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Impacts of dietary restriction on a Drosophila model of Werner syndrome

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Impacts of Dietary Restriction on a Drosophila Model of Werner Syndrome

By

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Abstract

Werner syndrome (WS) is an autosomal recessive disorder that results in premature aging and occurs in 1 in 1,000,0000 to 1 in 10,000,000 people. In humans, WS is the result of mutations that render the WRN gene, that contains a helicase and an exonuclease domain, non-functional. Currently, there is no cure for WS in humans, making dietary and lifestyle interventions attractive for increasing the quality and longevity of lives. Diet restriction (DR) has been shown to extend the lifespan of several model organisms, including *Drosophila melanogaster*, making it a strong candidate for WS treatment. In this thesis, mutant flies for the gene *WRNexo*, homologous to the exonuclease domain of WRN in humans, were used to examine the effect of DR on lifespan and the DR mediated physiological, and behavioral characteristics of starvation, oxidative stress, and sleep/activity. Serial yeast dilution medias (1%, 5%, and 20% yeast w/v), were used to evaluate the effects of DR on WS lifespan and physiological and behavior characteristics. Surprisingly, it was found that DR did not extend the lifespan of WS flies and even had a detrimental effect in females, but early life yeast supplementation was shown to partially rescue the early life mortality observed. DR was also observed to have a sex-dependent effect on WS flies that results in a reduced resistance to both starvation and oxidative stress while creating more sleep/activity disruption. Deleterious effects of DR on lifespan, physiological, and behavioral characteristics, suggest that the WRN protein is necessary for longevity benefits traditionally observed in DR as well as the WRN protein having a function in metabolic control. Future studies could identify and evaluate the mechanisms that control the effects of DR on the $WRNexo^{\Delta}$ mutants.

Lay Summary

Werner syndrome is a rare genetic disease that results in the premature death of the patients. This genetic disease is caused by mutations in the WRN gene that cause the gene to be nonfunctional. Currently there is no cure for this disease in humans, which makes dietary and lifestyle interventions attractive options for increasing life expectancy. Dietary restriction is a process where calories or nutrients are limited in the diet and has been shown to reverse the signs of aging and extend lifespan in several animal models including flies.

In this thesis, a fly model of Werner syndrome, *WRNexo*$^{\Delta}$, was used to evaluate the effects of dietary restriction on Werner syndrome. It was initially thought that dietary restriction could be a possible lifestyle intervention for patients, but DR was not found to increase the lifespan of the Werner syndrome model flies and even had a negative effect on the lifespan. Additionally, dietary restriction mediated physiological characteristics such as starvation, oxidative stress, and sleep/locomotion showed disruption in Werner syndrome model flies. Dietary restriction negatively impacting Werner syndrome model flies indicates that WRN protein has a function in metabolic control outside of its currently known function.

Introduction

Werner Syndrome (WS) is an autosomal recessive disease that results in premature aging and death and is often characterized by increased susceptibility to cancer and heart disease as well as low muscle mass, and metabolic syndromes including type II diabetes, dyslipidemia, and fatty liver disease (Epiney et al., 2021; Oshima et al., 2017). While many of these symptoms are common in people with WS, the leading cause of death in patients is cancer and myocardial infarction (Huang et al., 2006). Currently it is estimated that patients with WS have a mean life

expectancy of 54 years old which is significantly less than the national average of 77 years (Murphy, 2021; Oshima et al., 2017). While WS is classified as a rare genetic disease and only occurs in 1 in 1,000,000 to 1 in 10,000,000 births, there is currently no cure, and the best treatment options are currently lifestyle interventions (Shamanna et al., 2017; *Werner Syndrome*, 2022).

WS is caused by mutations in the WRN gene that results in a nonfunctional WRN protein. In humans, the WRN protein contains both 3'→5' ATP dependent helicase activity and 3'→5' exonuclease activity (Bolterstein et al., 2014). Currently it is known that the WRN protein is a member of the RecQ family of helicases and plays an important role in DNA structure and function including roles in repair, replication, recombination, and telomere maintenance (Epiney et al., 2021). More than 80 different types of mutations have been seen in various regions of WRN, including substitutions mutations within the exonuclease domain (Cassidy et al., 2019; Huang et al., 2006; Oshima et al., 2017).

Several models of WS have been created in mice, *C. elegans*, and *Drosophila melanogaster* (fruit fly) (Cassidy et al., 2019). While these models differ slightly with loss of function in one or both domains, they have been observed to have a shortened lifespan compared to controls (Aumailley & Lebel, 2021; Harkema et al., 2016). The mouse model $Wrn^{\Delta hel/\Delta hel}$ deletion null mutant removed function in both the helicase and the exonuclease domain (Aumailley et al., 2018; Aumailley et al., 2015) and showed a mean reduction of lifespan of 16.5% (Massip et al., 2010). A different mouse model that solely knocked down function in the helicase domain saw a milder reduction in lifespan of 10~15% (Lebel et al., 2003; Lebel & Leder, 1998). Although milder, this reduction clearly suggests that the exonuclease domain also plays an important role in lifespan of WS mutants.

Flies and worms differ from mice in that the exonuclease and helicase domains of the WRN gene are found on independent genes (Bolterstein et al., 2014; Lee et al., 2004). While there are several models of WS that investigate the function of the helicase domain, significantly less in known about the functions of the exonuclease domain. In flies, the exonuclease domain of the WRN protein is encoded by the gene *WRNexo* (Bolterstein et al., 2014; Boubriak et al., 2009). The deletion null mutant that was used in this project was the WRNexo/DmWRNexo (*WRNexo*$^{\Delta}$) and was created by removing most of the gene sequences. Previously, this model has been shown to be viable and fertile with some developmental defects, as well as having similar premature aging phenotypes to other models, with an increased tumor incidence and shortened lifespan (Bolterstein et al., 2014). These mutants have also been shown to have reduced fat content, abnormal activity/sleep patterns, and reduced resistance to some environmental stressors (Cassidy et al., 2019; Epiney et al., 2021). These observations suggest that the exonuclease domain may also play an important role in metabolic control in addition to its known roles in genome stability and maintenance.

While WS has been shown to cause premature aging and death in both humans and models, dietary restriction (DR) has been shown to be one of the most robust non-genetic interventions that delays aging and extends the lifespan of a variety of model organism (Fontana & Partridge, 2015). Previous studies have also shown that DR can be used to mitigate disease symptoms in both models and humans (Wu et al., 2022). DR is a reduction in food intake without causing malnutrition (Min & Tatar, 2006). In flies, DR is generally performed by limiting either sucrose or protein in the diet, and it has been shown that limiting the protein source is sufficient to increase lifespan (Gallinetti et al., 2013; Grandison et al., 2009; Hoedjes et al., 2017; Ja et al., 2009; Katewa et al., 2012; Lee et al., 2014). These previous results suggest

that protein sensing and metabolism must be intact in order for DR to extend lifespan and DR could potentially extend the lifespan of WS model flies if their protein sensing and metabolism are still intact.

In this project, I used the *WRNexo*$^{\Delta}$ null mutant fly model of WS to investigate the DR-mediate lifespan and physiological effects of variable protein DR on the exonuclease activity of WRN (Bolterstein et al., 2014; Cassidy et al., 2019; Epiney et al., 2021). I used variable protein DR, where the concentration of sucrose was kept constant while the concentrations of yeast were varied, to investigate these effects (Bass et al., 2007). Initially, I hypothesized that the exonuclease activity of WRN would not affect protein sensing and metabolism, and that DR would extend the lifespan of these flies. This extension of lifespan could then be used as a possible lifestyle intervention to extend the lifespan of patients with WS. However, it was shown that DR fails to extend the lifespan of *WRNexo*$^{\Delta}$ null mutant flies in both males and females, indicating a functioning WRN exonuclease is required for DR-mediated lifespan extension. I also found that these mutants had lower resistance to both starvation and oxidative stress, as well as dysregulated sleep and activity patterns. While these results show that DR may be deleterious in *WRNexo*$^{\Delta}$ mutants, my results suggest that the WRN protein plays an important role in DR-mediated physiological effects such as lifespan, stress response, and behavior through pathways involved in protein/amino acid sensing and metabolism.

Materials and Methods

Fly Stock and Maintenance

For all experiments, homozygous *WRNexo*$^{\Delta}$ null mutants (w^{1118}/ w^{1118}; +/+ ; WRNexo$^{\Delta}$/TM6, Sb, GFP) and their isogenic w^{1118} wildtype control flies were used. Both the

WRNexo$^{\Delta}$ null mutants and their isogenic w^{1118} wildtype control were obtained as generous gifts from Elyse Bolterstein (Bolterstein et al., 2014; Epiney et al., 2021). Before experimentation, the flies were expanded in vials (23mm X 95mm) containing ~5ml of a standard cornmeal media (Genesee Scientific, Cat#: 66-113) that is used for larval growth. The media is a variant from the one used in the Bloomington Stock Center. For all experiments as well as expansion, all flies were kept at 25˚C and 40~60% humidity on a 12hr light-12hr dark schedule. In order to obtain the flies used in each experiment, both *WRNexo*$^{\Delta}$ and w^{1118} flies were collected in ~48-hour cohorts and were allowed to mate for ~ 24 hours post-eclosion in order to ensure all flies were mated. Both mutants and controls were then separated by sex and genotype (homozygous *WRNexo*$^{\Delta}$ mutants) under light CO_2 anesthesia. Once separated, the flies were sorted by sex into their respective diet media with constant 5% [w/v] sucrose (Genesee Scientific, Cat#: 62-112) and variable yeast (1%, 5%, and 20% [w/v]) (Brewer's yeast, MP Biomedicals) (Bass et al., 2007). Throughout all experiments, each yeast concentration was labeled as 1Y, 5Y, and 20Y for 1%, 5%, and 20% yeast respectively and 5Y is considered to be DR. For the yeast supplementation shown in figure 9, yeast paste was prepared using a 1:1.25 [w/v] ratio of Brewer's yeast to water. Food for all experiments were made in batches, wrapped for storage, and kept in a 4˚C refrigerator until use. All food used was less than 2 weeks old.

Lifespan Assay

Lifespan assays were performed in order to observe the effects of variable protein DR on the lifespan of WS flies as well as the effects of early protein supplementation on lifespan. For both lifespan assays performed, flies were reared and separated as described above and then around 7-10 replicate vials of ~25 flies each were set up for each sex, genotype, and diet combination. These flies were transferred to new vials of food every 2-3 days in Figure 6 and

every 2 days in Figure 9. During each transfer, the number of dead flies was recorded and flies that were lost or damaged/stuck to cotton balls during each transfer were censored and not included in any analysis. In Figure 9, additional yeast supplementation was used. The yeast paste was created using the protocol described above and half of a spatula of refrigerated yeast paste was taken and smeared on the side of 5Y vials several hours before each transfer. These transfers were performed between the hours of 8 AM and 10 AM every two days to ensure an accurate mortality pattern analysis. The yeast paste used was always less than 1 week old at the time of application.

Figure 1

Diagram of lifespan assay performed in Figure 6

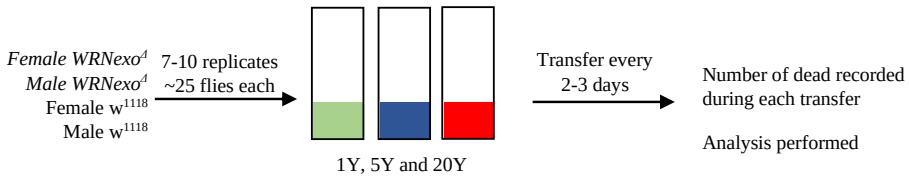
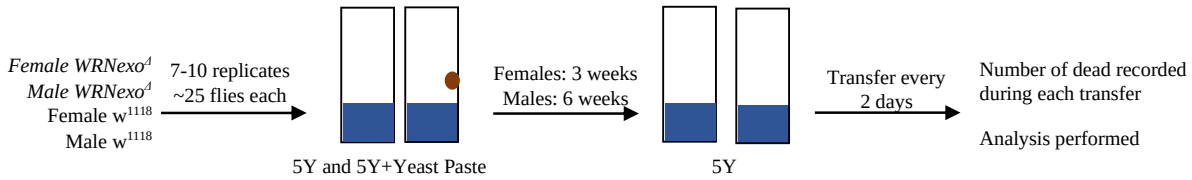


Figure 2

Diagram of lifespan assay performed in Figure 9



Starvation and Oxidative Stress

Both mutant and control flies were reared and collected according to the protocol described above (~48-hour cohorts with one day of post eclosion mating). Once separated, flies were transferred to 5Y and 20Y diets for 9~10 days until the time of experiments. Each

combination of sex, genotype, and diet for both oxidative stress and starvation had 6 replicates with 10-20 flies in each replicate. These flies were transferred to starvation media (1.5% agar) (Figure 3) while oxidative stress vials were created using a paraquat solution (10 mM paraquat in 10% [w/v] sucrose solution). Oxidative stress vials were created by placing one Kimwipe (Kimberly-Clark Professional™) into each vial and soaking it with 2ml of paraquat solution. Two round filter papers (23 mm, Whatman) were then placed in each vial until both filters were slightly damp with paraquat solution. The insides of each vial were wiped down to remove any excess parquet solution. Flies in the oxidative stress experiment were placed in starvation media for ~5 hours before being placed into oxidative stress vials in order to control for any feeding rhythm differences that may be present between the two diets (Figure 4). For both oxidative stress and starvation, the number of dead flies in each replicate was counted and recorded every 2-8 hours until all flies were dead.

Figure 3

Diagram of starvation assay in Figure 7

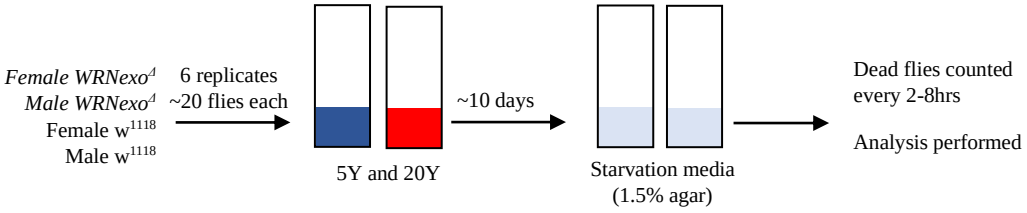
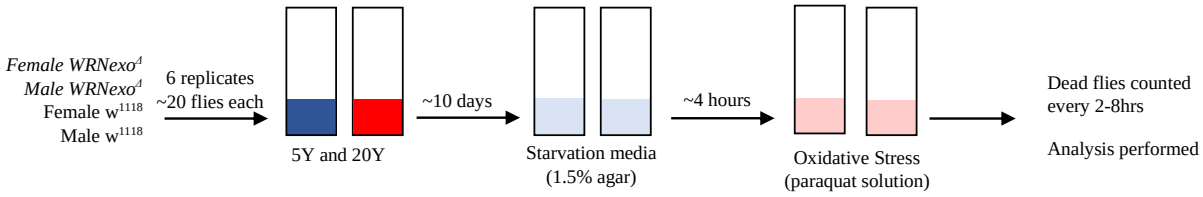


Figure 4

Diagram of oxidative stress assay in Figure 7

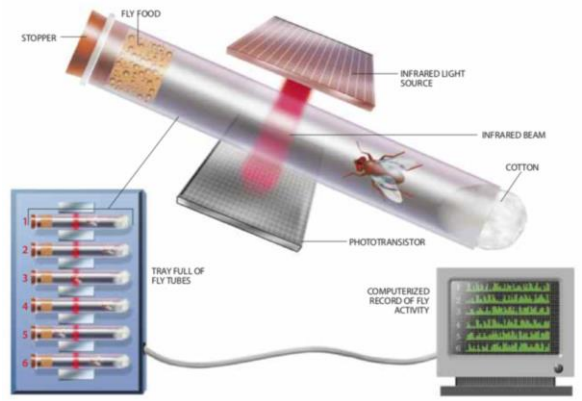


Sleep/Activity

In flies, sleep is defined as inactivity for more than five minutes (Hendricks et al., 2000) Sleep and activity were measured using the Drosophila Activity Monitors (DAM, Trikinetics) with standard established protocols (Pfeiffenberger et al., 2010a, 2010b). The DAM system works by loading glass tubes containing food and flies (each tube contains a singular fly) into the DAM2 monitor (Figure 5). This monitor shines an infrared beam through the center of each of the glass tubes and then counts the number of beam breaks each tube experiences. Once separated, flies were kept on 5Y and 20Y diets for ~ 7 days before being loaded into glass tubes with the corresponding diet. Flies were loaded while under light CO_2 anesthesia. DAM monitors collecting the sleep and activity data for each fly were run for ~4 days. By the end of monitoring, flies had been kept on 5Y or 20Y diet for ~10 days, which is similar to the amount of time the stress assay flies were kept on their diets before the assay was run. The first day of data was removed from analysis in order to eliminate any potential side effects from the CO_2 anesthesia, and any flies with less than 10 beam breaks during the last day were also removed to account for potential damage caused by condensation in the glass tubes.

Figure 5

Diagram of DAM2 system from Trikinetics ("Drosophila Activity Monitoring System," 2018)



Statistics

OASIS2 was used to obtain the descriptive survival statistics (such as mean lifespan and mortality rates) and pair-wise comparison of survival curves between groups (log-rank test) for all lifespan and stress resistance experiments (Figure 6,7, and 9) (https://sbi.postech.ac.kr/oasis2/) (Han et al., 2016). One-way ANOVA following a Tukey HSD post-hoc test was used to determine statical significance for sleep parameters (Figure 8).

Results

Lifespan

In order to test the effects of DR on the *WRNexo*$^{\Delta}$ model of WS and determine whether DR could extend the lifespan of the mutants, I used variable protein DR. In this type of DR, the concentration of sucrose is kept constant while the concentrations of yeast, the main protein source, is diluted (Bass et al., 2007; Ja et al., 2009; Min et al., 2007). Specifically, a reaction norm approach (Tatar, 2007) of three serial dilutions was used to create the media used in this

experiment (1% yeast, 5% yeast, and 20% yeast) with 5% sucrose being held constant between the three dilutions. This is a traditional method of DR and has been proven not to cause desiccation (Ja et al., 2009) or obesity in flies (van Dam et al., 2020).

Overall, it was observed that DR did not delay aging or extend the lifespan of *WRNexo*$^{\Delta}$ mutant flies, and there were significant gene and diet affects in a sex-dependent manner. In the 1Y, malnutrition media, a significant lifespan reduction was observed in both the WT (female: 15.8 days vs 43.7 days; -63.9%, $\chi 2$ =422.3, $p < 0.001$; male: 18.8 days vs 46.9 days; -59.9%, $\chi 2$ =369.5, $p < 0.001$) and WS mutants flies (female: 12.5 days vs 42.9 days; -70.8%, $\chi 2$ =417.7, $p < 0.001$; male: 15.8 days vs 47.3 days; -66.5%, $\chi 2$ = 379.1, $p < 0.001$) compared to WT and mutants in 20Y, likely due to malnutrition. The *WRNexo*$^{\Delta}$ mutants also experienced a greater reduction in lifespan compared to the WT flies in 1Y media (female: 15.8 days (WT) vs 12.5 days (*WRNexo*$^{\Delta}$); -20.6%, $\chi 2$ =38.9, $p < 0.001$; male: 18.8 days (WT) vs 15.8 days (*WRNexo*$^{\Delta}$); -15.8%, $\chi 2$ =41.5, $p < 0.001$). This result suggests that the accelerated aging phenotype that is seen in the WS mutants still occurs when under malnutrition conditions. In the 20Y control diet, *WRNexo*$^{\Delta}$ did not show a mean reduction in lifespan compared to WT flies under the same conditions (female: 43.7 days (WT) vs 42.9 days (*WRNexo*$^{\Delta}$), -1.7%, $\chi 2$ =0.1, $p = 0.705$; male: 46.9 days (WT) vs 47.3 days (*WRNexo*$^{\Delta}$), 0.9%, $\chi 2$ =0.3, $p = 0.589$). This result indicates that diet can have an impact of the lifespan of the *WRNexo*$^{\Delta}$ mutants as well as showing that the macro-/ micro- nutrients in the yeast, such as protein, may have the potential to extend the lifespan and delay aging in the mutants.

As expected, 5Y DR media was able to significantly extend the lifespan of WT flies compared to WT flies on 20Y media (female: 15.5%, $\chi 2$ =62.2, $p < 0.001$; male: 6.4%, $\chi 2$ =6.8, $p = 0.009$). It was also observed that, DR media was more effective at extending the lifespan of

female WT flies, which is consistent with previous research that shows that female flies often have a greater lifespan extension by DR than males (Magwere et al., 2004; Vargas et al., 2010). While DR extended the lifespan of WT flies, DR failed to extend the lifespan of *WRNexo*$^{\Delta}$ flies. In both sexes, the mean lifespan was shorter than on 20Y media with little to no statistical significance (female: -11%, $\chi 2$ =0.9, p = 0.335; male: -0.9%, $\chi 2$ = 3.2, p = 0.074). The failure of DR to extend the lifespan of the *WRNexo*$^{\Delta}$ mutants suggests that the mutants have an impaired response to changes in dietary yeast and that a functional WRN protein is necessary for DR-mediated lifespan extension.

Figure 6

DR fails to extend lifespan of WRNexo$^{\Delta}$ mutant flies

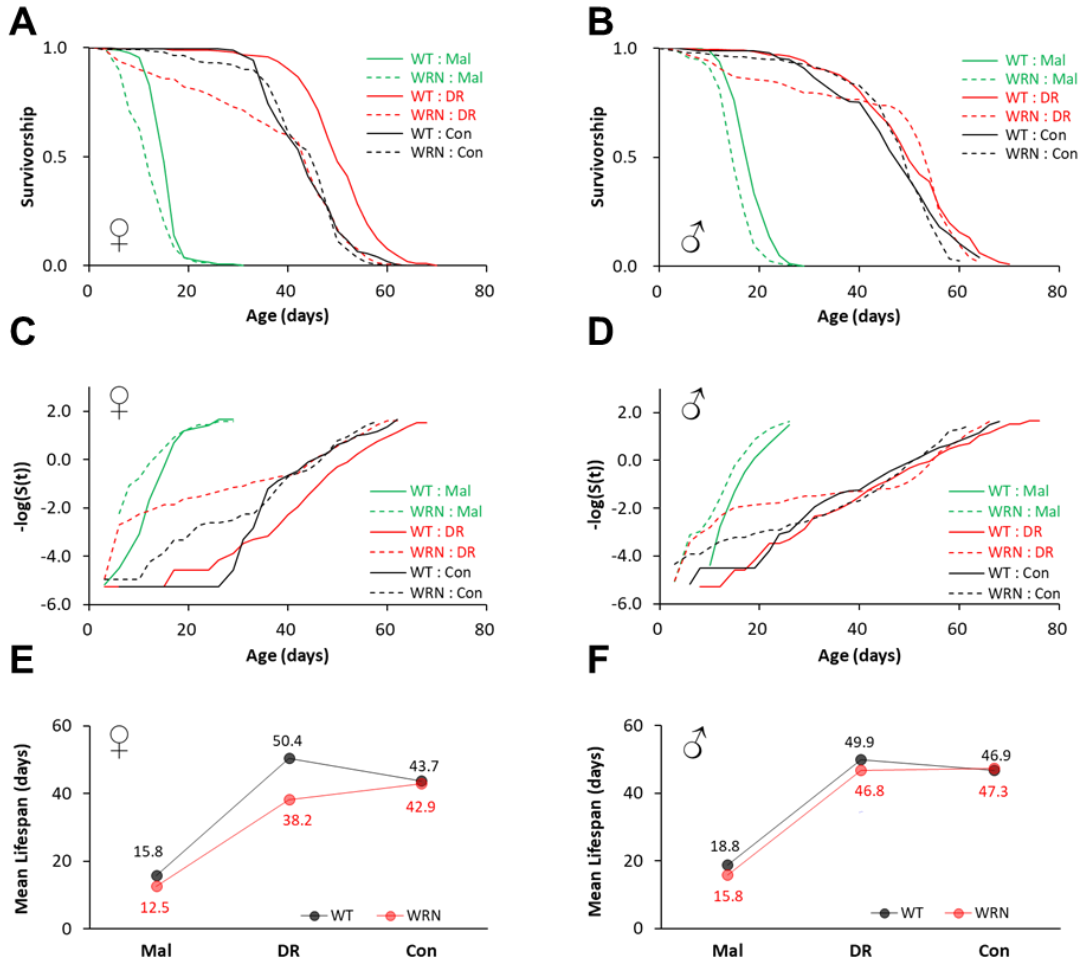


Figure 6. Diet-dependent lifespan of wild-type and WRN$^{\Delta}$ mutant flies in yeast-restriction diets. (A, B) Survival curves (S(t)), (C, D) log mortality rate (-log(S(t)), (E, F) Mean lifespan response to three different yeast-restriction diets (A, C,E : males; B,D,F: females). Mal (1Y): 1% yeast with 5% sucrose, DR (5Y): 5% yeast with 5% sucrose, Con (20Y): 20% yeast with 5% sucrose.

Stress Resistance: Starvation and Oxidative Stress

Once I observed that DR was incapable of extending the lifespan of the *WRNexo*$^{\Delta}$ mutants in general and caused increased early mortality, I hypothesized that physiological and metabolic effects of DR, such as starvation and oxidative stress, may also be impaired in the mutants. Starvation resistance was determined by transferring both WT and *WRNexo*$^{\Delta}$ mutants to starvation vials (only 1.5% agar without any nutrients) after ~10 days on 5Y and 20Y diets. *WRNexo*$^{\Delta}$ flies were observed to be more sensitive to starvation stress compared to WT flies in 5Y (female: 102.0 hours (WT) vs 65.6 hours (*WRNexo*$^{\Delta}$); -35.7%, $\chi2 = 136.4$, $p < 0.001$; male: 46.6 hours (WT) vs 30.8 hours (*WRNexo*$^{\Delta}$); -33.9%, $\chi2 = 148.0$, $p < 0.001$) and 20Y (female: 63.0 hours (WT) vs 51.3 hours (*WRNexo*$^{\Delta}$); -35.7%, $\chi2 = 136.4$, $p < 0.001$; male: 30.8 hours (WT) vs 23.0 hours (*WRNexo*$^{\Delta}$); -25.1%, $\chi2 = 93.8$, $p < 0.001$). While the effects of DR on starvation resistance have never been tested in *WRNexo*$^{\Delta}$ mutants, these results were similar to a previous study that tested the starvation resistance of *WRNexo*$^{\Delta}$ flies on a standard cornmeal-based diet (Epiney et al., 2021).

Previous studies in flies have shown that DR conditions increase starvation resistance (Katewa et al., 2016; Katewa et al., 2012). As expected, DR conditions increased starvation resistance in WT flies with a 64.6% mean survival increase in females (mean survival: 62.0 hours (20Y) vs 102.0 hours (5Y), $\chi2 = 138.5$, $p < 0.001$) and a 51.6% increase in males (mean survival: 30.8 hours (20Y) vs 46.6 hours (5Y), $\chi2 = 149.6$, $p < 0.001$). *WRNexo*$^{\Delta}$ mutants in DR conditions also had an increased resistance to starvation compared to 20Y, but the increase was much smaller than what was observed in WT flies with an increased mean survival of 27.9% in females (mean survival: 51.3 hours (20Y) vs 65.6 (5Y) hours, $\chi2 = 44.3$, $p < 0.001$) and a 30.8% increase in males (mean survival: 23.0 hours (20Y) vs 30.8 (5Y) hours, $\chi2 = 30.9$, $p < 0.001$).

This difference in starvation resistance corresponds to the results that were observed in the Figure 6 lifespan and indicate that a functional WRNexo protein is needed for DR mediated physiological changes or benefits.

In addition to starvation resistance, the effects of DR on oxidative stress in *WRNexo*$^{\Delta}$ mutants were also determined. For oxidative stress, flies were reared under the same conditions as starvation resistance with ~10 days on 5Y and 20Y media before experimentation. Previously, it was shown the female *WRNexo*$^{\Delta}$ have higher oxidative stress resistance (5% H_2O_2) compared to controls, which is partially explained by increased antioxidant defense system activity (Epiney et al., 2021). Instead of H_2O_2, a paraquat solution was used to test oxidative stress resistance (Epiney et al., 2021). This solution was used because our initial RNA-Seq analysis showed that cytochrome p450 genes, that help to detoxify exogenous chemicals, were highly dysregulated. Given the findings from the starvation resistance analysis and lifespans assays, I hypothesized that *WRNexo*$^{\Delta}$ under DR conditions may be more susceptible to oxidative stress compared to the 20Y diet.

In males, there was no significant difference in oxidative stress resistance between genotypes or diets. While the males did not show any significant differences, female *WRNexo*$^{\Delta}$ showed a higher oxidative stress resistance than WT flies in both 5Y (28.4 hours (WT) vs 32.5 hours (*WRNexo*$^{\Delta}$); 14.7%, $\chi 2 = 8.7$, $p = 0.003$) and 20Y diets (27.7 hours (WT) vs 37.5 hours (*WRNexo*$^{\Delta}$); 35.4%, $\chi 2 = 21.2$, $p < 0.001$). In WT females, there was no significant difference in oxidative stress resistance between 5Y and 20Y diets (28.4 hours (20Y) vs 27.7 hours (5Y); $\chi 2 = 0.1$, $p = 0.737$). However, female *WRNexo*$^{\Delta}$ did show a difference between diets and mutants on DR showed a survival rate that was ~13% lower than female mutants on 20Y (37.5 hours (20Y) vs 32.5 hours 5Y); $\chi 2 = 7.2$, $p = 0.007$). This difference in female mutants suggests that while

diets do not affect oxidative stress resistance in WT, a lower yeast concentration reduced the resistance to oxidative stress in the mutants. These results correspond to both the lifespan in Figure 6 and starvation and indicate that *WRNexo*$^{\Delta}$ flies exhibit impaired physiological response to diets.

Figure 7

WRNexo$^{\Delta}$ *mutants display altered diet-dependent stress resistance*

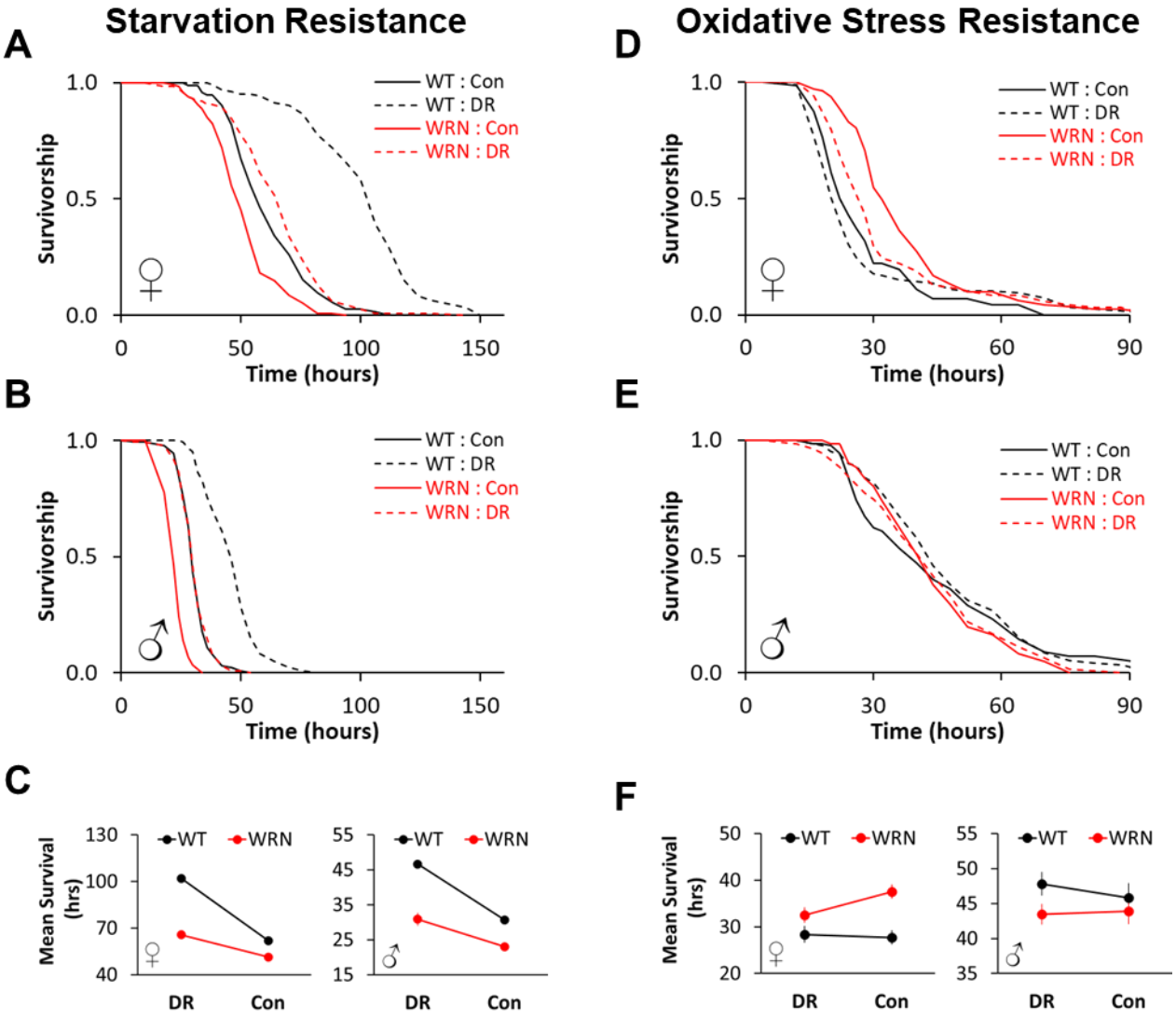


Figure 7. Diet-dependent stress responses of wild-type and *WRN$^{\Delta}$* mutant flies. (A-C) Starvation resistance. (B-D) Oxidative stress resistance. A,D: females, B,E: males. C,F: mean survival response (in hours by Kaplan-Meier estimation) in starvation and oxidative stress to diets in both females and males. DR (5Y): 5% yeast with 5% sucrose, Con (20Y): 20% yeast with 5% sucrose. Flies were fed 5Y or 20Y diets for ~ 10 days before the assays.

Sleep/Locomotive Activity

Previous research has shown that changes in diet composition can affect the locomotor activity and sleep patterns of flies (Brown et al., 2020; Linford et al., 2012). It has also been shown that *WRNexo$^{\Delta}$* flies display abnormal sleep patterns in standard media, but the effects of dietary composition have never been tested in *WRNexo$^{\Delta}$* mutants (Cassidy et al., 2019; Epiney et al., 2021). I hypothesized that sleep patterning in *WRNexo$^{\Delta}$* would follow a similar pattern to lifespan and stress resistance and mutants would have a disrupted sleep pattern compared to WT. To test this hypothesis, the sleep and activity of the flies on 5Y and 20Y diets were monitored using the Drosophila Activity Monitors (DAM) for 3 days (Pfeiffenberger et al., 2010a, 2010b). As expected, both sexes of WT flies in 5Y displayed an increase in sleep amount during the day compared to WT in 20Y (Females: the 12 hours of light-on phase; total of 720 minutes) in both females (475.1 minutes (20Y) vs 546.3 minutes (5Y), $p = 0.0013$ and Males 505.2 minutes (20Y) vs 623.6 minutes (5Y), $p < 0.0001$). However, this repatterning was not seen in *WRNexo$^{\Delta}$* and was repressed. Both sexes of *WRNexo$^{\Delta}$* mutants had very little difference in sleep patterning between 5Y and 20Y diets (females: 621.4 minutes (20Y) vs 639.6 minutes (5Y), $p = 0.7567$ and males: 613.5 minutes (20Y) vs 605.0 minutes (5Y), $p = 0.9565$). This failure to sleep repattern in 5Y DR media suggests that, in addition to the previously observed baseline sleep disruption in

WRNexo$^{\Delta}$ mutants (Cassidy et al., 2019; Epiney et al., 2021), a functional WRN protein is necessary for DR-dependent sleep regulation.

Figure 8

WRNexo$^{\Delta}$ *mutants display altered diet-dependent sleep repatterning*

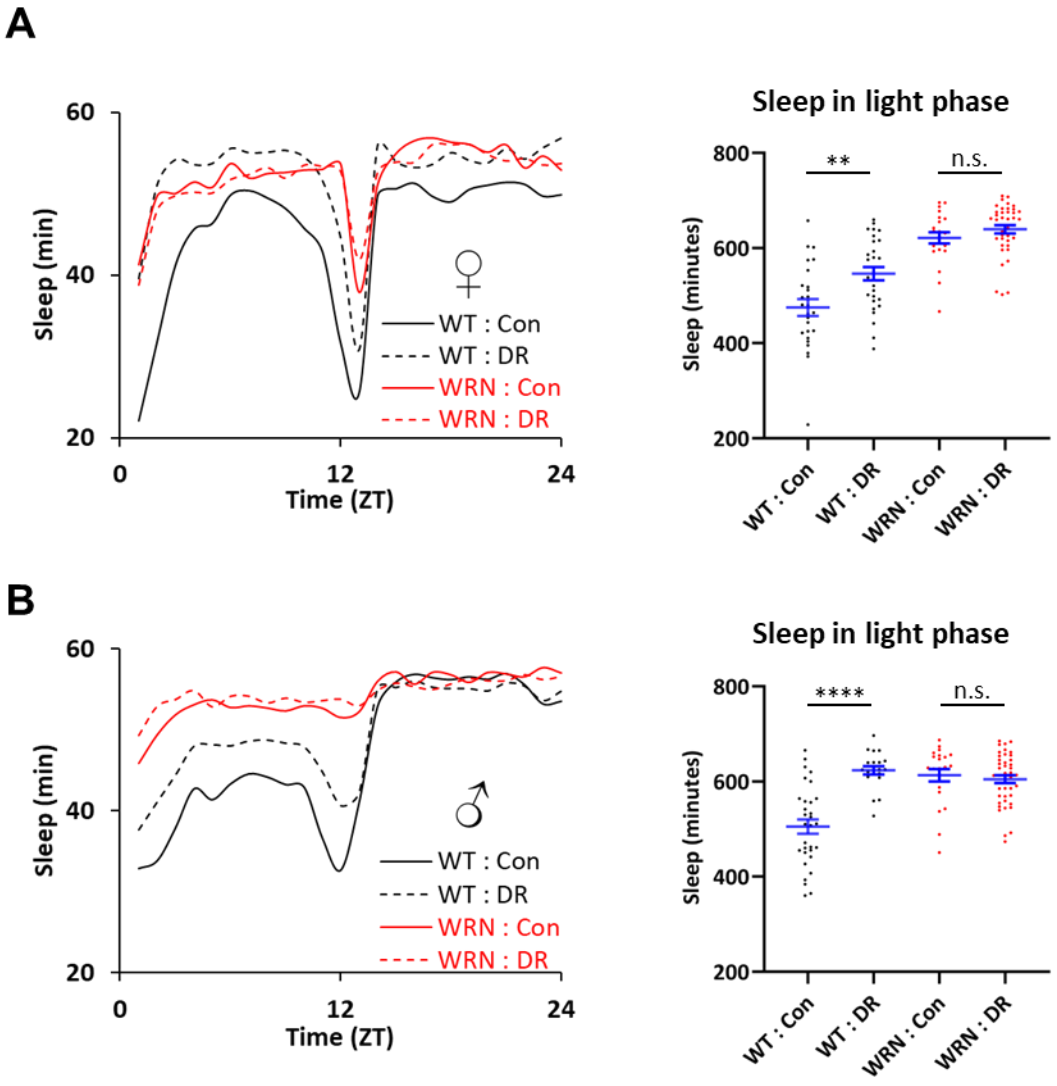


Figure 8. Diet-dependent sleep patterns of wild-type and *WRN*$^{\Delta}$ mutant flies. Daily sleep graphs (left) and sleep minutes of 12 hours of the light cycle (right). (A) females, (B) males. DR (5Y): 5% yeast with 5% sucrose, Con (20Y): 20% yeast with 5% sucrose. Flies were fed 5Y or

20Y diets for ~ 7 days in vials before the sleep analysis. Sleep was measured for 3 days on each diet using the DAM system (see methods). ZT0: light on, ZT12: light off. The 1st day of sleep was removed from analysis due to unstable sleep/activity pattern due to CO_2 anesthetization. ANOVA *$p \leq 0.01$, **** $p \leq 0.0001$.

Yeast Supplementation Lifespan

The diet dependent results observed in the lifespan (Figure 6), stress resistance (Figure 7), and sleep assays (Figure 8) indicate that mutations in the WRN protein may contribute to impaired protein metabolism. The lifespan data (Figure 6) revealed that the highest morality of the mutants in 5Y DR diet occurred in their early life, prohibiting beneficial effect of DR in lifespan extension. I hypothesized that early life supplementation of the nutrients in yeast may be able to reduce early life mortality in the mutants. To test this hypothesis, the effects of early life yeast supplementation were examined. Flies were supplemented with a concentrated yeast paste for ~3 weeks in females and ~6 weeks in males until an increase in mortality was observed. This supplementation was observed to delay and protect against early mortality in *WRNexo*$^{\Delta}$ mutants in 5Y. Female mutants exhibited an increased survival of 14% and males exhibited an increased survival of 6% by day 20. It was also observed that early yeast supplementation delayed the day of 25% mortality in mutants from day 18 to 28 in females, while males had the same day of 25% mortality at day 40. While mutants exhibited a delay in early in mortality, WT were unaffected by the early life yeast supplementation. This result suggests that protein supplementation can partially reverse accelerated aging in *WRNexo*$^{\Delta}$.

Figure 9

Yeast supplementation delays accelerated mortality of WRNexo$^{\Delta}$ mutants

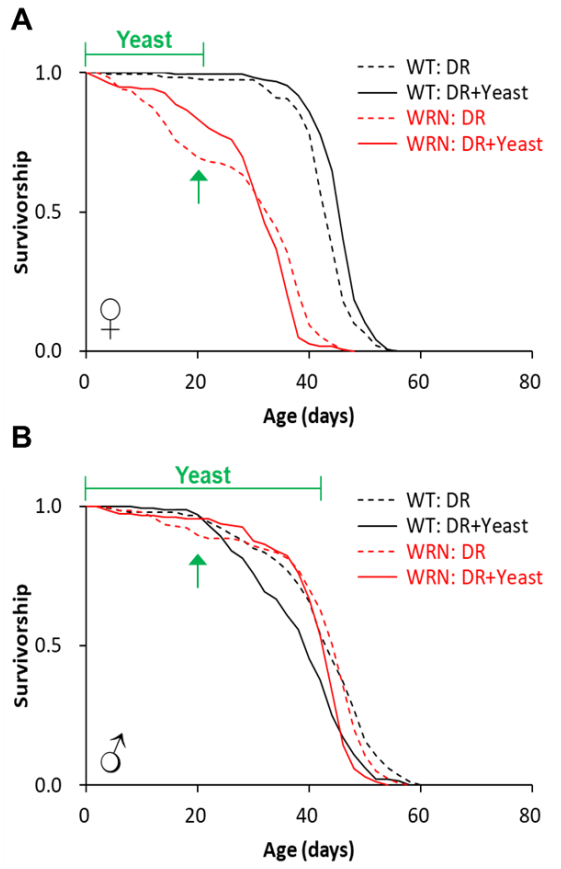


Figure 9. Impact of yeast supplementation on lifespan of wild-type and *WRN$^{\Delta}$* mutant flies. DR (5Y): 5% yeast with 5% sucrose, DR + Yeast: DR diet supplemented with yeast paste (Brewer's yeast mixed with water in 1: 1.25 ratio). Fresh yeast paste was supplemented on the side of lifespan vials every 2 days when dead flies were counted.

Discussion

WS is an autosomal recessive genetic disease that results in premature death. DR has been shown to be one of the most robust non-genetic interventions for increasing the lifespan of a variety of model organisms (Fontana & Partridge, 2015; Oshima et al., 2017). The goal of this

thesis was to evaluate the lifespan and physiological effects of DR on the *WRNexo*$^{\Delta}$ fly model of WS. It was observed that DR does not have the ability to extend the lifespan of these mutants and even decreased the lifespan of female mutants (Figure 6). However, early life yeast supplementation helped to partially rescue early life mortality in the mutants in 5Y DR diet (Figure 9). Stress responses such as starvation and oxidative stress were also shown to be negatively affected by DR by lowering resistance (Figure 7), while sleep patterning was also disturbed by DR (Figure 8). Initially, I hypothesized that DR could be used as a possible lifestyle intervention to extend lifespan in WS patients, but the findings of this thesis indicate otherwise. While DR might not be a viable treatment option for patients with WS, the results of this thesis indicate that the WRN protein may play a role in metabolism outside of its currently known functions in genome stability and maintenance (Epiney et al., 2021).

Results of the DR lifespan (Figure 6) show that DR does not extend the lifespan of *WRNexo*$^{\Delta}$ mutants and decreased the lifespan of the female mutants. This result indicates that the WRN protein must be functional in order to observe DR mediated lifespan extension. The effects of DR on starvation, oxidative stress, and sleep also support that a functional WRN protein is needed for DR-mediated lifespan extension because each of these physiological characteristics have been shown to be important for or at least linked to DR (Brown et al., 2020; Katewa et al., 2016; Katewa et al., 2012; Linford et al., 2012). *WRNexo*$^{\Delta}$ mutants in DR showed that disruptions in these characteristics, indicating that a functional WRN protein is required for DR-mediated physiological characteristics. In addition to showing that a functional WRN protein is needed for DR-mediated lifespan extension, the failure to extend lifespan, reduced resistance to starvation and oxidative stress, and abnormal sleep patterning all suggest that the WRN protein plays a role in metabolism. The function of WRN protein in DNA repair,

replication, recombination, and telomere maintenance is fairly well understood, but metabolic functions of the protein are poorly understood (Epiney et al., 2021). While the mechanism of the role of the WRN protein in metabolism is currently unknown, the results of this thesis clearly indicate that it participates in metabolism. The WRN protein having a functional role in metabolism may also explain some of the clinal metabolic symptoms of WS patients including type II diabetes, dyslipidemia, and fatty liver disease (Epiney et al., 2021; Oshima et al., 2017).

The findings of these experiments are also supported by the current literature on other fly models and the *WRNexo*$^{\Delta}$ model of WS in flies. Several physiological characteristics have already been tested in WS, including starvation, oxidative stress, sleep, and thermal stress (Cassidy et al., 2019; Epiney et al., 2021). These physiological characteristics all showed altered function including decreased resistance to starvation, increased resistance to oxidative stress in females, and disrupted sleep patterning (Cassidy et al., 2019; Epiney et al., 2021). However, physiological characteristics associated with DR mediated lifespan extension as well as DR lifespan have never been tested in *WRNexo*$^{\Delta}$ flies. Our results in DR mediated characteristics correspond and align with what is currently known about WS and these previous observations also support the observation that the WRN protein plays a role in metabolism. The sex differences that were observed in the WS lifespan and physiological assays are also consistent with the current literature. It has been previously shown that female flies are more likely to show effects of DR mediated lifespan extension (Magwere et al., 2004; Vargas et al., 2010). This corresponds to our finding in both lifespan assays (Figure 6 and 9) where female mutants were more effected. It has also been previously shown that female *WRNexo*$^{\Delta}$ mutants had a higher resistance to oxidative stress (Epiney et al., 2021). This is mirrored in our findings, while DR in the female mutants decreases oxidative stress resistance.

There is one caveat to the findings of this research that will need future testing to verify. Our conclusions suggest that mutations in the WRN exonuclease domain may alter protein metabolism, leading to the observations in DR-mediated physiological characteristics and DR lifespan. However, there is a possibility that these results are a consequence of additional macronutrients and micronutrients, such as vitamins and fats, that are also present in the Brewer's yeast at smaller concentrations. For example, vitamin C was shown to be capable of rescuing accelerated aging phenotypes in mouse and worm models of WS (Aumailley et al., 2018; Aumailley & Lebel, 2021; Massip et al., 2010). While it is mostly likely the concentrations of protein that are mediating the results observed in this thesis because the micronutrients in the Brewer's yeast are at very low concentrations, the possibility of these micronutrients contributing to our results cannot be ruled out. In order to ensure that it is the protein causing these results, further testing will be required.

Conclusion

This research reveals the effects of DR on the *WRNexo*$^{\Delta}$ fly model of WS. Surprisingly, DR was found to not extend the lifespan of these mutants and lead to disrupted stress resistance and sleep pattering. These results can be used to explain a novel function of the WRN protein and establish that a functional protein is needed for DR mediated lifespan extension. Future research should be conducted using only amino acid sources of protein to ensure that it is the protein that led to these results and not small amounts of micronutrients such as vitamin C in the yeast, which was shown to protect WRN mutants from premature aging in others models of WS (Aumailley et al., 2018; Aumailley & Lebel, 2021; Massip et al., 2010). Additionally, more

research should be conducted to determine the mechanism of the WRN protein in metabolism control as well as investigating tissue specific WRN mutation effects.

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