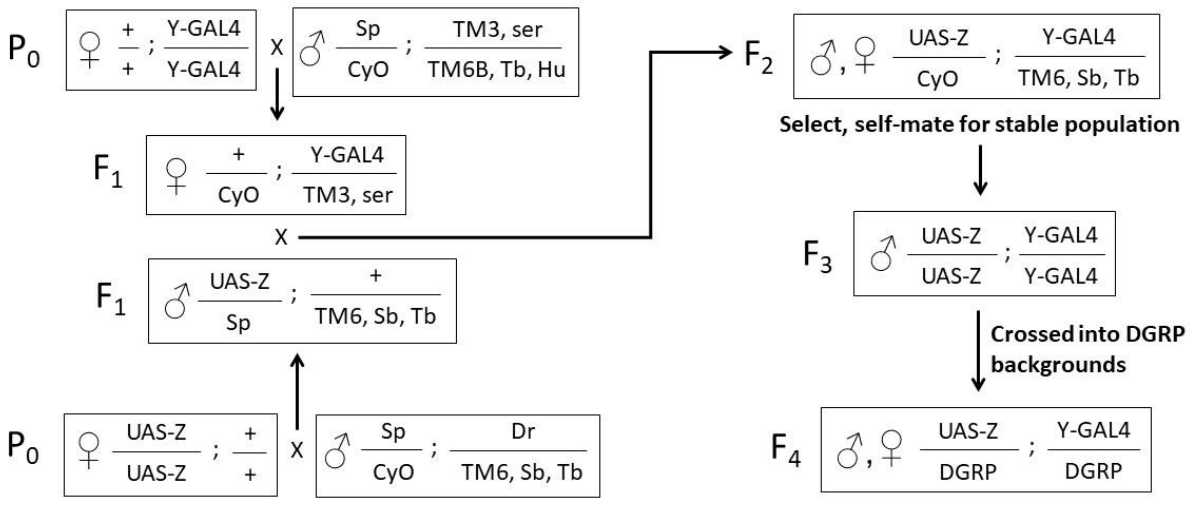


Supplemental Figure 8: Overview of the GAL4/UAS system

Image from Biorender. A GAL4 driver line is created wherein the Gal4 gene is inserted downstream from a promoter only active in a specific cell type of choice. A UAS line is created wherein the gene of interest and an upstream activation sequence (UAS) are inserted such that the UAS prevents transcription of the gene of interest unless bound by GAL4. When the GAL4 driver line and UAS lines are crossed, progeny containing both parts of the system are generated and exhibit cell

Gclc RNAi). These lines were crossed with two short-lived (911, 356) and two long-lived (821, 335) DGRP lines in order to create heterozygous DGRP backgrounds containing our transgenic constructs. Control crosses in which the DGRP lines or double-transgenic lines were crossed with w^{1118}, a standard Drosophila control line, were also generated. The progeny of all crosses were aged on standard yellow food that was changed twice weekly, at which time mortality data collections were conducted. Once all the flies had perished, survival analyses were performed to assess for significant correlations.

Figure S9



Supplemental Figure 9: Mating scheme for generating double-transgenics to be crossed into DGRP backgrounds

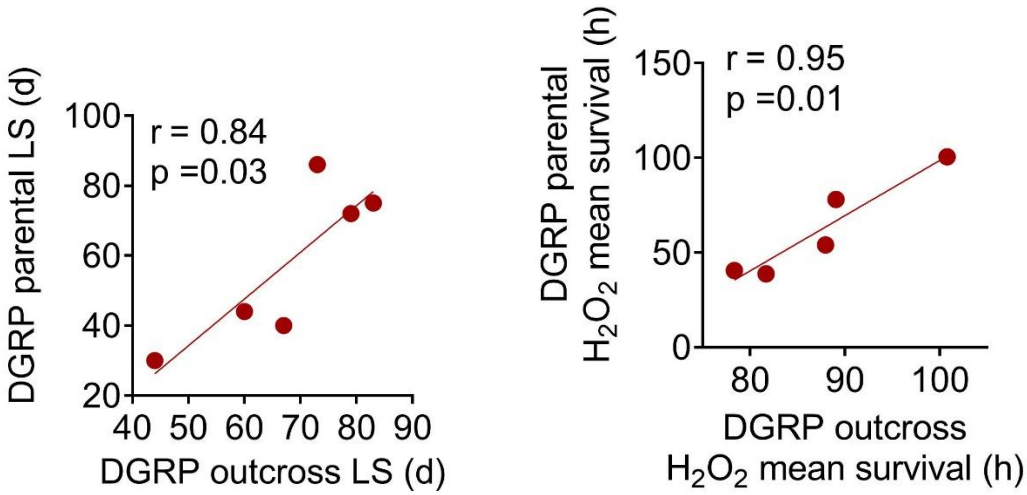
Y-GAL4 represents Da-GAL4 or TH-GAL4, UAS-Z represents GCLC or GCLC RNAi. First, lines containing the GAL4 and UAS constructs of interest were crossed with double-balancer lines (G_0). Next, offspring of those crosses (G_1) were selected for the presence of the transgene along with a specific set of balancers, so that the UAS+ and GAL4+ G_1 flies could be effectively crossed in a way that allows for the selection and generation of G_2 offspring able to self-mate and create a stable double-transgenic population (G_3). With the stable double-transgenic lines created for each desired GAL4+UAS combination, they were then crossed into DGRP backgrounds to assess their impact on lifespan.

An unfortunate occurrence worth noting is that approximately 10 weeks into the aging of the flies, I observed several flies with the curly-wing (CyO) phenotype across the various aging groups. The presence of this balancer indicates that such flies are lacking the UAS construct and therefore not regulating glutathione as desired. I believed that I appropriately selected against CyO+ offspring during the collection stage of the experiment, but this observation proved that was not the case. At that stage of the

experiment, after a majority of the flies had already died, it was impossible to know what percentage of the flies being used were CyO+. This meant that the null hypothesis (that glutathione overexpression or knockdown has no effect) could neither be confirmed nor denied.

Despite the curly-wing complication, analyses of the survival data revealed that male and female flies react differently to glutathione manipulation, but not necessarily in ways that align with my hypothesis (Figure 3). In males, both short- and long-lived flies

Figure S10



Supplemental Figure 10 – Heterozygous DGRP backgrounds maintain relative average lifespans and peroxide vulnerabilities

Data and graphs generated by Alicia Arreola-Bustos and Dr. Judit Pallos. The main three short-lived (356, 727, 911) and three long-lived (335, 379, 821) DGRP lines were crossed with w^{1118} flies to create heterozygous DGRP genetic backgrounds. These heterozygote offspring were then assessed for longevity and peroxide vulnerability, and then such values were compared to the homozygous parental DGRP background values. The loss of homozygosity did not result in a loss of longevity or peroxide vulnerability disparity between the short- and long-lived flies, providing confidence for the use of double-transgenic systems in a heterozygous DGRP format as described in Figure S9. n = 15-20 flies/genotype

with Gclc upregulation showed increased lifespans compared to the w^{1118}-crossed controls, however this same lifespan extension was also seen in the Gclc knockdown flies. In females, the opposite was the case: all double-transgenic crosses, both Gclc upregulation and knockdown, had shorter lifespans than the control crosses. There was also variation of effect between the neuronal and ubiquitous upregulation of Gclc across the lines tested. In some crosses, such as with the male 911 (short-lived) flies, the neuronal Gclc overexpressing flies lived significantly longer than the ubiquitous Gclc overexpressing flies. In others, such as the female 821 (long-lived) flies, the ubiquitous Gclc overexpressing flies lived significantly longer than the neuronal Gclc overexpressing flies. There was no discernable overall correlation between the cell type scope of overexpression and lifespan impact in the crosses tested for this experiment, however the experiment would undoubtedly been strengthened by the use of a ubiquitous GCLC RNAi line. Such a line was unable to be properly generated in time for this experiment, but is in development for future iterations.

Figure 3

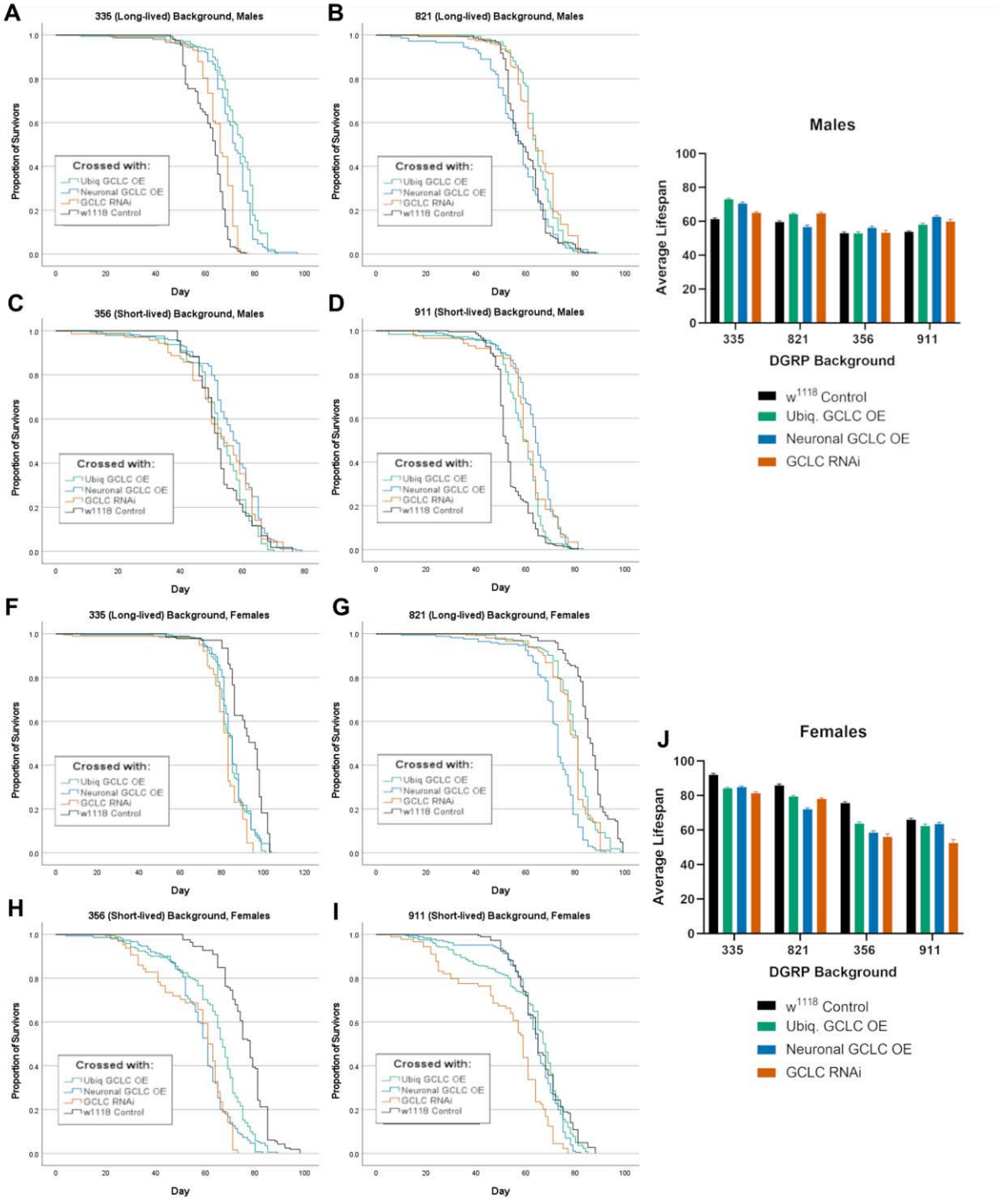


Figure 3: GCLC overexpression and knockdown impact DGRP lifespans in a gender-specific pattern

(Continued on next page)

A-E) Nearly all the male progeny of GCLC overexpression (OE) or RNAi constructs crossed into DGRP backgrounds had longer lifespans than the control DGRP heterozygote flies. The effects of tissue specificity (ubiquitous vs. neuronal drivers) for GCLC OE was inconsistent across the DGRP backgrounds. Chi-square and associated significance values from log-rank pairwise comparison tests within each DGRP background can be found in Supplemental Figure 11. Mean survival, SEM, and total N values can be found in Supplemental Figure 12. **F-J)** In contrast to the male results, all the female progeny of GCLC OE and RNAi lines crossed into DGRP backgrounds had shorter lifespans than the control DGRP heterozygote flies. In two lines, 821 and 356, ubiquitous GCLC overexpression resulted in a longer average lifespan than pan-neuronal overexpression. Chi-square and associated significance values from log-rank pairwise comparison tests within each DGRP background can be found in Supplemental Figure 11. Mean survival, SEM, and total N values can be found in Supplemental Figure 12.

Figure S11

Males

DGRP Background	Transgenic Cross	Ubiq GCLC OE		Neuronal GCLC OE		Neuronal GCLC RNAi		w1118 Control	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
335	Ubiq GCLC OE			9.819	0.002	120.180	< 0.001	188.225	< 0.
	Neuronal GCLC OE	9.819	0.002			73.757	< 0.001	132.291	< 0.
	Neuronal GCLC RNAi	120.180	< 0.001	73.757	< 0.001			37.005	< 0.
	w1118 Control	188.225	< 0.001	132.291	< 0.001	37.005	< 0.001		
821	Ubiq GCLC OE			18.330	< 0.001	3.458	0.063	13.780	< 0.
	Neuronal GCLC OE	18.330	< 0.001			28.128	< 0.001	0.607	0.
	Neuronal GCLC RNAi	3.458	0.063	28.128	< 0.001			23.691	< 0.
	w1118 Control	13.780	< 0.001	0.607	0.436	23.691	< 0.001		
356	Ubiq GCLC OE			13.706	< 0.001	2.348	0.125	0.094	0.
	Neuronal GCLC OE	13.706	< 0.001			1.537	0.215	11.948	0.
	Neuronal GCLC RNAi	2.348	0.125	1.537	0.215			1.246	0.
	w1118 Control	0.094	0.760	11.948	0.001	1.246	0.264		
911	Ubiq GCLC OE			33.096	< 0.001	4.862	0.027	33.772	< 0.
	Neuronal GCLC OE	33.096	< 0.001			1.653	0.199	100.261	< 0.
	Neuronal GCLC RNAi	4.862	0.027	1.653	0.199			37.728	< 0.
	w1118 Control	33.772	< 0.001	100.261	< 0.001	37.728	< 0.001		

Females

DGRP Background	Transgenic Cross	Ubiq GCLC OE		Neuronal GCLC OE		Neuronal GCLC RNAi		w1118 Control	
		Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.	Chi-Square	Sig.
335	Ubiq GCLC OE			1.462	0.227	10.572	< 0.001	78.581	< 0.
	Neuronal GCLC OE	1.462	0.227			16.021	< 0.001	47.383	< 0.
	Neuronal GCLC RNAi	10.572	0.001	16.021	< 0.001			129.518	< 0.
	w1118 Control	78.581	< 0.001	47.383	< 0.001	129.518	< 0.001		
821	Ubiq GCLC OE			58.761	< 0.001	2.414	0.120	40.509	< 0.
	Neuronal GCLC OE	58.761	< 0.001			41.074	< 0.001	155.255	< 0.
	Neuronal GCLC RNAi	2.414	0.120	41.074	< 0.001			53.197	< 0.
	w1118 Control	40.509	< 0.001	155.255	< 0.001	53.197	< 0.001		
356	Ubiq GCLC OE			23.857	< 0.001	26.225	0.000	78.765	< 0.
	Neuronal GCLC OE	23.857	< 0.001			0.529	0.467	168.717	< 0.
	Neuronal GCLC RNAi	26.225	< 0.001	0.529	0.467			160.039	< 0.
	w1118 Control	78.765	< 0.001	168.717	0.000	160.039	< 0.001		
911	Ubiq GCLC OE			5.558	0.018	42.331	< 0.001	1.249	0.
	Neuronal GCLC OE	5.558	0.018			28.248	< 0.001	6.676	0.
	Neuronal GCLC RNAi	42.331	< 0.001	28.248	< 0.001			41.167	< 0.
	w1118 Control	1.249	0.264	6.676	0.010	41.167	< 0.001		

Supplemental Figure 11: Extended pairwise comparison data for the effect of GCLC overexpression or knockdown on DGRP lifespans.

Data was generated in SPSS and transferred into Microsoft Excel for aesthetic formatting. Results are from log-rank (Mantel-Cox) pair-wise comparison tests between the transgenic conditions: ubiquitously expressed GCLC overexpression (Ubiq GCLC OE), neuronally expressed GCLC overexpression (Neuronal GCLC OE), neuronally expressed GCLC knockdown via RNAi (Neuronal GCLC RNAi), and wild-type control cross lacking any transgenic constructs (WT Control).

Figure S12

Males					Females				
DGRP Background	Transgenic Cross	Total N	Mean Survival	SEM	DGRP Background	Transgenic Cross	Total N	Mean Survival	SEM
335	Ubiq GCLC OE	169	73.012	0.727	335	Ubiq GCLC OE	219	84.269	0
	Neuronal GCLC OE	150	70.560	0.816		Neuronal GCLC OE	189	84.905	0
	Neuronal GCLC RNAi	173	65.098	0.591		Neuronal GCLC RNAi	177	81.475	0
	w1118 Control	151	61.457	0.573		w1118 Control	137	92.117	0
	No cross (DGRP LS)		53.99			No cross (DGRP LS)		61.99	
821	Ubiq GCLC OE	159	64.346	0.622	821	Ubiq GCLC OE	163	79.429	0
	Neuronal GCLC OE	145	56.738	1.089		Neuronal GCLC OE	171	72.018	0
	Neuronal GCLC RNAi	155	64.677	0.779		Neuronal GCLC RNAi	152	78.007	0
	w1118 Control	157	59.752	0.731		w1118 Control	124	85.984	0
	No cross (DGRP LS)		44.51			No cross (DGRP LS)		55.14	
356	Ubiq GCLC OE	145	52.993	0.834	356	Ubiq GCLC OE	171	63.684	1
	Neuronal GCLC OE	169	56.320	0.787		Neuronal GCLC OE	150	58.607	1
	Neuronal GCLC RNAi	71	53.254	1.450		Neuronal GCLC RNAi	64	56.109	1
	w1118 Control	112	53.018	0.778		w1118 Control	165	75.788	0
	No cross (DGRP LS)		40.85			No cross (DGRP LS)		35.19	
911	Ubiq GCLC OE	181	58.160	0.790	911	Ubiq GCLC OE	245	62.482	1
	Neuronal GCLC OE	178	62.809	0.771		Neuronal GCLC OE	185	63.638	0
	Neuronal GCLC RNAi	87	59.874	1.320		Neuronal GCLC RNAi	89	52.584	1
	w1118 Control	222	53.901	0.496		w1118 Control	185	66.151	0
	No cross (DGRP LS)		40.41			No cross (DGRP LS)		21.62	

Supplemental Figure 12 – Summary data for the glutathione double-transgenic experiment

Additional data from the glutathione double-transgenic experiment is presented here, including total number of flies counted, mean survival, and standard error of the survival mean for each cross. The DGRP average lifespan for each sex is also provided with the “No cross” heading (data generated previously in our lab).

Chapter 4: Discussion

A common saying that has been used innumerable times the world over is that "age is just a number." Unfortunately, from a biological standpoint, that statement could not be further from the truth. Aging is one of the most complex biological phenomena to study, but in using the DGRP as a sample population, I have been able to investigate how antioxidation may play a role in Drosophila aging. Such efforts have generated novel results and insights that contribute to the ever-growing and ever-important field of aging research

One of the first experiments I conducted – using respirometry to measure and compare mitochondrial function in older short- and long-lived DGRP flies – revealed that peroxide production rates were negatively correlated with lifespan, meaning that the mitochondria of shorter-lived flies generated more oxidative stress than longer-lived flies at the aged 4-week timepoint used. When this experiment was simplified and performed later in our lab to validate the results, it was found that the significant difference in peroxide levels only exists for aged flies and not young flies, a distinction that would be seen throughout my experiments. Nevertheless, the results showing that lifespan being correlated to peroxide levels encouraged me to begin investigating oxidative stress and antioxidation differences that may be contributing to lifespan variance in our DGRP model. This investigation would soon focus on a single key antioxidant: glutathione.

Our original data on glutathione abundance in DGRP fly heads came from a metabolomics experiment that we ran in partnership with the Promislow lab at the University of Washington. In this experiment, we sent them the heads (due to our lab's PD-motivated interest in CNS phenotypes) of 4-week-old female flies from three short-

lived and three long-lived DGRP lines for analysis. In the end, we were able to determine the levels of over 100 metabolites across the six tested lines, spanning many critical biochemical pathways, and were able to see which ones were correlated with lifespan. One such metabolite was glutathione, because both the reduced and oxidized versions of this metabolite were lower in the short-lived flies compared to the long-lived flies. These data, in conjunction with the previous data highlighting oxidative stress differences between the short- and long-lived DGRP lines, provided me a strong candidate for more targeted investigation.

To assess the correlation of glutathione levels with lifespan in an extended sample, I decided to measure glutathione in the fly heads of 13 DGRP at 1-week, 2-weeks, and 4-weeks post-eclosion to see not just whether this antioxidant's presence correlates with line lifespan, but also to see how those levels change over time. That experimental design proved useful when my analysis showed that glutathione levels were significantly correlated with lifespan, but only at the one- and two-week-old timepoints; no significant correlation was seen with four-week-old flies. This confirms the four-week-old data from the metabolomics experiments and expands the scope with earlier timepoints, highlighting that glutathione levels change over time and that the critical antioxidant differences might only be apparent early in the fly lifespan.

The age-dependent nature of the glutathione differences connects with our lab's previous peroxide experiment in intriguing ways: in that experiment, the peroxide differences were not seen in younger flies (less than a week old), while here I observed that glutathione differences were not seen in flies older than two weeks. Perhaps the short-lived DGRP lifespans are driven by a progressive oxidative stress phenotype that

begins with glutathione impairment or inhibition in youth, consequently followed by increased peroxide production or impaired peroxide neutralization later in adulthood that results in reduced lifespan overall (Figure 4). While required timepoints and sample sizes in order to further test this hypothesis is beyond the scope of this thesis, the underlying theory of a stepwise biological mechanism explaining, at least in part, the variation in lifespan between the short- and long-lived DGRP flies is worth additional consideration.

Figure 4

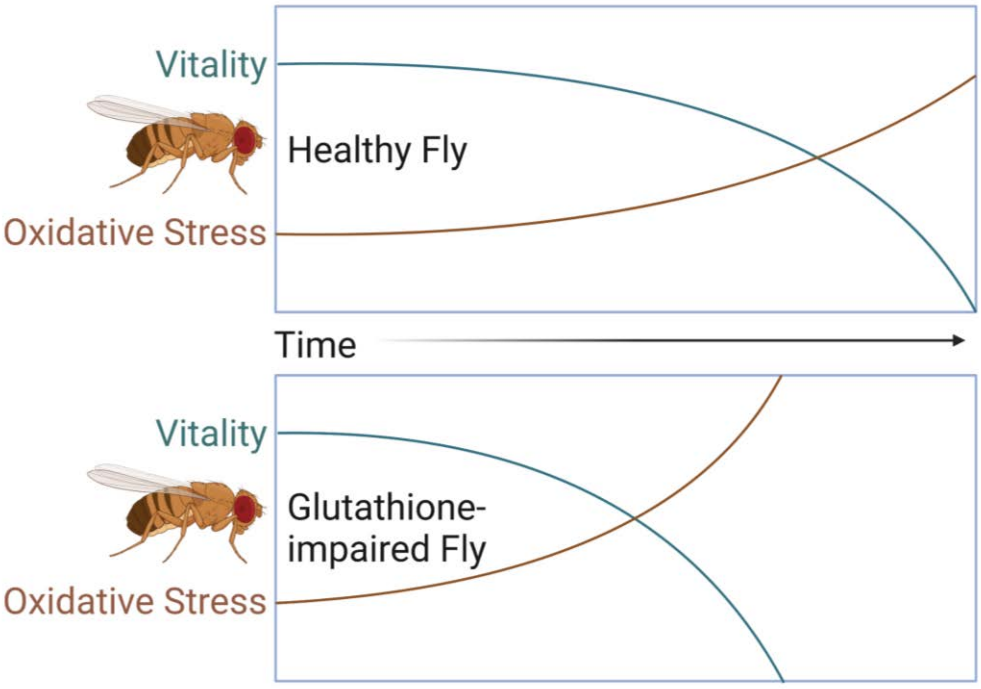


Figure 4: Proposed two-stage process for glutathione impairment-induced lifespan reduction

Made in BioRender. Healthy flies with fully-functional glutathione synthesis and regulation pathways are able to cope with naturally-produced oxidative stress over time until inevitable accumulations of dysfunctions reduce such capacity, resulting in increasing oxidative stress leading to eventual death. In glutathione-impaired flies, such decline begins much earlier in life, with oxidative stress ramping up more quickly without a functional glutathione antioxidation system and resulting in a quicker death and shorter lifespan.

After the extensive amount of *observation* conducted in the previous experiments, at this point I was eager to engage in some *manipulation*, in particular genetic manipulation of glutathione synthesis. I was able to do so utilizing the Drosophila GAL4/UAS system to induce targeted expression of either glutamate-cysteine ligase (GCL, the rate-limiting enzyme for glutathione synthesis), or RNAi against GCL. My hypothesis for all such experiments was that glutathione synthesis upregulation would lead to increased lifespans in the affected DGRP flies, with the greatest increases seen in the short-lived lines as they are rescued from the glutathione impairments contributing to their short-lived nature. Conversely, I also hypothesized that glutathione synthesis inhibition would lead to decreased lifespans, with affected long-lived DGRP flies being "rescued" from their glutathione-mediated longer lifespans.

After generating the double-transgenic GAL4-UAS lines that would allow upregulation or inhibition of glutathione synthesis either ubiquitously or pan-neuronally, I crossed those double-transgenic systems into the DGRP backgrounds and measured the lifespans of the offspring. Previous work in our lab showed that heterozygous DGRP genetic backgrounds still maintained their short- and long-lived natures, complete with lifespan-correlated peroxide vulnerabilities, which gave me confidence in the biological merits of this experimental design.

The takeaways from the data are complex, to say the least, with the most striking observation being the sex-specific lifespan changes. All double-transgenic female flies lived shorter lifespans than their control counterparts – DGRPs crossed w^{1118} control flies to isolate any heterozygosity effects – regardless of whether they were upregulating or inhibiting GCL. A mirrored effect was seen in the males, where all

double-transgenic male flies lived longer than the controls. This stark sex-specific difference echoes both previous data I've collected as well as what has been shown in the clinical literature: in my experiments measuring glutathione levels in the fly heads, female DGRP flies consistently had higher levels of glutathione compared to their male equivalents. This might influence how most DGRP female flies live longer than their male counterparts (Figure S10). Clinically, it has been reported that human males are twice as likely as females to develop PD[55], and a preterm infant study found significantly higher levels of key glutathione enzymes glutathione reductase (GR) and peroxidase (GPX) in newborn girls than matched newborn boys, although interestingly their glutathione levels were similar[56]. It is clear that there is some critical sex-specific differences involved in the glutathione pathway and are important for awareness and investigation with any future studies involving glutathione.

The other element that stuck out from the data was that the effect tissue-specific GCL upregulation was inconsistent from line to line, and sometimes from male to female within the same line. These discrepancies may very well be due to the curly-wing error, described in more detail in the Results section but in short: curly wings, a balancer phenotype that were supposed to be selected against since they indicated a lack of the UAS construct, were detected in some of the flies very late into the experiment. This called into question how many flies being measured were unintentionally lacking the full GAL4-UAS double transgene. However, if we assume that a relatively equal number of CyO+ flies ended up in each of the experimental groups, that error would not explain how certain lines had such dramatic differences between their ubiquitous and pan-neuronal overexpression lifespans, while in other lines those groups had similar

lifespans. In both male and female 821 flies, a long-lived line, the ubiquitously-expressing GCL flies lived significantly longer that then pan-neuronal expression flies; in fact, the GCL knockdown flies had similar lifespans to the ubiquitous flies, raising even more questions.

While I based my confidence in the RNAi and overexpression constructs on the previous work in which they were used successfully, due diligence and the puzzling results from this experiment warrant more definitive data showing their efficacy. This would require performing qRT-PCRs on the overexpressor flies to ensure that they are expressing GCL genes at a higher level than their control counterparts, and qRT-PCRs on the knockdown lines to confirm the efficacy of the RNAi. Western blots could also be done on all experimental lines to measure the effect of such manipulations on glutathione levels to further confirm the validity of the experimental design, or lead to its reassessment.

The future directions of this work are diverse, as the implications of glutathione activity potentially influencing aging are vast and the required scientific steps to explore, validate, and take advantage of such phenomena are equally as extensive. First, generating and utilizing a larger suite of GCL double-transgenics would considerably increase our understanding of how GCL upregulation and knockdown impacts lifespan within DGRP contexts. With data from novel crosses utilizing GCL manipulation in dopamine neurons using a TH driver, as well as additional quality control ensuring that all flies tested do in fact contain the genetic regulation construct, we would be able to more accurately hone in on where glutathione impairment is most impactful and adjust our follow-up experiments accordingly. Additionally, measuring the dopamine neuron

viability of the double-transgenic lines crossed into the DGRP backgrounds at the key aging timepoints will also yield valuable insights into glutathione's impact on neuronal health. By observing how such manipulations potentially rescue the neurodegenerative phenotypes seen in the short-lived DGRP lines, we could draw more direct connections between the antioxidant and it's relevance in aging-related diseases like PD.

An additional pilot experiment I conducted that illustrates another future direction for this work involves comparing the oxidative stress environments of CNS cells between short- and long-lived DGRP flies using dihydroethidium (DHE). DHE is a membrane-permeable reagent that can be used to visualize oxidative stress present within cells with fluorescence microscopy. DHE reacts with superoxide anions to form 2-hydroxyethidium, a red fluorescent protein. The intensity of fluorescence can be used to quantify cellular oxidative stress and when combined with additional staining to identify specific cell types, DHE can be an effective way to compare tissues-specific changes in oxidative stress. Since I was most interested in the redox state – a general term for the homeostatic balance of oxidants and antioxidants – of dopamine neurons in those flies, I used a dopamine neuron reporter line (UAS-GFP-nls; TH-GAL4) to label those neurons with green fluorescence, which could then be used as a filter to measure just the DHE-created fluorescence within those cells. For this pilot experiment, I crossed this reporter line to one long-lived DGRP line (821) and one short-lived DGRP line (911), performing the DHE staining on the brains of the one-week-old offspring.

In total, I was able to successfully stain and image using a confocal microscope three long-lived 821 brains and four short-lived 911 brains. I decided to look specifically at the PPL1 neuron clusters as they were easiest to identify and simplified the analysis.

No significant difference in DHE fluorescent intensity was observed between the 821 and 911 brains, indicating that the oxidative stress levels within their PPL1 dopamine neurons were similar at this measured 1-week-old timepoint (Figure 5). This lack of difference may be due to the underpowered nature of the pilot experiment, but it may also fall in line with the previously mentioned theory of delayed oxidative stress impacts: if glutathione impairments early in life take time to result in measurable oxidative stress phenotypes later in life, then that would explain why these short- and long-lived brains have similar oxidative stress profiles early in life. While the limited strength of my experiment prevented the formation of any solid data-based conclusions, the effort was worthwhile and validated the protocol's future use. We plan on incorporating later time points and additional DGRP lines with more brain samples analyzed to boost the power of our experiment. Generating a comprehensive picture of redox changes in the fly brain over aging could provide fascinating insights into the oxidative stress landscape affecting these fragile neurons.

Figure 5

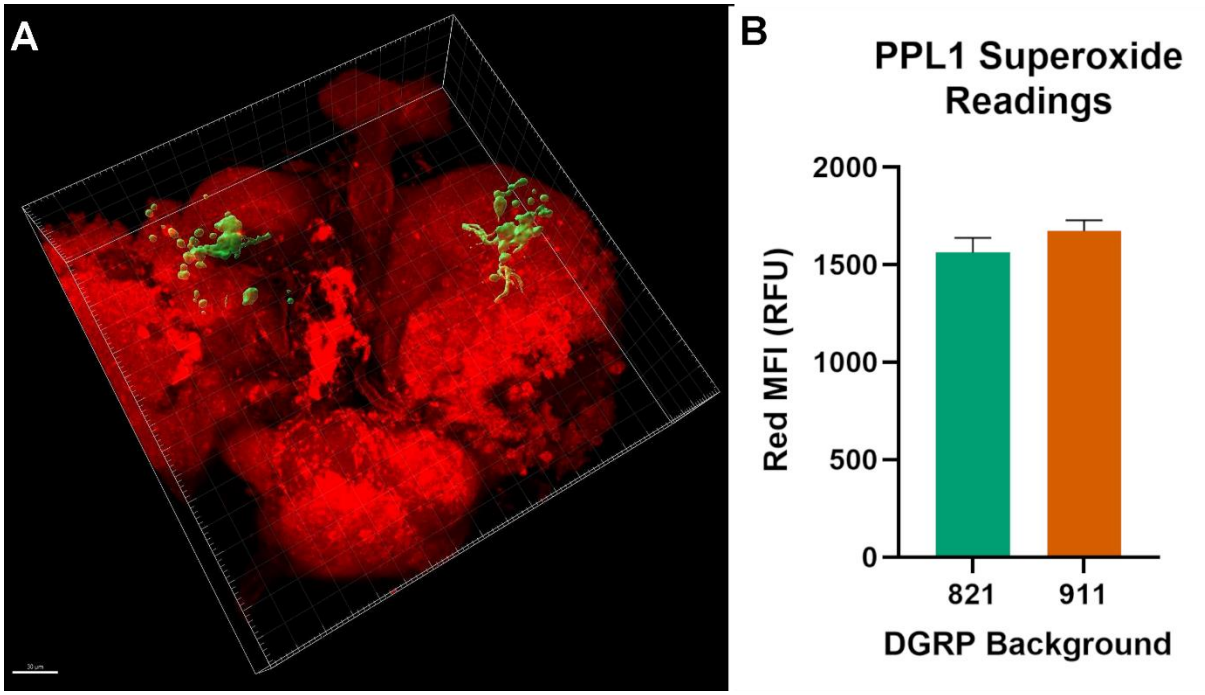


Figure 5: Pilot experiment for visualizing oxidative stress differences in DGRP fly brains using DHE

A) A representative image of a Drosophila brain stained for superoxide using DHE (red) and the PPL1 dopamine neuron clusters created into 3D surfaces (green) for measuring mean fluorescence intensity (MFI) within those neurons. Scale bar = 30um. **B)** MFI data was measured and averaged for the PPL1 dopamine neurons of three long-lived 821 fly brains and four short-lived 911 fly brains. No significance difference of DHE MFI was seen between the 821 and 911 fly brains at one week post-eclosion. Mean and standard error of the mean shown.

Summary and Conclusions

The 19th century English novelist Samuel Butler once wrote that “Life is like music, it must be composed by ear, feeling, and instinct, not by rule.”[57] In a similar vein, studying aging is like understanding an orchestra, albeit one with an unknowable number of musicians and whose composition may vary from species to species. In this thesis, I have decided to focus on the antioxidation section in this orchestra of aging with the goal of gaining insight into glutathione’s potentially instrumental role. Through my experiments, I have determined that glutathione levels correlate with lifespan in our DGRP fly model, however such correlations drop off as the flies get older. I have also observed that in older flies, there is a correlation between lifespan and hydrogen peroxide generation, a correlation that does not exist in younger flies. This led to the proposition that a stepwise oxidative stress progression may be contributing to the short-lived flies reduced lifespan. Such progression begins with glutathione impairments early in life, followed by increased oxidative stress generation that appears later in life. While there were several issues with the experiment, when glutathione synthesis was manipulated in the DGRP genetic backgrounds using GAL4-UAS constructs, additional sex-specific differences were seen that add further nuance to discussion of antioxidation state contributing to lifespan determination. Finally, imaging and quantifying oxidative stress in young DGRP fly brains using DHE yielded promise for the protocol’s ability to uncover more insights into the oxidative environments of neurons as they age in these short- and long-lived flies. My hope is that the work described in this thesis will resonate with future aging and PD-related research in the field, providing some notes of insight into glutathione’s role in this grand symphony.

Appendix

In addition to the work described above, in the past two years I worked with my mentor Dr. Ian Martin to write a review article titled “Unraveling Parkinson’s Disease Neurodegeneration: Does Aging Hold the Clues?” This manuscript has been submitted to the Journal of Parkinson’s Disease and is currently under review. The following represents the sections that I wrote and contributed to the article, with editing by Dr. Martin.

1. Aging is the biggest risk factor for PD

Development and progression of Parkinsonism, the movement disorder of PD, is driven primarily by the degeneration of dopaminergic neurons within the substantia nigra (SN)[9,10] . Established contributors to this progressive neurodegeneration include mitochondrial dysfunction[58] , oxidative stress[59], inflammation[60], and the misfolding and aggregation of α-synuclein into protein deposits such as Lewy bodies[61].

It is now clear that aging is the greatest risk factor for developing PD[5-7]. While this connection had long been presumed, detailed studies characterizing the aging-PD link emerged in the 1980’s and 1990’s. One of the most influential studies from that time was the Rotterdam Study, a door-to-door population-based survey of nearly 7000 persons of 55 years of age or older living in a Netherlands suburb[62]. The authors reported quantitative evidence of the connection between aging and PD, with prevalence in males and females combined increasing from 0.3% in those aged 55-64 to 1.0% in those aged 65-74, 3.1% in those aged 75-84, and 4.3% in those aged 85-94. Census studies like the Rotterdam study were conducted in several other countries as well, including Italy[63], Greece[64], Spain[65], France[66], China[67], Taiwan[68], and USA[69], all of

which reported similar age-associated increases in PD prevalence. These studies consistently confirmed the close association of aging with PD development.

Besides aging, PD has been associated with genetic risk factors including highly penetrant familial PD gene mutations and common risk variants, as well as environmental risk factors such as pesticide exposure[70]. Epidemiological evidence indicates that exposure to the pesticides paraquat or rotenone increases PD risk 2-3 fold due to the neurotoxic action of these hazardous materials[71]. However, the reported increase in PD risk associated with known environmental factors pales in comparison to that of aging. Recent analyses of older populations have observed that PD incidence increases approximately 100-fold when comparing people aged 45-49 to those aged 85-89[72].

Here, to examine the aging-PD link further, we will first assess points of convergence in the hallmarks of CNS aging and PD that constitute abundant correlative evidence linking the two. We will then discuss how metabolism has emerged as a potential nexus connecting aging to PD neurodegeneration, and evaluate evidence that altered expression of lifespan-regulating metabolic genes influences PD-related phenotype development, potentially demonstrating a causal link between aging and PD.

2. Aging and PD: Points of convergence

The process of aging is highly complex and spans many integral biological pathways and systems within organisms. While the details differ across species, the underlying phenomenon is always the same: a progressive accumulation of molecular and cellular dysfunction that ultimately results in the breakdown of tissues necessary to sustain life. Several major molecular hallmarks of brain aging overlap with mechanisms

implicated in PD neurodegeneration, including oxidative damage and mitochondrial dysfunction, loss of protein homeostasis, neuroinflammation, genomic instability and impaired stress responses. Accumulating age-related dysfunction in these areas likely renders neurons vulnerable to PD-associated environmental and genetic factors that affect the same processes, thus compounding dysfunction and promoting α-synucelin pathology. The convergence of aging hallmarks with PD is consistent with aging being the predominant risk factor for disease, and to an extent, PD-related neurodegeneration resembles an exacerbated form of aging. It is important to note, however, that the degree to which these functional deficits are actually causal in the physiological declines of aging and neurodegeneration of PD has not yet been resolved. Yet, compelling evidence from cell and animal disease models supports convergent roles for (i) mitochondrial dysfunction and oxidative stress; (ii) loss of protein homeostasis and protein aggregate formation; and (iii) chronic inflammation in aging and PD. Similarities between aging and PD for each of these areas are discussed below.

2.1 Mitochondrial dysfunction and oxidative stress

Mitochondrial oxidative phosphorylation relies on the electron transport chain (ETC) utilizing oxygen molecules for their electron accepting potential. This use of oxygen creates the opportunity for the formation of superoxide ($O_2^{\cdot -}$) radicals that can give rise to other reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and reactive nitrogen species (RNS) such as peroxynitrite ($ONOO^-$). While recent research has supported that these ROS or RNS molecules may have essential signaling functions within the cell[19-21], their high biochemical reactivities can also cause disruption through oxidative or nitrosative stress, respectively[22]. Mitochondria harboring

dysfunctional ETC complex component(s) have increased potential for generating $O_2^{\cdot-}$ (produced predominantly by complexes I and III) which can elevate overall oxidative stress in the cell.

The accumulation of oxidative stress has been implicated in the normal processes of aging. In fact, the oxidative stress theory of aging was one of the most popular until recent evidence has begun to cast doubt on its validity[14,22,73]. Originally conceived as the mitochondrial free radical theory of aging, the oxidative stress theory of aging posits that the growing presence of reactive species within the cell derived from both endogenous (e.g. mitochondrial) and exogenous sources causes structural damage to macromolecules including lipids, proteins, and nucleic acids. Since these ROS/RNS could target the mitochondria themselves, a putative feed-forward cycle of mitochondrial dysfunction and ROS/RNS generation has been proposed to account for the progressive nature of cellular aging. The widely-held hypothesis that oxidative damage alone is sufficient to account for functional losses associated with aging has recently been challenged[23]. An alternative hypothesis posits that an age-related shift toward a pro-oxidizing cellular redox state via mitochondrial ROS production leads to a disruption of redox-regulated signaling pathways that in turn promotes cell senescence and death[23].

Mitochondrial dysfunction has been implicated in PD for over thirty years, initially arising from studies examining the effects of exposure to MPTP[74,75] and certain pesticides such as rotenone and paraquat that selectively inhibit mitochondrial ETC complex I[76,77]. This evidence was supported by the identification of reduced complex I levels in PD patient dopamine neurons[78] and more recently reinforced by studies on

familial PD-linked genes and from the PD-like phenotypes arising from genetic deletion of a catalytic ETC complex I subunit[79], thus establishing mitochondrial dysfunction and bioenergetic failure as a primary candidate mechanism for PD development.

Familial PD genes and mitochondrial dysfunction

Investigations into highly penetrant mutations in genes including *LRRK2*, *Parkin*, *PINK1*, *SNCA*, and *DJ-1* have revealed a robust association of neurodegeneration with markers of mitochondrial damage, dysfunction and oxidative stress in genetic disease models, marking a clear parallel with mitochondrial hallmarks of aging (Table). *Parkin* encodes an E3-ubiquitin ligase that can monoubiquitinate and polyubiquitinate a number of cellular substrates and PD-associated *Parkin* mutations are generally thought to result in a loss of function via one of several mechanisms[80-82]. PINK1 is a mitochondria-targeted kinase whose functional silencing has also been linked to PD. Recent work has shed light on a coordinated role for PINK1 and Parkin in regulating mitochondrial quality control via mitophagy and in mitochondrial biogenesis via transcriptional control of PGC-1α[83]. Quality control deficits occurring through loss of Parkin or PINK1 function enables dysfunctional mitochondrial to remain within the overall mitochondrial pool thus promoting ROS generation and bioenergetic deficits[84,85] reminiscent of aging cells.

Mutations in the leucine-rich repeat kinase 2 (*LRRK2*) gene cause late-onset PD (>60 years-old) consistent with neurodegeneration occurring following a major intersection with aging. The common LRRK2 G2019S mutation increases LRRK2 kinase activity which appears to be a key route to LRRK2-induced neurotoxicity. Cells

expressing pathogenic LRRK2 mutations exhibit mitophagy deficits through unclear mechanisms[83,86,87], hence impaired clearance of damaged mitochondria may be a common feature of multiple familial PD genetic mutations. Pharmacologically inhibiting LRRK2 kinase activity restores mitophagy, thus linking aberrant LRRK2 kinase activity to altered mitochondrial homeostasis[83,86]. iPSC-derived neural cells from PD patients carrying LRRK2 G2019S mutations or at-risk individuals harboring the R1441C mutation display elevated levels of mtDNA damage that can be blocked by correcting the gene mutation[88]. LRRK2 G2019S was also reported to hyper-phosphorylate the essential H_2O_2 scavenger peroxiredoxin 3 (PRDX3) in *Drosophila* brains, reducing overall peroxide activity and resulting in skeletal muscle mitochondrial degeneration and dopaminergic neurodegeneration that could be reversed by PRDX3 overexpression or treatment with the peroxidase mimic ebselen[89]. Lastly, LRRK2 G2019S overexpression can induce mitochondrial fragmentation in cultured neurons, severely impairing their energetic homeostasis and increasing ROS levels[90].

Further intersection between aging and PD within the context of mitochondrial health can be found in studies of α-synuclein and DJ-1. Point mutations and common variants in *SNCA*, the gene encoding for α-synuclein are linked to familial and sporadic forms of PD, respectively[91,92]. α-synuclein is a small, natively unfolded, cytosolic protein that has been observed to cause mitochondrial fragmentation in DA neurons *in vivo* that may be dependent on its binding to and disruption of mitochondrial membranes[93,94]. These effects were reported to occur independently of the mitochondrial fission protein Drp1[93], although in *Drosophila*, α-synuclein overexpression led to disruption of the spectrin cytoskeleton resulting in actin-mediated Drp1 mislocalization and aberrant

mitochondrial dynamics[95]. In transgenic mice expressing the PD-linked α-synuclein A53T mutation, degenerating mitochondria were observed harboring significant mitochondrial DNA (mtDNA) damage[96]. DJ-1 is a ubiquitous and highly-conserved protein critical for cellular responses to oxidative stress, where upon it becomes relocalized to mitochondria[97]. While the specific mechanism of action is still obscure, loss of DJ-1 results in mitochondrial fragmentation in a human dopaminergic cell line[98]. Specific pathogenic mutations in DJ-1 have been associated with autosomal recessive early-onset Parkinsonism, likely due to a lack of protection from oxidative stress-induced cytotoxicity[99-101]. In mice, overexpression of wild-type DJ-1 has conferred resistance to MPTP, a mitochondrial complex 1 inhibitor that can cause a PD-like clinical syndrome with dopaminergic neurodegeneration[102]. Conversely, mice lacking DJ-1 show increased susceptibility to MPTP toxicity and display nigrostriatal dopaminergic deficits[103,104]. Despite its effectiveness at neutralizing oxidative stress threats in the cell, over time DJ-1 undergoes oxidative modifications that impair its functionality in the cell[105]. One study found that the percentage of modified DJ-1 in fly brains increased from 0.8% in 1-day-old flies to 17.9% in 40-day-old flies; similar significant age-related changes were also observed in mouse and human brain samples[106]. Therefore, both aging-related modification and PD-associated mutations have the potential to impair DJ-1 functionality and lead to the development of PD.

POLG and mtDNA mutations

Aging cells accumulate mtDNA mutations[107], potentially via oxidative damage and clonal expansion of mtDNA replication errors that occur early in life[108]. Deficiencies

in the *POLG* gene, encoding mtDNA polymerase gamma results in accelerated parameters of aging and reduced lifespan in mice accompanied by mutations in mtDNA[109,110]. Likewise, *POLG1* mutations are associated with PD in humans[111,112] and *POLG* mutator mice that express a proofreading-deficient variant of POLG exhibit comparable mtDNA mutations in SNpc neurons by 1 year of age to that of aged human SNpc[113-115]. Despite the manifestation of brain metabolic and neurotransmitter abnormalities[116], these mice do not develop overt dopaminergic neurodegeneration[113,117], even when challenged with loss-of-function in the familial PD gene DJ-1[117] or the PD-associated toxin MPTP[118]. Closer examination indicates that mtDNA deletions in these mice trigger a neuroprotective response within DA neurons that may stave off neuronal death[113]. This does not appear to be the case in flies, however, where expression of proofreading-deficient mtDNA polymerase leads to somatic mtDNA mutations, mitochondrial dysfunction and PD-related phenotypes such as dopamine neuron loss and locomotor dysfunction[119].

2.2 Loss of protein homeostasis and protein aggregate formation

Protein homeostasis is achieved upon the balance and fidelity of protein synthesis, folding, and degradation. Impaired proteolysis and the accumulation of misfolded protein aggregates, implicated in both aging and PD etiology, can cause significant stress on cell resources and ability to function. The aggregation of specific proteins are directly linked to aging-related diseases such Aβ-containing amyloid plaques in Alzheimer's disease[120] and α-synuclein-containing Lewy bodies in Parkinson's disease[61]. While it still remains to be conclusively determined if these

protein aggregates are the cause of their respective disease's clinical progression, it is clear that such a phenomenon can be promoted by the aging process.

Healthy cells produce vast amounts of proteins on a daily basis to maintain homeostasis amid constant protein turnover. Errors in translation or folding can render proteins dysfunctional or actively disruptive via toxic gain of function. These misfolded proteins are dealt with by molecular chaperones and degradation machinery within the cell to prevent damage and stress. As cells age, however, these proteome-stabilizing elements lose effectiveness which results in a growing loss of protein homeostasis[121,122]. Additionally, levels of protein oxidation and nitrosylation, modifications that can impair protein folding and increase aggregation potential, increase over aging and add additional pressure to the proteostasis machinery[123,124]. Given enough time and accumulation, aging cells may reach their threshold of proteome stress tolerance leading to cell death[125,126].

While protein aggregation is commonly associated with age-related disease, recent studies have shown that the phenomenon occurs during normal aging as well. In C. elegans, it was shown that hundreds of proteins become insoluble upon aging, spanning a wide range of biological systems including proteostasis itself[127]. Other studies in C. elegans as well as cultured mammalian cells suggests that osmotic stress results in the formation of age-related unstable polyglutamine (polyQ)-repeat-containing proteins that have the potential to self-aggregate, without any mutation needed[128,129]. These data led to even further work that found that for a large portion of these unstable polyQ proteins, the increased aggregation potential is conferred by aging-related post-translational carbonyl modification[130,131].

α-synuclein point mutations or gene multiplications linked to PD promote the formation of oligomeric and higher-order aggregates within neurons[132-134]. Interestingly, brainstem Lewy Bodies and Lewy Neurites have been repeatedly documented in a small subset (~10%) of aged individuals without PD[135], consistent with a baseline degree of synuclein aggregation during normal aging. Haploinsufficiency of *GBA1,* the gene that encodes for glucocerebrosidase (GCase), is one of the most common genetic risk factors for PD[136-138]. GCase is a lysosomal hydrolase critical for the metabolism of glycosphingolipids, and recent studies have suggested that its impaired activity can contribute to increased aggregation of α-synuclein and the formation of Lewy bodies within the cell[139-141]. This impairment can be caused by PD-associated *GBA1* mutations, but may also appear as a natural result of aging: In wild-type mice, levels of GCase activity in the brain were found to decrease with aging and were correlated with an increase in glycosphingolipids[139]. Patients with a PD-linked *GBA1* mutation exhibit earlier age of onset and a more rapid disease progression compared to non-carrier PD patients[142,143].

Chaperones assist in intracellular protein folding by providing the space and protection for proteins to undergo the conformational changes needed to achieve the proper configuration and functionality. Chaperones are also able to recognize misfolded proteins that are beyond repair and target them for degradation, a process known as chaperone-mediated autophagy (CMA)[144]. Several PD-related mutations interfere with CMA, likely contributing to the disease pathology. The disease-associated mutants of alpha-synuclein and LRRK2, while recognized by chaperones and marked for CMA, fail to be fully degraded by the lysosomes due to their mutation-derived association with

lysosomal-associated membrane protein 2a (Lamp2a)[145]. Even more interestingly, this degradation failure instead results in mutant alpha-synuclein accumulating at lysosomal surfaces, promoting its subsequent aggregation and further impairing the overall CMA proteostasis of the cell[146]. Overexpression of Lamp2a was found to be neuroprotective against alpha-synuclein aggregation, as CMA function was maintained[147]. Taken together, multiple lines of evidence indicate that the ability of a cell to maintain proper protein folding and correct for misfolding is impaired similarly with old age and with PD-associated mutations, illustrating the convergence between the two and highlighting the relevance for investigation.

2.3 Chronic inflammation

A chronic low-grade inflammation associated with aging, dubbed “inflammaging” may result from elevated innate immune system triggers such as pro-inflammatory damaged or dysfunctional cells[148]. Aging also increases the likelihood of developing conditions that promote chronic inflammation, such as obesity, cardiovascular disease, and diabetes[149]. A major biochemical root of aging-induced chronic inflammation has to do with immune response resolution. In the prime of adult life, the human immune system can clear out threats efficiently while also ramping down the inflammatory response to reduce unnecessary stress on the body. With aging, however, inflammatory triggers from damaged and dying cells are chronically elevated resulting in prolonged inflammation with increased collateral damage to the body, damage that can leave the immune system hypervigilant and overreactive to future insults[148].

The role of inflammation in PD has been extensively studied since the connection was first posited several decades ago and has recently garnered even greater attention as a causative factor with the proposed influence of intestinal inflammation on disease development via a putative gut-brain transmission of α-synuclein[150]. PD genetic studies support a role for inflammation, at the level of penetrant familial PD genes and low-risk common variants. GWAS studies have linked late-onset PD with variants at the *HLA* (*human leukocyte antigen*) locus[151,152] and several of the familial PD genes discussed above are expressed in immune cells and involved in their function[153], particularly *LRRK2*. LRRK2 is highly expressed in cells of the innate immune system and is elevated in the peripheral immune cells of idiopathic PD patients and in inflamed colonic tissue of Crohn's disease patients[154]. Inflammatory triggers such as lipopolysaccharide or IFN-γ induce LRRK2 expression in several immune cells[155-157] implicating its role in inflammation. In the CNS, LRRK2 is highly expressed in microglia[155] and gain-of-function mutations found in PD such as G2019S may promote hyperactive or inappropriate microglia-mediated inflammation[158,159]. When activated, microglia upregulate a variety of receptors and pro-inflammatory cytokines that stimulate the immune response and associated phagocytosis[160]. This is beneficial for the clearance of dead cells or debris, yet trouble arises when the microglia remain in an activated state for too long, as their activity also produces ROS that can damage neurons[60]. Evidence of activated microglia has been observed both in the aging brain[161,162] and in the brain or CSF of PD patients[163,164] characterized by upregulation of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα, implying chronic innate immune activation. Additionally, microglia in both aging mammals and PD display an active (deramified)

morphology[165,166], increased phagocytic activity[166,167] and inflammatory marker levels[168-171]. In PD, they are found in post-mortem brain surrounding degenerating dopaminergic neurons[172] consistent with a potential role in neurodegeneration. While there is compelling evidence supporting microglial-induced inflammation in the brains of PD patients and aging mammals, it remains to be determined whether this is causal vs. consequence of age-related cognitive changes or PD onset and progression.

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