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Genotype And Sex Specific Effects Of Methionine Restriction On The Life And Healthspan Of Drosophila Melanogaster

Joshua Robert Smith
University of Alabama at Birmingham

GENOTYPE AND SEX SPECIFIC EFFECTS OF METHIONINE RESTRICTION ON THE LIFE AND HEALTHSPAN OF *DROSOPHILA MELANOGASTER*

by

JOSHUA R. SMITH

STEVEN AUSTAD, COMMITTEE CHAIR
ANDREW PICKERING
STEPHEN WATTS

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BIOLOGY

ABSTRACT

Reduced dietary intake of the amino acid methionine has been reported to significantly extend the lifespan of common laboratory model organisms including *Saccharomyces cerevisiae*, *Mus musculus*, and *Drosophila melanogaster*. Methionine restriction (MR) has also been achieved via non-dietary means such as metabolite supplementation and CRISPR-Cas9 gene editing. MR along with protein and calorie restriction are known as dietary interventions capable of producing pro-longevity benefits through regulation of nutrient sensing pathways. Recent studies in model organisms have also identified beneficial effects of a low methionine diet on aging-associated phenotypes and markers of overall healthspan. However, the effects of this intervention on genetically diverse populations have yet to be explored.

In this study, we investigate the relationship between sex and genetic factors on determining the outcomes of a long-term MR diet given to multiple strains of the fruit fly *D. melanogaster*. We found significant variation in the survival response, including lifespan shortening in addition to previously reported lifespan extension. Additionally, we report an observed lack of correlation between resistance to oxidative stress and pro-longevity benefits. These results suggest a significant genetic component to the longevity phenotype of MR as well as context-dependent mechanisms involved in its regulation.

Keywords: Methionine Restriction, Dietary Restriction, *Drosophila melanogaster*, Aging, Geroscience, Nutrition

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CHAPTER 1

INTRODUCTION

Global human life expectancy has increased consistently over the last 150 years, driven predominately by advances in quality and accessibility of medicine and sanitation, though localized exceptions caused by events such as wars and pandemics (e.g. the 1918 flu pandemic and COVID-19), have caused life expectancy to decline for brief periods of time (Andrasfay & Goldman, 2022). This increase in life expectancy has led to an exponential increase in older adults worldwide, and accompanying this aging population is an increase in chronic aging-associated diseases such as cancer and neurodegenerative disease. This has spurred an increased interest in research on interventions that directly target the basic biology of aging as a means to robustly extend life and healthspan. The mechanisms by which reduced methionine extends lifespan at the cellular and molecular level are still predominantly unknown, though like CR and other dietary interventions it may involve the regulation of nutrient sensing signaling pathways such as mTOR (Kaeberlein & Kapahi, 2009), or a reduction in oxidative stress levels (Caro et al., 2009; Naudi et al., 2007; Plummer & Johnson, 2019; Sanz et al., 2006). Regardless, research has demonstrated that lifespan is a modifiable trait, with some interventions extending longevity as much as 2-3x compared to controls in laboratory organisms (Gems & Riddle,

2000). Over just the last couple of decades, numerous studies have found that certain dietary interventions, such as overall reduction of calorie intake without inducing malnutrition, have the ability to reproducibly extend lifespan and markers of healthy aging in a number of model organisms, including fruit flies (Metaxakis & Partridge, 2013), nematodes (Lakowski & Hekimi, 1998), rodents (Weindruch, Walford, Fligiel, & Guthrie, 1986), and primates (Mattison et al., 2017). CR through dietary or other means can modulate key aging-associated life history traits, such as sarcopenia, cancer, and age-related decline in physical activity (Rhoads et al., 2020), usually at the cost of early life fitness (Austad & Hoffman, 2018). As interventions such as caloric restriction (Strekalova, Malin, Good, & Cryns) have shown, alterations in nutrient intake or nutrient sensing pathways can delay not only mortality but aging-associated illnesses and life traits as well, suggesting that these dietary interventions may act directly on fundamental aging processes.

More recent studies have determined that alterations in levels of individual macro- and micro-nutrients, including total protein and individual amino acids, can mimic many of the lifespan extension and health benefits of caloric restriction (Plummer & Johnson, 2019; Solon-Biet et al., 2020). The mechanisms of these interventions and their relationships to general caloric restriction is not fully understood, but restriction of essential amino acids (EAA) (Emran, Yang, He, Zandveld, & Piper, 2014, Richardson et al., 2021), has been shown to extend lifespan in a broad range of model organisms. Studies using the nutritional geometry framework suggest that the complex interactions between relative levels of macronutrients such as protein and carbohydrates can play an important role in determining life and healthspan (Moatt et al., 2019). Interestingly, while restriction of the individual sulfur-containing amino acid methionine (Met) is sufficient to

improve lifespan and provide a number of metabolic benefits, increased methionine has been shown to abrogate many geroprotective benefits of dietary restriction (Elshorbagy et al.; Kitada, Ogura, Monno, Xu, & Koya, 2020). There is growing interest in the potential of dietary methionine restriction (MR) and MR mimetics to safely regulate key attributes of the aging process, from longevity to prevention of neurodegenerative disease and cancer. Here we briefly cover the role of MR on different physiological processes, with a focus on healthy aging and disease.

Methionine Metabolism is Implicated in Aging Physiology

Methionine Metabolism

L-methionine is a proteogenic (or protein forming) and sulfur-containing essential amino acid that is necessary for normal growth and development in most species. The other enantiomer, D-methionine, is rarely found in nature and is not necessary for protein synthesis as is L-methionine. As L-methionine is generally encoded in most organisms by the AUG codon which signals ribosomes to begin protein translation, dietary methionine plays a central role in protein synthesis and proteostasis. Methionine is an essential amino acid: it is not produced *de novo* in most animals but is ingested either directly or through consumption of animal or plant protein, after which it is predominantly metabolized in the liver. Methionine metabolism can be separated into three distinct phases: the transsulfuration pathway and the methionine and salvage cycles, all of which have been shown to modulate aging-related processes. Regulation of many important metabolites of these pathways, such as S-adenosyl methionine (SAM), S-adenosyl homocysteine (Rajabian et al., 2023), and the antioxidant glutathione, can alter physiological processes and improve negative aging-related phenotypes.

In the first step of the methionine cycle, the enzyme methionine adenyl transferase catalyzes the conversion of L-methionine to S-adenosylmethionine (SAM), an important methyl donor necessary in the methylation of proteins and nucleic acids that is implicated in a number of aging processes (Parkhitko, Jouandin, Mohr, & Perrimon, 2019, Uthus & Brown-Borg, 2006). Certain studies suggest that reduction in SAM levels is responsible for much of the pro-longevity benefits of methionine restriction (Obata & Miura, 2015; Xiao et al., 2022). Following this reaction, S-adenosylhomocysteine (Rajabian et al., 2023) is generated from SAM. SAH inhibits DNA methylation by SAM by binding to the active site of DNA methyltransferases. A recent study by Ogawa et al. indicates that SAH supplementation is sufficient to induce lifespan extension through MR effects in worms and yeast (Ogawa et al., 2022). SAH is reversibly converted by hydrolysis to L-homocysteine and adenosine by SAH hydrolase, from which the methionine cycle is completed by the regeneration of methionine from homocysteine. This conversion can happen either through the folate cycle using methionine synthase, or by betaine homocysteine methyltransferase, both of which remethylate homocysteine, forming methionine.

Additionally, homocysteine can progress into the transsulfuration pathway, where the biosynthesis of the amino acid cysteine occurs by the donation of the sulfur atom from homocysteine via an intermediate known as cystathionine. Cystathionine is synthesized through a condensation reaction from homocysteine by an enzyme known as cystathionine-β-synthase, or CBS. Cystathionine-γ-lyase catalyzes the hydrolysis of cystathionine into cysteine. The sulfur containing amino acid cysteine is a product of methionine metabolism and is implicated in many age-related processes, and in some cases, it has been shown that supplementation of cysteine to low methionine diets

improve some aspects of metabolic health (Elshorbagy et al., 2011). However, recent studies using defined diets that include cysteine while restricting methionine suggest that many of the pro-longevity and healthspan benefits of MR are independent of cysteine restriction (Lee et al., 2014).

Methionine can also be regenerated through the 5′-methylthioadenosine cycle, also called the methionine salvage pathway. The salvage pathway is involved in the production of powerful antioxidants known to be associated with aging called polyamines, which are involved in many important biological processes such as cell proliferation, protein and nucleic acid synthesis, and regulation of genes. The most common of these are Spermine, Spermidine, and Putrescine, which have been shown to regulate lifespan (Eisenberg et al., 2016). Putrescine is synthesized by ornithine decarboxylase from a starting molecule of ornithine derived from arginine. At the beginning of the pathway, adenosylmethionine decarboxylase decarboxylates SAM resulting in dcSAM, which acts as an aminopropyl donor in the production of Spermine and Spermidine from Putrescine by Spermine synthase and Spermidine synthase, respectively **(Figure 1)**. Simultaneously dcSAM is converted elsewhere in the pathway to MTA and then used to regenerate methionine. There are a few minor paralogous pathways such as 5′deoxyadenosine metabolism that contribute to methionine metabolism (Sekowska, Ashida, & Danchin, 2019).

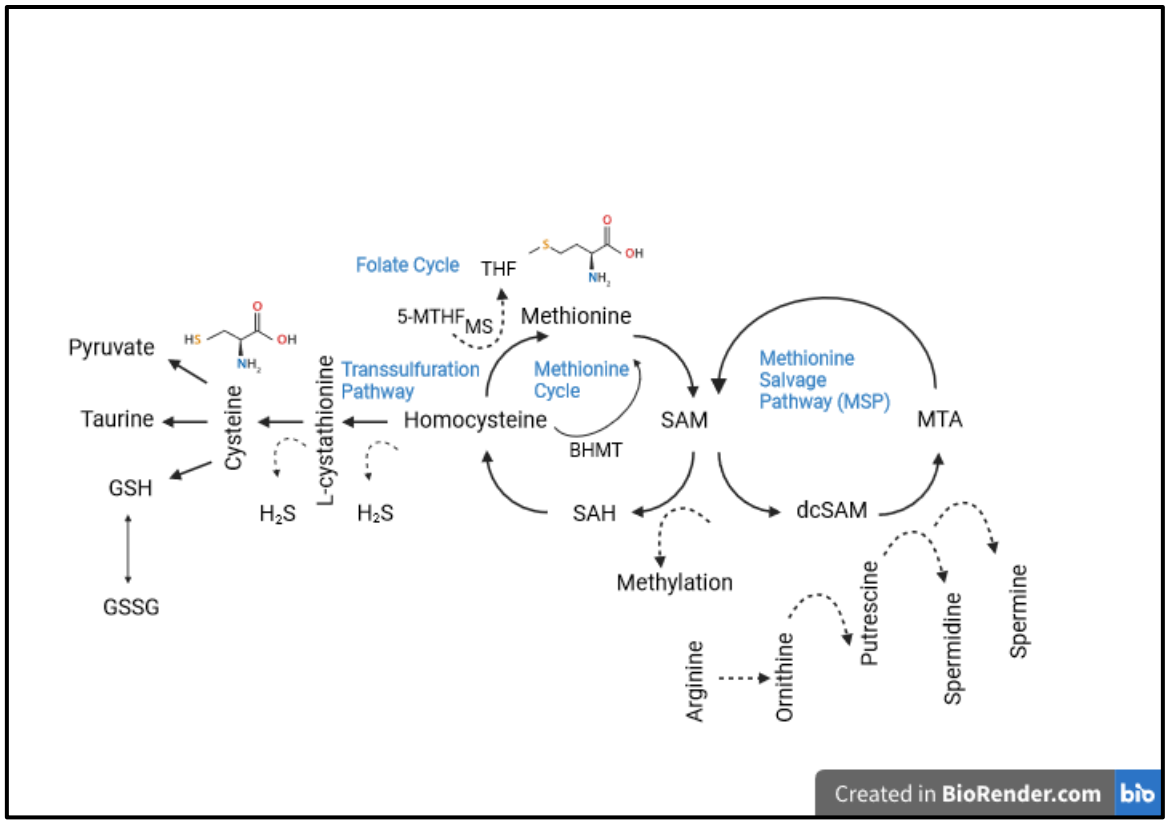

Figure 1. Methionine metabolism consists of three separate pathways: the methionine cycle, and the transsulfuration and salvage pathways, which can produce intermediates that feed back into and drive the methionine cycle. Many of the metabolites and regulatory genes involved in methionine metabolism affect aging related processes and pathologies. Created with BioRender.com.

Regulation of Mechanisms Influencing Life and Healthspan

Decreased levels of methionine have been shown to alter several other signaling pathways, many of which experience crosstalk that can make understanding the source of MR induced life and healthspan benefits difficult. There is evidence suggesting many of the longevity and metabolic health benefits of MR are influenced by increased autophagy and reduction of mitochondrial ROS production and oxidative damage. Manipulation of methionine metabolism by MR also modulates other important signaling pathways in the body with known pronounced effects on age-related phenotypes that are conserved across species such as mTORC1, insulin/IGF-1, and AMPK (Ogawa et al., 2016; Plummer &

Johnson, 2022). Several studies implicate fibroblast growth factor 21 (FGF21) as a possible mechanism of MR induced amelioration of age-related diseases and pro-longevity benefits (Lees et al., 2014; Ren et al., 2021; Sharma et al., 2019; Wanders et al., 2017).

One process that contributes to the pro-longevity benefits of MR is reduction in oxidative stress through multiple mechanisms. Autophagy, the process by which cells break down damaged, no longer useful macromolecular components and plays a critical role in the aging process and normal homeostasis and cell death, has been shown to be upregulated in several models of MR and reduces oxidative stress (Laxman, Sutter, & Tu, 2014; Plummer & Johnson, 2019). The longevity and metabolic health benefits of increased autophagy appear to be independent of FGF21 and adiponectin but may be associated with consequentially reduced levels of the methionine metabolite, S-adenosyl-homocysteine. Additionally, MR ameliorates oxidative stress through increased H_2S production (Yang et al., 2019). MR may also regulate autophagy, and by extension oxidative stress, via regulation of another of its metabolites, S-adenosyl-methionine, a precursor of SAH, both of which have been implicated in longevity extension in numerous animal models including *C. elegans* and *D. melanogaster* (Lim et al., 2023; Ogawa et al., 2016; Sutter, Wu, Laxman, & Tu, 2013; Yang et al., 2019). Methyltransferases, products of methionine metabolism that transfer methyl groups during protein synthesis, such as leucine carboxyl methyltransferase 1 (LCMT-1), have also been demonstrated to lead to an upregulation in autophagy. A number of studies have implicated the transsulfurration pathway as a potential mechanism in the lifespan extension effects of MR (Kabil, Kabil, Banerjee, Harshman, & Pletcher, 2011; MotaMartorell et al., 2021).

Insulin-like growth factor 1 (IGF-1) is a key determinant of lifespan across species and like other nutrient sensing pathways is regulated by MR. Reduction in IGF-1 levels is sufficient to extend lifespan and enact numerous metabolic health benefits (Plummer & Johnson, 2022). A pair of studies on the role of growth hormone (Rhoads et al., 2020) and IGF-1 signaling found that MR did not further extend the life and healthspan of GH/IGF-1 impaired mice, suggesting a primary role for these nutrient sensing hormones in the MR response. Additionally, male and female C57BL/6J mice subjected to continuous and intermittent methionine-restricted diets exhibit reduced insulin/IGF-1 levels compared to control mice and improved glucose tolerance. Diets low in methionine seem to produce comparable metabolic health benefits to CR in at least one study in humans (Plummer & Johnson, 2022). MR is also known to reduce mechanistic target of rapamycin (mTOR) signaling (Kitada, Xu, Ogura, Monno, & Koya, 2020; Lauinger & Kaiser, 2021; Zhao et al., 2022). Downregulation of nutrient sensing by the mTOR pathway is implicated in many of the life and healthspan benefits of CR and MR.

Lifespan Extension by MR

Methionine availability and perception can regulate numerous age-related phenotypes, such as resistance to oxidative stress and induction of autophagy. The cellular microenvironment of diseases commonly associated with aging, such as cancer and Alzheimer's, is also methionine-dependent, with much research underway to elucidate the potential of MR as a therapeutic target for these illnesses (Gao et al., 2019). Reduced intracellular methionine has also been correlated with reduced hallmarks of aging such as oxidative stress (Ying et al., 2015) and adiposity (Malloy et al., 2006) in addition to lifespan extension (Kitada, Ogura, Monno, & Koya, 2019). Though the

proximate mechanisms behind such physiological changes and their relationship to methionine metabolism are only poorly understood, MR has been demonstrated to improve a number of negative age-related metabolic phenotypes (Lees et al., 2014).

In addition to reversing many negative aging related phenotypes, the evidence that MR can robustly extend lifespan is increasingly compelling. Over the last decade, emerging research has shown that not just total caloric intake, but the source and overall amount and composition of amino acids, carbohydrates, and other macronutrients plays a significant role in determining lifespan. A significant portion of the current literature consists of studies in the inbred laboratory mouse model, *Mus musculus*, and brown rat, *Rattus norvegicus*. Orentreich et al. in 1993 were the first to demonstrate that restricting the levels of the essential amino acid methionine from 0.86% to 0.17% in the diet impaired growth and extended lifespan by up to 30% in Fischer 344 rats (Orentreich, Matias, DeFelice, & Zimmerman, 1993). Notably, these studies were performed only in male rats, potentially obscuring sex-specific effects of MR. Other studies have reported similar sex-specific life and healthspan effects in animals kept on methionine-restricted diets without malnutrition suggesting males respond more robustly to dietary MR, but the exact cause and extent of these effects is unknown (Thyne & Salmon, 2022).

Several studies have since focused on investigating the relationship between MR and aging, especially the effect of MR on lifespan. Another such study, by Sun et al. published in 2009 showed that a methionine-restricted diet initiated at midlife- roughly 12 months of age in mice, could significantly extend healthy lifespan in male CB6F1 mice (Sun, Sadighi Akha, Miller, & Harper, 2009). Multiple other studies have demonstrated pro-longevity benefits in rodents under 80% MR continuously from early and middle age (Miller et al., 2005; Mladenovic, Radosavljevic, Hrncic, Rasic-Markovic, & Stanojlovic,

2019; Richie et al., 1994). Interestingly, some long-lived organisms, such as the naked mole rat (McIsaac, Lewis, Gibney, & Buffenstein, 2016), the long-lived Ames dwarf mouse (Uthus & Brown-Borg, 2003), and long-lived mutant fly strains such as *Chico*, have altered methionine metabolism that suggests a reduction in the levels of methionine and its metabolites may contribute to increased longevity in these organisms.

Decreased intracellular methionine has been shown to be a key regulator of lifespan and pro-longevity interventions in invertebrate models, such as yeast (Z. Wu, Song, Liu, & Huang, 2013; Zou et al., 2020), fruit flies (Grandison, Piper, & Partridge, 2009), and the nematode, *C. elegans* (Cabreiro et al., 2013). Like caloric restriction, lifespan extension by MR has some caveats- in one study, only *Drosophila* kept under low amino acid conditions experienced the pro-longevity benefits of reduced methionine, while those kept on a high amino acid diet did not, in contrast to studies performed in mammals (Lee et al., 2014). Genetic models of MR induced by methioninase expression in *Drosophila* did not require low amino acid status to extend lifespan (Parkhitko et al., 2021). However, studies performed in other invertebrate models, such as *S. cerevisiae* and *C. elegans*, have shown significant increases in lifespan under conditions of low methionine (Lee et al., 2014; Lee et al., 2018). Total MR or modulation of methionine pathway metabolites such as *sams-1* has been shown to extend longevity in the roundworm *C. elegans* (Chen, Lim, Lee, Hsu, & Ching, 2020; Choi et al., 2023*)*. Two studies have shown that dietary MR and induction of a MR state by stimulated production of the primary methionine metabolite, S-Adenosyl L-methionine, extends the lifespan of *C. elegans* (Cabreiro et al., 2013; Ogawa et al., 2022). At the cellular level, a number of studies using both dietary and genetic means of inducing MR found that both dietary dilution and genetic knockdown of Met genes such as MET2 and MET15 improved yeast

chronological lifespan of the budding yeast *S. cerevisiae*, as well as replicative doubling time in mouse and human cells (Johnson & Johnson, 2014; Nacarelli et al., 2022). The above studies demonstrate the potential of MR to regulate a key aging phenotype, lifespan, as measured by different survival analyses.

Oxidative Stress Resistance is Increased by MR

Alterations in the levels of individual amino acids can also result in significant changes to other important age-related phenotypes, such as stress resistance. Increased oxidative stress is a hallmark of the aging process and age-related diseases, one which can be altered through modulation of methionine metabolism. MR reduces oxidative stress through different mechanisms: MR induces autophagy which initiates the removal of reactive oxygen species from mitochondria (Ying et al., 2015), though this effect is reversed with cysteine supplementation (Gomez et al., 2015). Additionally, MR increases production of antioxidants including glutathione, via the upregulation of the transsulfuration pathway and its principal product, Hydrogen Sulfate (H_2S). Reduction in oxidative stress may play a key role in the lifespan extension effects of MR in addition to its other benefits.

Reactive oxygen species accumulate in cells throughout the aging process from normal cellular processes as well as through endogenous errors such as mitochondrial dysfunction, and exogenous sources like radiation, causing DNA and RNA damage as well as a feedback loop of damage to the mitochondria. Though ROS are implicated as necessary in some core cellular functions such as programmed cell death and activation of the immune system, the decreased elimination of free radicals that comes with age is associated with an increase in negative aging-associated phenotypes, such as cancer and

oxidative damage causing cellular dysfunction. Numerous studies have shown that dietary MR reduces oxidative stress in a variety of animal models (Pang et al., 2023; Plummer & Johnson, 2019; Yang et al., 2023). One way that MR reduces oxidative stress is through increased autophagy, and one recent study indicates that mitophagy is required for many of the life and healthspan benefits of MR (Plummer & Johnson, 2019). Several methionine metabolites are implicated in the upregulation of autophagy (Lim et al., 2023). Cysteine synthesis via the transsulfuration pathway leads to the production of H_2S and glutathione, which function to ameliorate oxidative stress through inhibition of ROS overproduction in the mitochondria. Additionally, biosynthesis of the polyamines spermine and spermidine via the methionine salvage pathway have been shown to induce autophagy, suppress oxidative stress, and extend longevity (Hofer et al., 2021).

MR Ameliorates Age-Related Disease Pathology

Numerous studies have investigated the potential of MR to ameliorate pathologies associated with age-related diseases such as cardiovascular disease (CVD), neurodegenerative diseases, muscle loss, and cancer/tumor development. These studies are spurred by observations that L-methionine is an essential component in the production of proteins implicated in age-related diseases as well as its necessity for the growth of malignant tumors and certain kinds of cancer cells. Similar to caloric restriction or single-gene mutations that extend longevity, this is to be expected if MR is acting on one or more fundamental drivers of aging.

Reduced methionine has been shown to be associated with improved cardiac function and lower risk factors associated with cardiovascular disease (Mota-Martorell et al., 2022). One study by Ables et al. showed that dietary MR in male C57BL/6J mice

increased the production of hormones with cardioprotective functions such as adiponectin and FGF21, the latter of which has been shown to be an essential component of the metabolic benefits of MR (Ables et al., 2015). A methionine-restricted diet was shown to reduce apoptosis and alleviate the extent of damage to myocardial tissue in a mouse model of ischemia/reperfusion-induced myocardial injury through regulation of H_2S production (Pan et al., 2020). Additionally, an 80% methionine-restricted diet improved cardiac dysfunction in obese C57BL/6J mice through elevated cardiac H_2S production (Han et al., 2020). One recent notable study investigated the effects of MR and sulfur-containing amino acid restriction (SAAR) in a small (n=10 per group) controlled feeding study of adult humans over a period of four weeks and found that both interventions were well tolerated and reduced biomarkers of cardiovascular disease, albeit to a lesser degree in the MR group than the SAAR group (Richie et al., 2023). These studies suggest that MR or low-methionine vegetarian and vegan diets may ameliorate damage caused by CVD and decrease its likelihood.

Methionine intake has been found to be negatively associated with higher levels of cognitive impairment in humans and animal models and may play a role in the development of age-related diseases such as Parkinson's and Alzheimer's Disease (AD). A study of cognitive health in 658 humans aged between 60-89 and control matched based on factors like age, socioeconomic status and sex found that high methionine intake is significantly positively associated with mild cognitive impairment in both sexes (Xi et al., 2023). Interestingly, the same study found that a methionine-restricted diet improved cognitive function more in males than in females in mouse models of AD, which may be explained by a sexually dimorphic response to MR in mice that is absent in humans (Xi et

al., 2023). This study found that MR elicited its neuroprotective effects through upregulation of the cystathionine-βsynthase/H_2S pathway.

A 2021 study found that 15 and 18-month-old C57BL/6J MR treated mice performed better on behavioral assays of cognitive performance including open-field, Morris water maze, and novel object recognition tests compared to control groups (Ren et al., 2021) Ren et al. also found that MR treated mice attenuated cognitive decline in a FGF21 dependent manner, which other studies have corroborated.

Multiple studies have shown that MR can reduce the risk of age-related neurodegenerative diseases in animal models (Xu et al., 2022), though data from humans is limited. One study suggests that lower levels of methionine is associated with lower levels of the protein amyloid-beta (Aβ) that forms plaques in the brains of animals with Alzheimer's (Xi et al., 2023). Lee et al. found in one study in mice that those on a diet low in methionine had less cognitive decline in old age compared to a control diet. Additionally, one study found that MR partially rescues age-related changes in the lipidome of the rat cerebellum and frontal cortex in male Wistar rats aged to 26 months over controls (Jove et al., 2021)

As methionine is essential for cancer cell growth, MR has been extensively studied for its potential to reduce cancer proliferation due to the methionine dependent nature of some cancer types, as well as enhance the chemotherapeutic benefits of cancer drugs (Miyake et al., 2023). There is increasing evidence that methionine deprivation suppresses tumor growth and improves outcomes in clinical settings (Calderon-Montano et al.,2022; Gao et al., 2019; Jimenez-Alonso et al., 2023; Li et al., 2023; Upadhyayula et al., 2023).

MR has the potential to alter cancer metabolism and gene expression in a number of methionine dependent types of cancer including colorectal cancer (Durando et al., 2010), pancreatic cancer (Kawaguchi et al., 2018), gastric cancer (Miyake et al., 2023), and breast cancer (Strekalova et al., 2015). A study using public data from the Cancer Genome Atlas found that MR affects the expression of 330 genes that are dysregulated in colon cancer and identified four genes associated with overall survival (Zhou, Chen, & Liu, 2022).

Lastly, a methionine-restricted diet has been shown to improve outcomes in other age-related illnesses such as sarcopenia (Rajabian et al., 2023). Several studies have shown that MR improves skeletal muscle health (Swaminathan, Cesanelli, Venckunas, & Degens, 2022) and reduces aging-associated inflammation (Swaminathan, Fokin, Venckunas, & Degens, 2021) in 26-month-old C57BL/6J mice, respectively. Another such study indicates that MR improves glucose metabolism in the skeletal muscle of the middle-aged mice gastrocnemius by promoting insulin section in the pancreas (Feng et al., 2023). Taken together, these studies suggest that MR alters aging processes associated with disease and longevity through multiple mechanisms **(Figure 2)**.

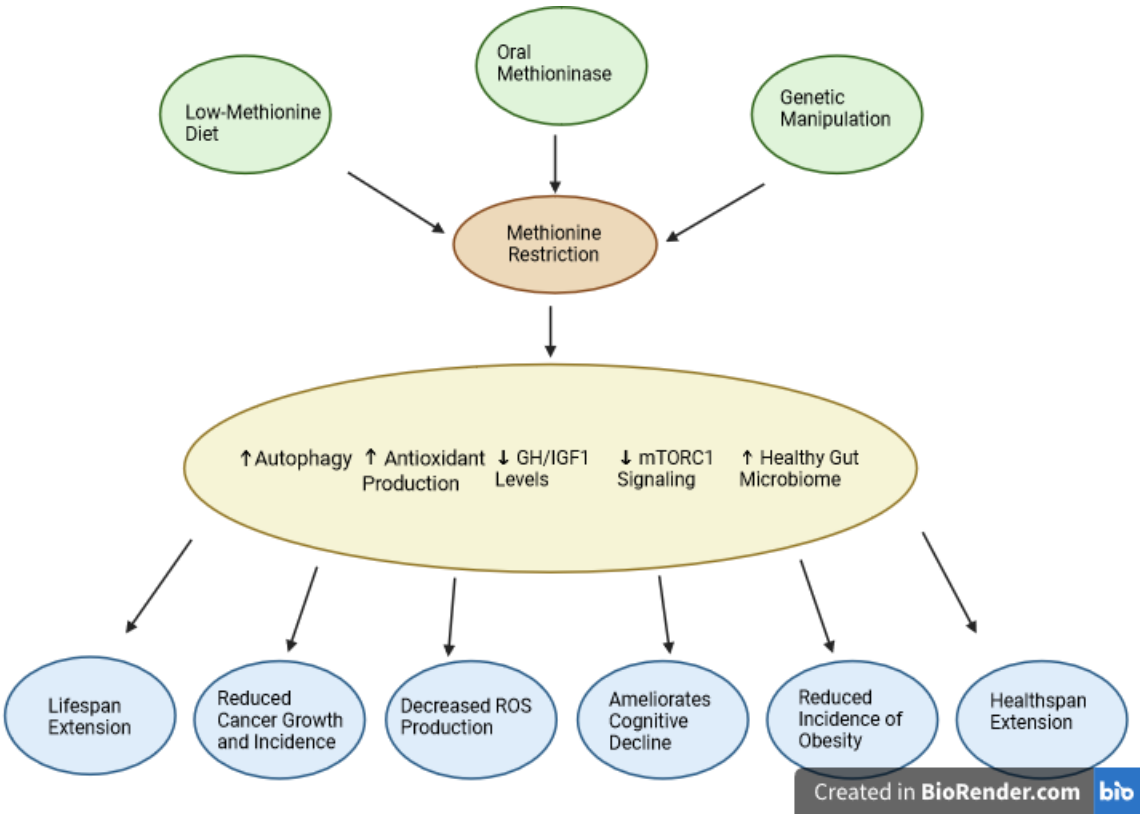

Figure 2. MR mechanisms and their potential outcomes. A wide range of dietary and metabolic manipulations affect methionine metabolism in such a way as to lead to a variety of beneficial changes to aging processes and illnesses. Improved health outcomes and lifespan extension associated with low-methionine diets have been shown to act partially through improved oxidative stress resistance and upregulated autophagy, as well as inhibition of nutrient signaling pathways like mTOR and IGF. Additional research is still necessary to unravel the complex interactions and crosstalk between these mechanisms to understand how they influence individual aging phenotypes. Created with BioRender.com

CONCLUSION

Restriction of methionine levels has been clearly shown to increase longevity and positively regulate health factors associated with aging in various animal models. However, the mechanisms that regulate life and healthspan are not clearly understood and there are multiple factors involved, from crosstalk between methionine levels and other nutrient sensing pathways to the role of the products of methionine metabolism. Thus far,

we have summarized the current state of research involving MR, the role of restriction in regulating the products of methionine metabolism, and their impact on age-related phenotypes such as lifespan and stress resistance. Recent findings indicate that genetic background, sex, and the timing of nutrient intake significantly impact the health and lifespan benefits of MR and other types of dietary restriction. Though methionine is required for normal development, the levels of methionine that are optimal for lifespan and healthy aging may vary based on these factors and may be significantly less than what is found in animal protein heavy western diets, compared to low methionine, plant-based diets such as those common among long-lived human populations such as the Japanese island of Okinawa (McCarty et al., 2009). In the future, such diets may be used for the general promotion of healthy aging or as targeted interventions in one or more aging-associated diseases.

CHAPTER 2

ABSTRACT

Methionine is an essential amino acid that has been found to be of great consequence to human health and aging. Methionine is ingested in the diet, but the amount of methionine that is optimal for aging may vary greatly depending on many factors. Restriction of this amino acid has been shown to have potential benefits for increased longevity and amelioration of age-related pathologies in a number of model organisms. However, the generality and degree to which sex and different genetic backgrounds influence the response to dietary MR remains unclear. Here we used the natural genetic variation of the Drosophila Genetic Reference Panel (DGRP) to investigate MR diet-dependent changes to survival and health. Furthermore, short and long-lived DGRP strains of both sexes were compared to a common wildtype lab strain, *w1118*. In addition to longevity, climbing ability, stress resistance, and metabolic rate were assessed. A low-methionine diet increased longevity in some, but not all, strains, suggesting that the optimal dietary methionine content may vary significantly among genotypes. Additionally, we observed a range of diet-dependent changes in weight, climbing ability, and stress resistance. No changes to resting metabolic rate were observed between control and restricted individuals within individual strains and sexes.

Interestingly, there was no clear association between beneficial health changes and survival, providing evidence that increased lifespan can be uncoupled from stress resistance and climbing ability under low-methionine diets. Broadly, this study suggests a fundamental role of sex and genotype in determining diet responsiveness among aging-associated traits in flies.

INTRODUCTION

Many important aging processes are influenced by diet and nutrient signaling, and it is now known that altering intake of even individual macromolecules is sufficient to enact large scale changes to lifespan and organismal health and fitness (Grandison, Piper, & Partridge, 2009; Juricic P, Grönke S, Partridge L 2020). The sulfur-containing amino acid methionine is one such dietary component that has been shown to have protective benefits against aging-related illnesses and to improve longevity across multiple laboratory animal models when its intake is restricted in the diet including in fruit flies (Lee et al., 2014) rats (Orentreich, Matias, DeFelice, & Zimmerman, 1993), and mice (Sun, Sadighi Akha, Miller, & Harper, 2009). However, the complex relationship between factors such as genetic background, sex, and environment and the mechanisms by which restriction of single amino acids such as methionine influence different aspects of aging is not well understood.

Previous studies have suggested MR seems to bear some similarities to other dietary interventions such as caloric restriction, in their modulation of nutrient signaling pathways like GH/IGF and mTOR, and reduction in reactive oxygen species that damage cells and tissues during aging (Gu et al., 2017). To answer questions about the role of methionine in regulating aging phenotypes, recent studies have used defined diets to allow the manipulation of individual dietary components including amino acids (Troen et

al., 2007; Lee et al., 2014). Several diets adjusted to the specific daily dietary needs of different species have been manufactured for use by different research companies and suppliers, including the diet used in the initial MR rat study by Orentreich et al. (1993), but most of these studies consistently utilize a similar ratio of approximately 0.86% methionine in the control diet and 0.017% methionine in the restricted group as determined by the Orentreich and later (Richie et al., 2014) studies that have proven to be effective at extending lifespan. Notably, previous research using defined diets in *Drosophila* have reported that low amino acid status is required in addition to methionine reduction to see similar lifespan effects shown in MR studies in other animal models (Lee et al., 2014), but some recent studies (Kosakamoto et al., 2023; (Parkhitko, Wang, Filine, & Perrimon, 2019) have disputed this.

Prior research has utilized the publicly available sequenced genotypes of the inbred *Drosophila* Genetic Reference Panel (DGRP) (Mackay et al., 2012) extensively to investigate the role of genetic variation in determining phenotypic variation in life history traits, including survival. These studies investigating the effects of genetic variation on aging-related traits have found that lifespan extension is not always associated with improved healthspan (Wilson et al., 2020; Bansal, Zhu, Yen, & Tissenbaum, 2015). One of the first studies to show that longevity can be uncoupled from stress resistance was Burger & Promislow (2006), who showed the phenotypic plasticity of longevity of *Drosophila* in cold-stressed conditions. However, multiple previous studies have suggested that MR extension of lifespan is associated with reduced oxidative stress (Ying et al., 2015; Yang et al., 2019; Pang et al., 2023). If the beneficial effects of MR are the

result, partially or otherwise, of improved oxidative stress resistance, it remains to be characterized in *Drosophila*.

In this study, we used 28 *D. melanogaster* strains from the DGRP to determine the role of genetic variation in mediating the survival response of both sexes to MR. We focused on three relatively short and long-lived strains from the DGRP and compared them to the commonly used lab strain, w^{1118}. To assess the role that genetic background and sex play in determining life and healthspan outcomes under MR, we utilized a fully defined medium to assay diet-dependent changes in health and longevity (Troen et al., 2007; Lee et al., 2014). Here, we demonstrate significant effects of genetic variation and sex on lifespan and phenotypes thought to be associated with MR-dependent lifespan extension such as oxidative stress resistance and oxygen consumption rate. The results of our study establish no clear correlation between these mechanisms and lifespan extension across different genetic backgrounds and sexes. We further report multiple diet-dependent changes in body weight and climbing ability, a measure of organismal health in fruit flies, that are genotype and sex-specific.

METHODS

Fly Stocks and Husbandry

Fully sequenced, well characterized inbred *Drosophila* strains derived from wild caught Raleigh, USA flies from the DGRP (Mackay et al., 2012) were used for this study. *D. melanogaster* w1118 and multiple DGRP lines (Mackay et al., 2012) were obtained from the Bloomington Drosophila Stock Center (BDSC) and are listed in the

supplemental information with their genotype and BDSC stock number. Flies were maintained on a standard corn meal diet and housed in a temperature-controlled Percival incubator at 25°C with a 12-hour light/dark cycle at 60% humidity. Fly strains were transferred to fresh corn meal food every two weeks and allowed to mate and lay eggs for 24-48 hours, after which adult flies were removed and larvae allowed to develop. Virgin experimental flies were collected within 8 hours of eclosion over a 24-48 hour period under light CO2 anesthesia, sorted by sex, and transferred to corn meal food until assays. Three-day old virgin flies were placed on the experimental diet and transferred to fresh vials every 48-72 hours until death.

Fly Media and Experimental Diet

Strains were raised on standard corn meal food common to the BDSC and Pletcher labs containing 10g agar (Genesee Scientific), 60g corn meal (Genesee Scientific), 25g yeast (Genesee Scientific), 55g Dextrose (Genesee Scientific), 30g Sucrose (Genesee Scientific), and 3ml propionic acid (Sigma) per liter of water. The experimental diet consisted of a fully defined custom diet (Diet TD.10417) ordered from Envigo Teklad based on a diet used by Troen et al. (2007) which contains exact proportions of amino acids minus methionine, to which is added 10g agar (Genesee Scientific), 45g dextrose (Genesee Scientific), 35g sucrose (Genesee Scientific), 500 mg ribonucleic acid from Torula yeast (Sigma), and 3ml propionic acid (Sigma). Components were boiled in 1L of water, with the propionic acid being added after cooling to below boiling to prevent loss due to vaporization. Corresponding amounts of L-methionine to

the amino acid ratio of the defined diet were added to the control (1mM) and Restricted (0.15mM) diets, respectively.

Table 1. Composition of *Drosophila* media fed to control and experimental animals

Ingredient	**0.4x AA Control (g/L)**	**0.4x AA Met Restricted (g/L)**
Agar	10.00	10.00
Sucrose	35.00	35.00
Dextrose	45.00	45.00
amino acid mix (w/o Met)	40.68	40.68
- Protein	15.17	15.17
- Dextrose Monohydrate	22.05	22.05
Propionic acid	3.00	3.00
Ribonucleic acid from Torula Yeast	0.50	0.50
L-Methionine	0.15	0.02
Total		
Total Protein (g/L)	15.17	15.17
Total Sugar (g/L)	102.05	102.05

Lifespan Analysis

For all cohorts, virgin flies of each treatment group were collected for each genotype. Newly eclosed virgin *D. melanogaster* were collected within 8 hours over a 24–48-hour period and separated by sex under light CO_2 anesthesia before being placed temporarily on standard corn meal food. Experimental flies were then transferred to control and restricted diets on day three with approximately 25 flies per vial. Flies were transferred to fresh food of the assigned diet every 2-3 days at which point deaths were recorded and removed. Fly deaths were recorded until all flies were censored or deceased. Lifespan assay flies were kept at 25°C in the same Percival incubator as stocks with a 12-hour light/dark cycle at 60% humidity.

Stress Resistance Assays

Oxidative stress resistance under MR was assayed across strains using a 1mL solution of 5% sucrose and 10mM of the oxidative stressor, paraquat (Sigma-Aldrich). Flies were placed into vials containing half a Kim wipe treated with the paraquat solution and transferred by flipping onto new vials every 8 hours, at which point deaths were recorded. This process was repeated until all flies were deceased. Oxidative stress resistance experiment flies were kept at 25°C/60% humidity.

Negative Geotaxis

Negative geotaxis assays for each strain were performed as follows. Climbing assays were performed once a week on days 7, 14, and 21. Randomized control and treatment flies were transferred to empty vials and the number of flies capable of climbing past a 5cm line on the vial within 10 seconds was assayed by gently tapping the flies to the bottom of the vial. The number of flies past the 5cm line after 10 seconds was recorded. Negative geotaxis assays were repeated three times for each vial. Flies were acclimatized for approximately 1 minute before and after each replicate.

Microplate Respirometry

Oxygen consumption rate (OCR) was measured using the Loligo Systems Microplate System. W^{1118}, *Ral913*, *Ral443*, and *Ral59* strains were measured at young (Days 5-6) and aged (20% mortality) time points. The OCR of individual flies from each of the four strains was measured by placing one fly into 20 different randomized 80 µl wells of a glass 24 well Loligo Microplate. Individual wells contain an oxygen sensor that records the concentration of oxygen in each well at numerous time points throughout each experiment. Four of the twenty-four wells were left blank as negative controls, with the difference between empty wells and wells containing Drosophila representing oxygen consumption by the organism. The MicroResp software reports the oxygen concentration over time in mmol/L.

Following transfer to the UAB Nathan Shock Center Comparative Organismal Energetics Core and a period of acclimation, Loligo plates were run for each of the four strains, with 20 flies per plate, 5 of each sex and treatment. To do this, vials of flies were

placed on ice to render them temporarily unconscious, then single flies were placed in wells according to randomization performed by the Loligo MicroResp software. Well plates were then sealed using waterproof, airtight sheets of PCR film and placed in an incubator at 25°C for the duration of the experiment. Microplates were allowed to record oxygen consumption for approximately 60 minutes. The first 15 minutes of measurements were excluded from the data analysis step due to past experiments optimizing the Micro Loligo System that determined this was sufficient to allow flies to acclimatize. Additionally, experiments were performed consistently during the hours of 11am-2pm to avoid confounds from time-of-day effects identified in past Microplate runs.

Statistical Analysis

All statistical tests were performed utilizing R statistical software (version 4.3.0), except where otherwise stated. For survival analysis of longevity and stress resistance, R packages 'survival,' 'survminer,' and 'ggplot2' were used to plot the survival function and log-rank tests were performed for statistical differences between survival curves. Analyses for survivorship were performed on pooled data from two cohorts for control and restricted diets. Sample sizes were based off similar previous experiments in the Austad lab and related literature, not predetermined using statistical software. Climbing ability and metabolic rate were plotted using Prism version 10.1.2 (GraphPad). Unpaired t-tests were used for the analysis of fly climbing ability across strains. Flies that escaped were censured from the final analysis.

RESULTS

Survival Response

First, we assessed the reported ability of MR to extend lifespan in 28 strains from the DGRP of different genetic backgrounds of both sexes. We found that a low-methionine diet elicits a diverse range of responses across the DGRP, from an approximately 14% increase to a 36% decrease in median longevity, to no significant effect whatsoever **(Figure 3A, 3B; Table 2).** Interestingly, short-lived (<35 days median longevity) flies from both sexes were not found to be significantly responsive to the intervention, despite previous studies showing larger effect sizes in short-lived flies. A comparison of all strains shows that generally, females were more responsive than males, with the highest increase and decrease in lifespan both belonging to females **(Figure 3A).** Interestingly, the Ral799 strain had one of the largest lifespan increases (in females), as well as the largest lifespan decrease (in males).

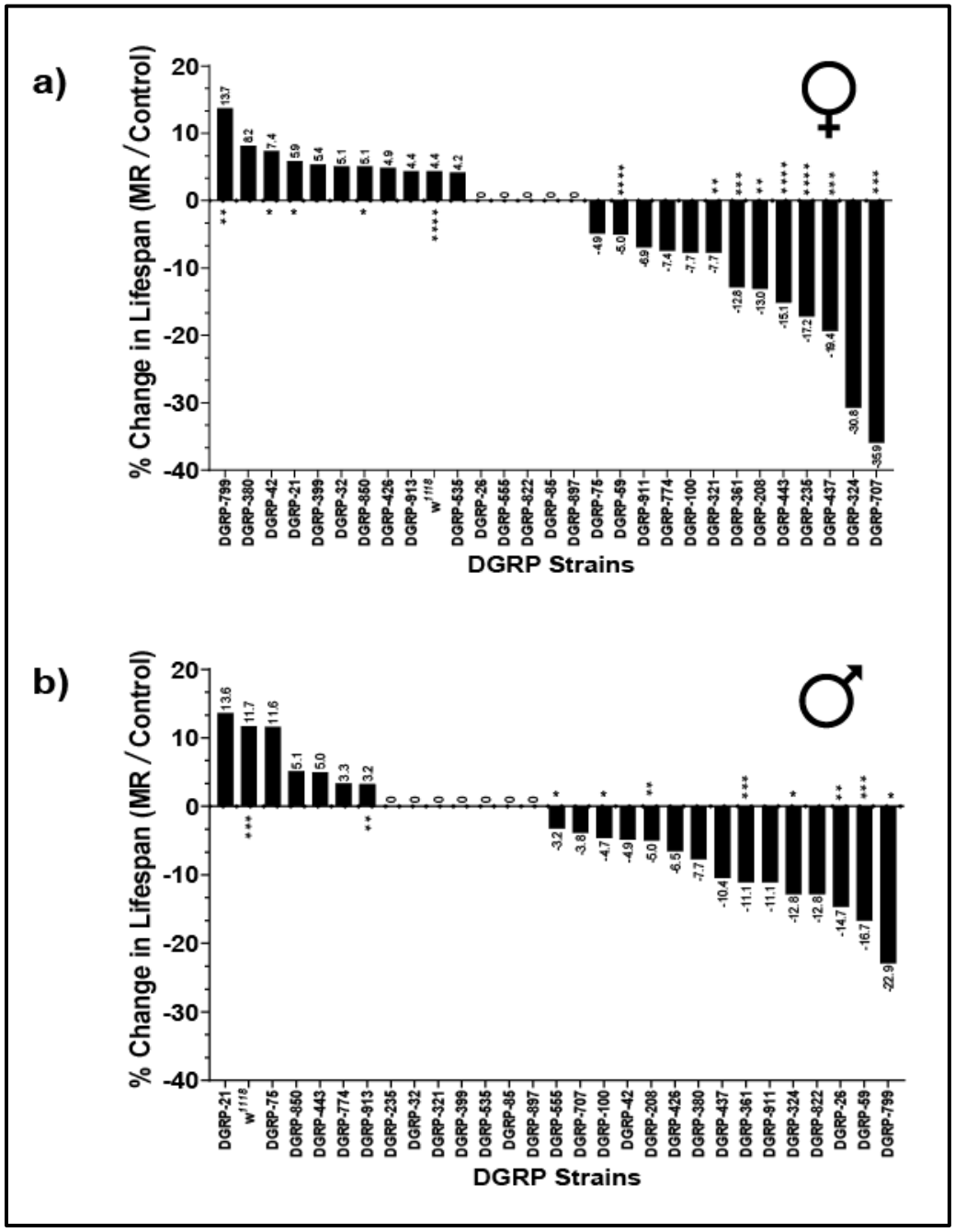

Figure 3. Genetic background and sex influence response to MR. Observed changes in median longevity across DGRP strains as well as w^{1118} when subjected to a low-methionine diet are shown in descending order. N = 100-175 per group. Data were analyzed via log-rank test.

Next, to assess how sex and genetics impact the pro-longevity benefits of MR, we further investigated the effects of a low methionine defined diet on the survival of four genotypes of both sexes, short-, medium-, and long-lived DGRP strains plus a commonly used lab strain. W^{1118}, *Ral 443*, *Ral 59*, and *Ral 913* strains were placed on long-term methionine-restricted diets beginning 3 days after eclosion. Males had higher median lifespans in each of the four genotypes tested. Median lifespan ranged from a low of 40 days to a high of 64 days in males and 38 days to 53 days in females. Survival curves show a significant decrease in lifespan for Ral443 females under MR ($p < 0.0001$), as well as for Ral59 males and females ($p < 0.0001$), suggesting that the longevity benefits of MR is not as ubiquitous as prior studies have suggested **(Figure 4).** However, Ral913 males and females had a significant positive effect of treatment on lifespan ($p = 0.007$ and $p = 0.05$ respectively), as well as the w^{1118} strain, highlighting the importance of testing interventions in more than just a few well-known genetic backgrounds **(Figure 4).**

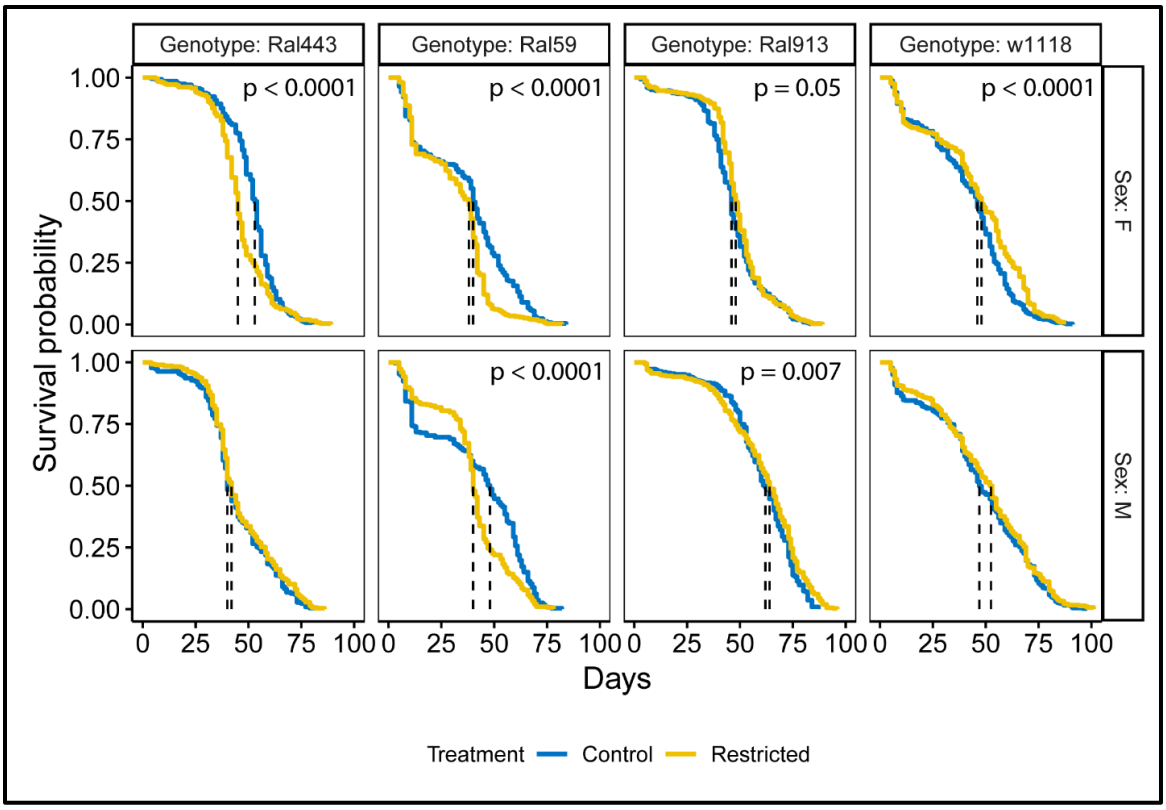

Figure 4. Variation in survival response to MR across strains and sexes. Survival probability is shown on the Y-axis and survival time in days on the X-axis. Significant differences were determined by log-rank test between control and restricted groups of each condition.

Table 2. Median Lifespan of Control and Restricted DGRP Flies

	Males			Females		
Strain	**Median MR Lifespan (days)**	**Median Control Lifespan (days)**	**% Change**	**Median MR Lifespan (days)**	**Median Control Lifespan (days)**	**% Change**
DGRP-100	41	43	-4.65	36	39	-7.69
DGRP-208	57	60	-5.00	60	69	-13.04
DGRP-21	25	22	13.64	36	34	5.88
DGRP-235	48	48	0.00	53	64	-17.19
DGRP-26	29	34	-14.71	32	32	0.00
DGRP-32	32	32	0.00	41	39	5.13
DGRP-321	39	39	0.00	36	39	-7.69
DGRP-324	34	39	-12.82	27	39	-30.77
DGRP-361	32	36	-11.11	34	39	-12.82
DGRP-380	36	39	-7.69	53	49	8.16
DGRP-399	39	39	0.00	39	37	5.41
DGRP-42	39	41	-4.88	29	27	7.41
DGRP-426	43	46	-6.52	43	41	4.88
DGRP-437	60	67	-10.45	50	62	-19.35
DGRP-443	42	40	5.00	45	53	-15.09
DGRP-535	25	25	0.00	37.5	36	4.17
DGRP-555	60	62	-3.23	41	41	0.00
DGRP-59	40	48	-16.67	38	40	-5.00
DGRP-707	25	26	-3.85	25	39	-35.90
DGRP-75	48	43	11.63	39	41	-4.88
DGRP-774	62	60	3.33	25	27	-7.41
DGRP-799	37	48	-22.92	58	51	13.73
DGRP-822	34	39	-12.82	25	25	0.00
DGRP-85	30	30	0.00	23	23	0.00
DGRP-850	41	39	5.13	41	39	5.13
DGRP-897	32	32	0.00	39	39	0.00
DGRP-911	32	36	-11.11	27	29	-6.90
DGRP-913	64	62	3.23	48	46	4.35
w^{1118}	52.5	47	11.70	48	46	4.35

MR Elicits Context Dependent Changes to Body Mass

Finally, we wanted to test whether MR is associated with decreased body weight in either sex. Prior studies have not shown any significant effects of MR on body mass in *Drosophila*. However, some dietary interventions have been shown to reduce body weight and can impact aging and health processes via this mechanism. To establish whether MR decreases body weight, we recorded body weight at approximately 1 week after eclosion as the average of five flies **(Figure 5A, B).** Females are generally larger than males, which is common in *Drosophila*. There was no effect of treatment across different genotypes of both sexes, except in w^{1118} females, which showed a small but significant ($p = 0.021$) decrease in body mass **(Figure 5B).** Our results suggest that while MR can alter body weight in some cases, genotype and sex have a stronger overall effect on body mass.

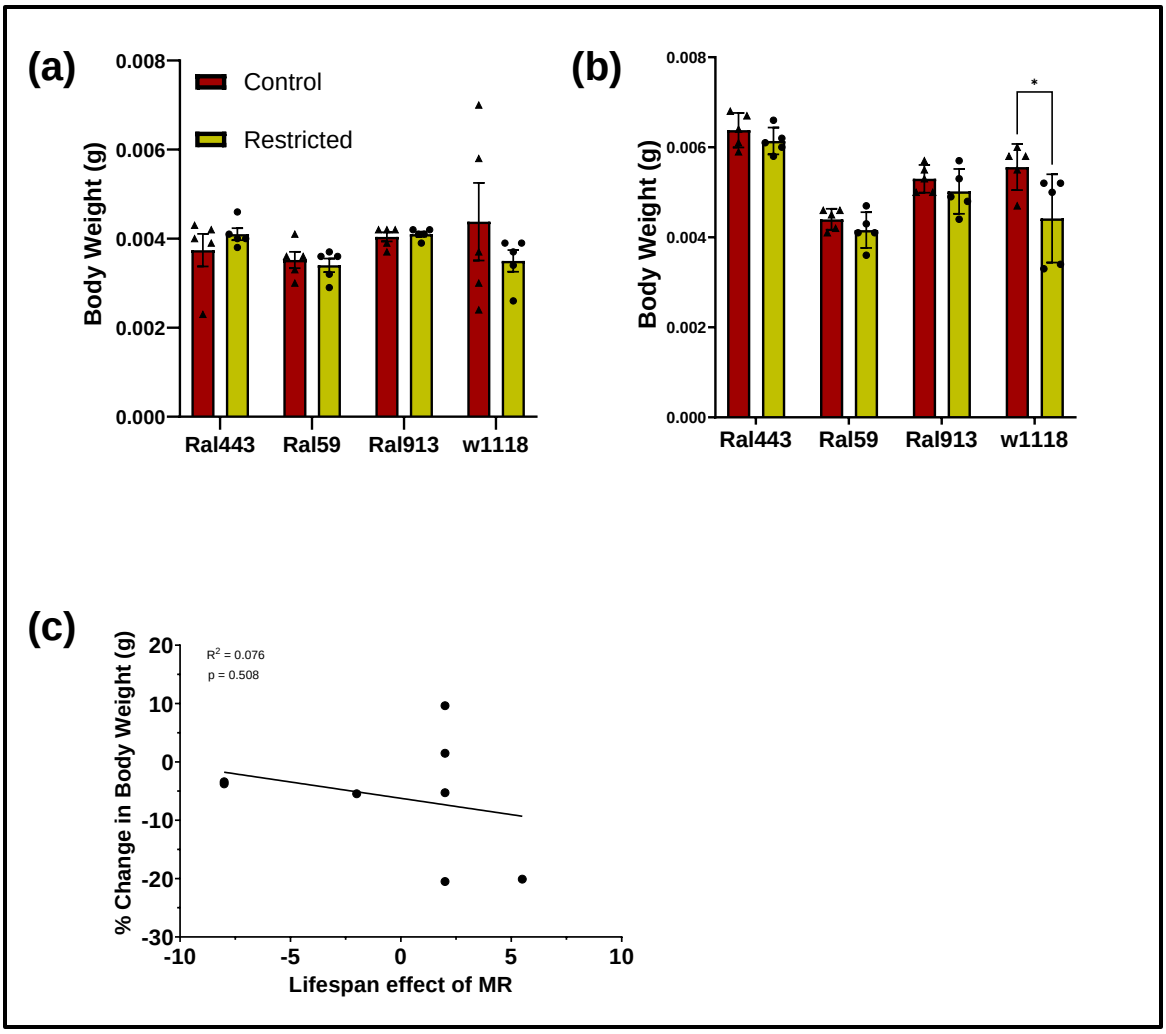

Figure 5. *w*^{1118} flies have reduced body weight under MR. a) no significant changes in male body weight on MR diet **b)** Females of the w1118 see a reduction in body weight when treated with a methionine-restricted diet (p = 0.0214). Individual data points represent the mean body weight of five flies. N = 5. Significance is determined by multiple unpaired t-tests. c) There was no significant overall impact of MR on body weight (p = 0.508).

Climbing Performance, Longevity, and MR

MR has been shown to replicate many of the beneficial health effects of other dietary interventions, such as caloric restriction. Caloric restriction improves markers of physical performance, including climbing ability in flies, which declines with age and is considered a common measure of *Drosophila* healthspan and physical function. To assess

climbing ability in the selected strains, we utilized Drosophila's inclination for negative geotaxis to stimulate their natural climbing reflex in response to the gentle tapping of an empty vial on the surface. In contrast to previous studies (Wilson et al., 2020; Bansal, Zhu, Yen, & Tissenbaum, 2015), improved lifespan was not associated with improved climbing ability in any of the four strains that we tested in either sex **(Figure 7).** Additionally, MR did not significantly ameliorate age-related decline in climbing ability in any of the conditions tested, in contrast to previous studies that have shown reduced age-related deficits in physical performance in MR flies **(Figure 6C, 6D)**. One genotype, *Ral443*, had reduced climbing performance ($p = 0.019$) in males on the low-methionine diet at day 5 **(Figure 6A).** Young female flies on the low methionine diet did not have significantly different climbing ability from controls **(Figure 6B).**

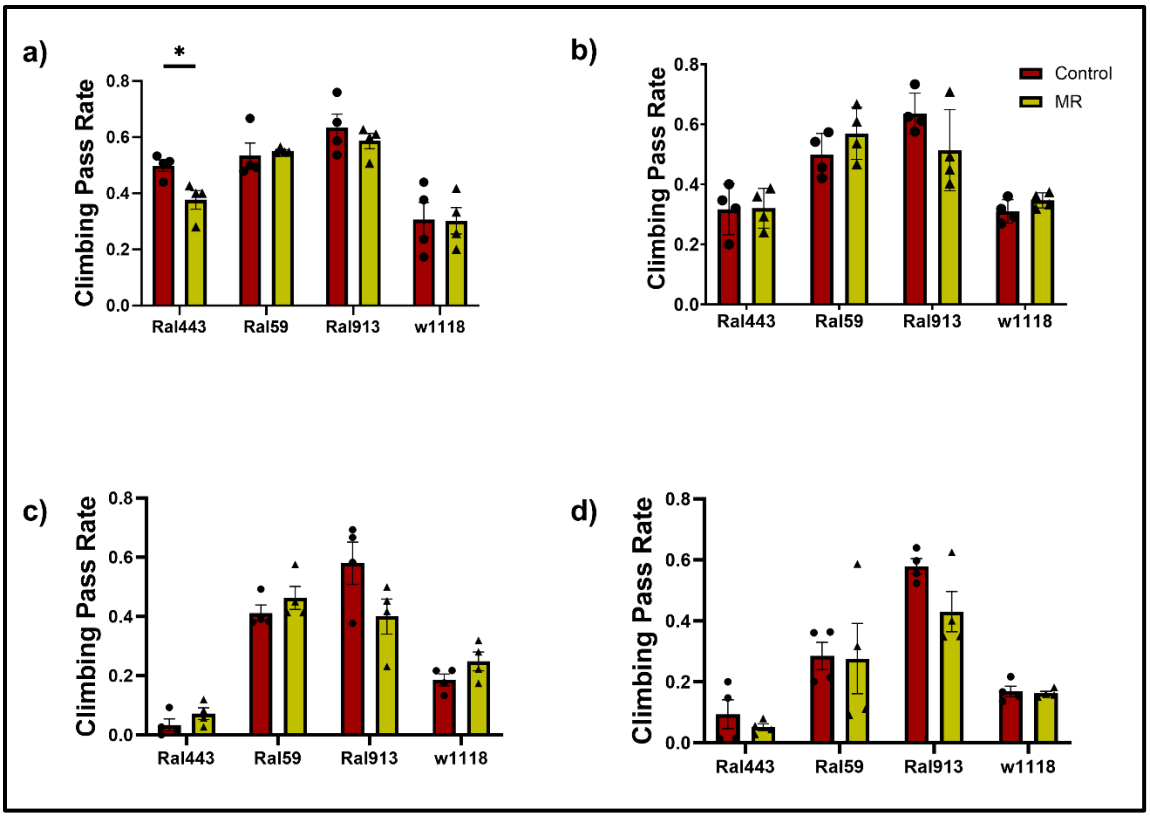

Figure 6. Climbing ability declines with age and is not ameliorated by a methionine-restricted diet. (A) Ral443 males on a restricted diet have decreased climbing ability at Day 5 compared to control flies. (B) climbing ability of female DGRP flies at Day 5. (C) Male climbing ability at Day 21. Climbing performance decreases with age in male DGRP flies but is not influenced by MR. (D) Female climbing ability at Day 21. Female DGRP flies also experience age-related declines in climbing ability, but no significant effect of treatment. N = 4-5 vials, individual points represent averages of vials. Significance is determined by multiple unpaired t-tests.

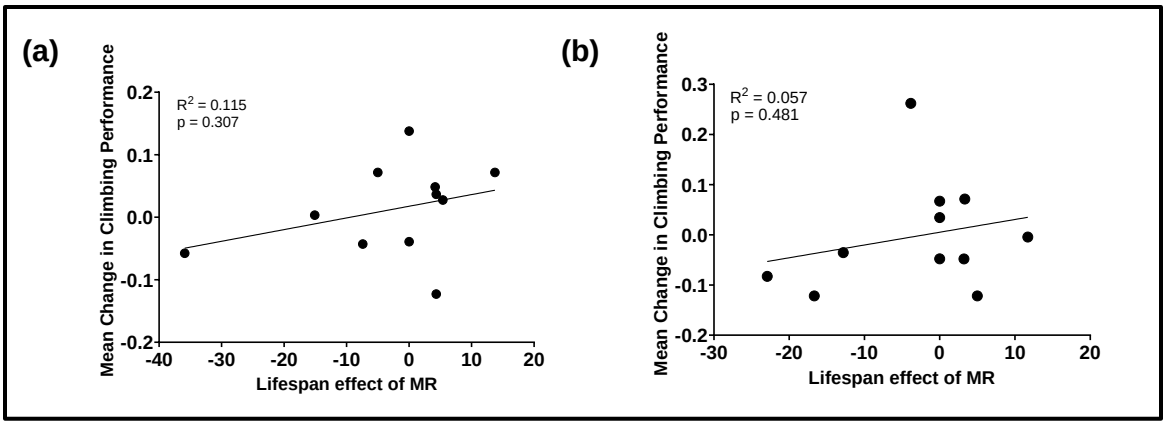

Figure 7. Relationship between the survival effect of MR and climbing performance in: (a) female and (b) male DGRP flies. As shown, there is a nonsignificant positive relationship between improved longevity and increased climbing performance. Each dot represents individual DGRP strains.

Effects of MR on Metabolic Rate

Additionally, we wanted to look at another potential mechanism involved in regulating changes in aging processes under MR, metabolic rate. Increased metabolic rate has been associated with reduced lifespan in past studies, and as such, is a proposed mechanism by which diet can act on aging-associated phenotypes. For this experiment, we compared the normalized oxygen consumption rate (OCR) of individual Drosophila measured by a microplate respirometry assay to determine if either diet resulted in altered metabolism at either 5 days of age or in an aged condition determined by recording fly deaths until 20% mortality was reached. There was no effect of treatment on OCR in any of the four genotypes tested **(Figure 8).** Sex and genotype showed a stronger impact on all three strains tested than did either the control or low-methionine diet at either timepoint. Irrespective of genotype and diet, there was a significant positive relationship between median longevity and OCR in the aged **(Figure 9b)**, but not young, flies **(Figure 9a)**, which is contrary to the rate of living theory of aging. Analysis of variance indicated

a significant effect of genetic background and sex at both Day 5 and 20% mortality, but not of genotype, sex, and treatment **(Table 1).**

Table 3. ANOVA results of oxygen consumption rate for control and MR flies

	DF	**F value**	**P value**
Age: 5 Days			
Genotype	2	10.798	**5.31E-05**
Sex	1	15.846	**0.000125**
Treatment	1	4.529	**0.0356**
Genotype:Sex	2	3.6383	**0.02835**
Genotype:Treatment	2	0.0422	0.437146
Sex:Treatment	1	0.1417	0.097231
Genotype:Sex:Treatment	2	0.0105	0.812734
Age: 20% Mortality			
Genotype	2	12.155	**1.73E-05**
Sex	1	21.763	**8.89E-06**
Treatment	1	2.427	0.1222
Genotype:Sex	2	3.378	**0.0377**
Genotype:Treatment	2	1.187	0.3089
Sex:Treatment	1	2.888	0.0921
Genotype:Sex:Treatment	2	1.397	0.2518

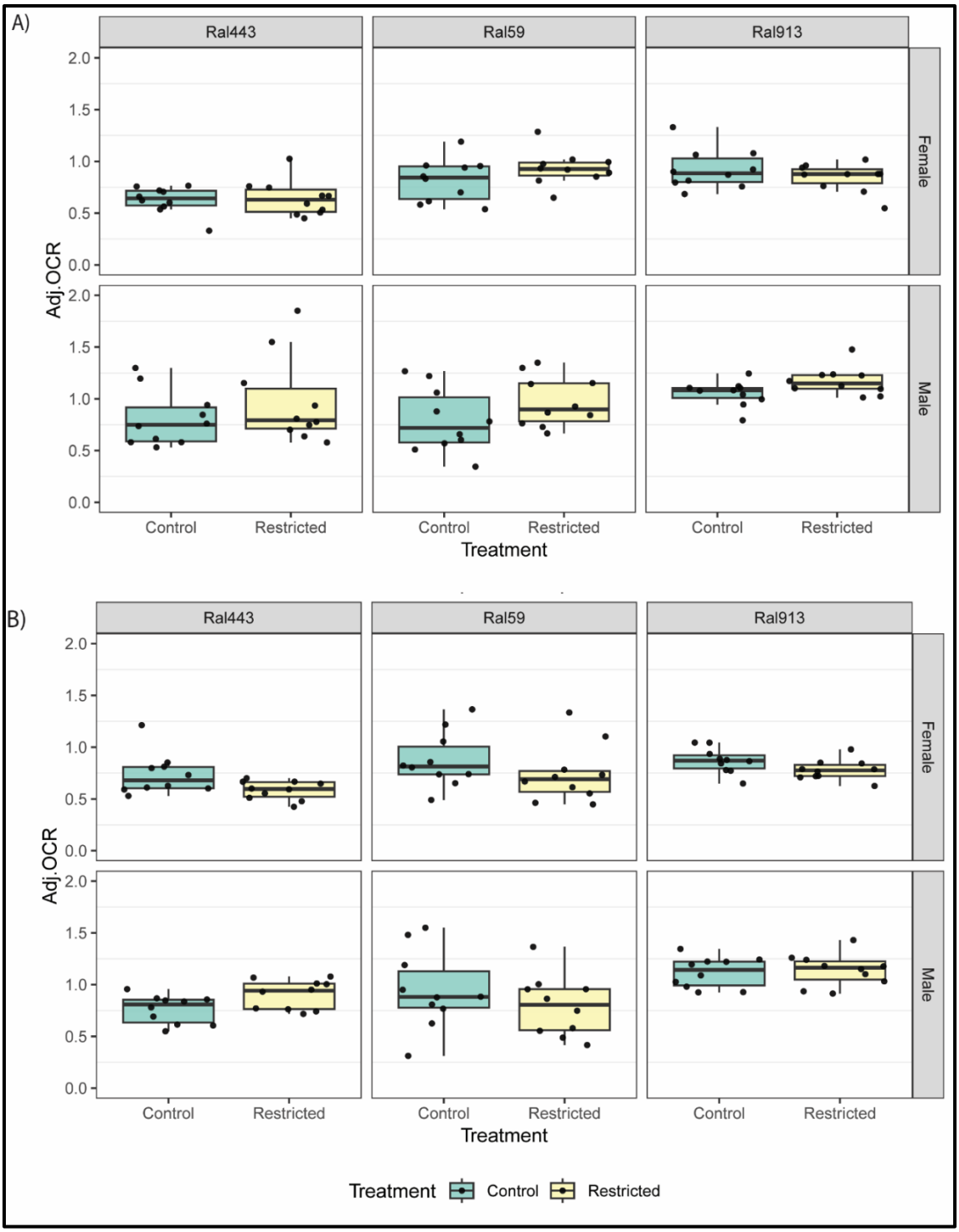

Figure 8. Oxygen consumption rate of methionine-restricted *D. melanogaster* is unaltered by MR. (A) Control flies had a consistently lower metabolic rate than methionine-restricted flies at 5 days of age, though the difference was small, and none approached significance. (B) Oxygen consumption rate of Drosophila at approximately 20% mortality. Two-way ANOVA showed significant effects of sex and genotype, but not treatment, at either timepoint. N = 10

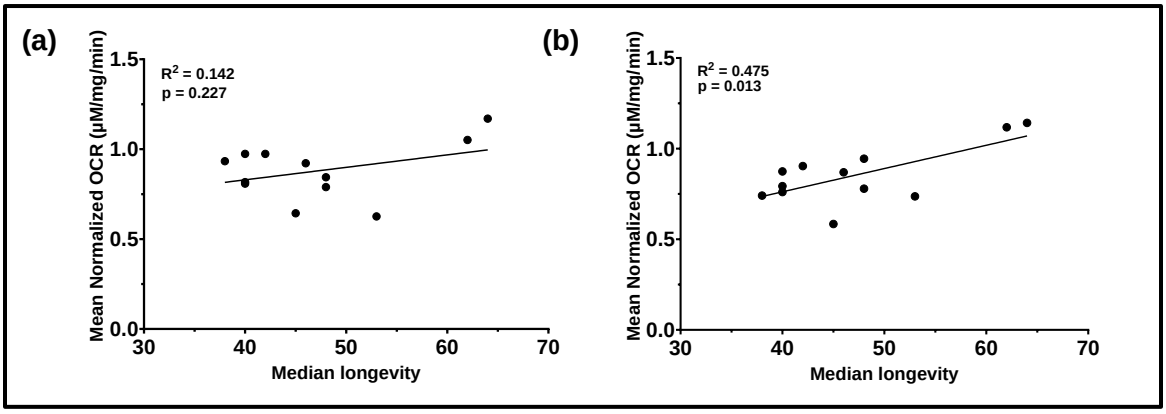

Figure 9. Effect of metabolic rate on longevity in: (a) young (Day 5) and (b) aged flies at 20% mortality. There was a significant positive relationship between median longevity and increased metabolic rate in the aged cohort.

Oxidative Stress Resistance

Next, we investigated the relationship between a methionine-restricted diet and oxidative stress resistance. Methionine is known to stimulate ROS production, and likewise restriction of methionine has been proposed to extend lifespan through the production of polyamine antioxidants and the inhibition of ROS overproduction in the mitochondria (Ying et al., 2015; Yang et al., 2019; Pang et al., 2023). Treatment of control and methionine-restricted flies with the oxidative stressor, paraquat, resulted in significant changes to multiple subpopulations, from increased stress resistance to decreased survival when treated with the stressor **(Figure 10).** Particularly, *Ral443* males ($p = 0.002$) and females ($p = 0.0001$), have reduced stress resistance on the MR diet, while *w1118* males have improved stress resistance ($p = 0.023$) in the young cohort **(Figure 10a)**. In the aged cohort, only *Ral443* experienced any amelioration of age-related decline in oxidative stress resistance ($p = 0.0002$) **(Figure 10b)**. Comparison of stress resistance with the survival effect of MR showed no positive relationship between

lifespan and oxidative stress resistance at 20% mortality, but a weak positive association in young flies **(Figure 11)**. Our results indicate that MR does improve oxidative stress resistance under some conditions, however, it can also result in lower stress resistance in some strains. This suggests that though MR may partially enact its effects on the aging process through mediating oxidative stress, this may not be the only mechanism involved in regulating life- and healthspan under these conditions.

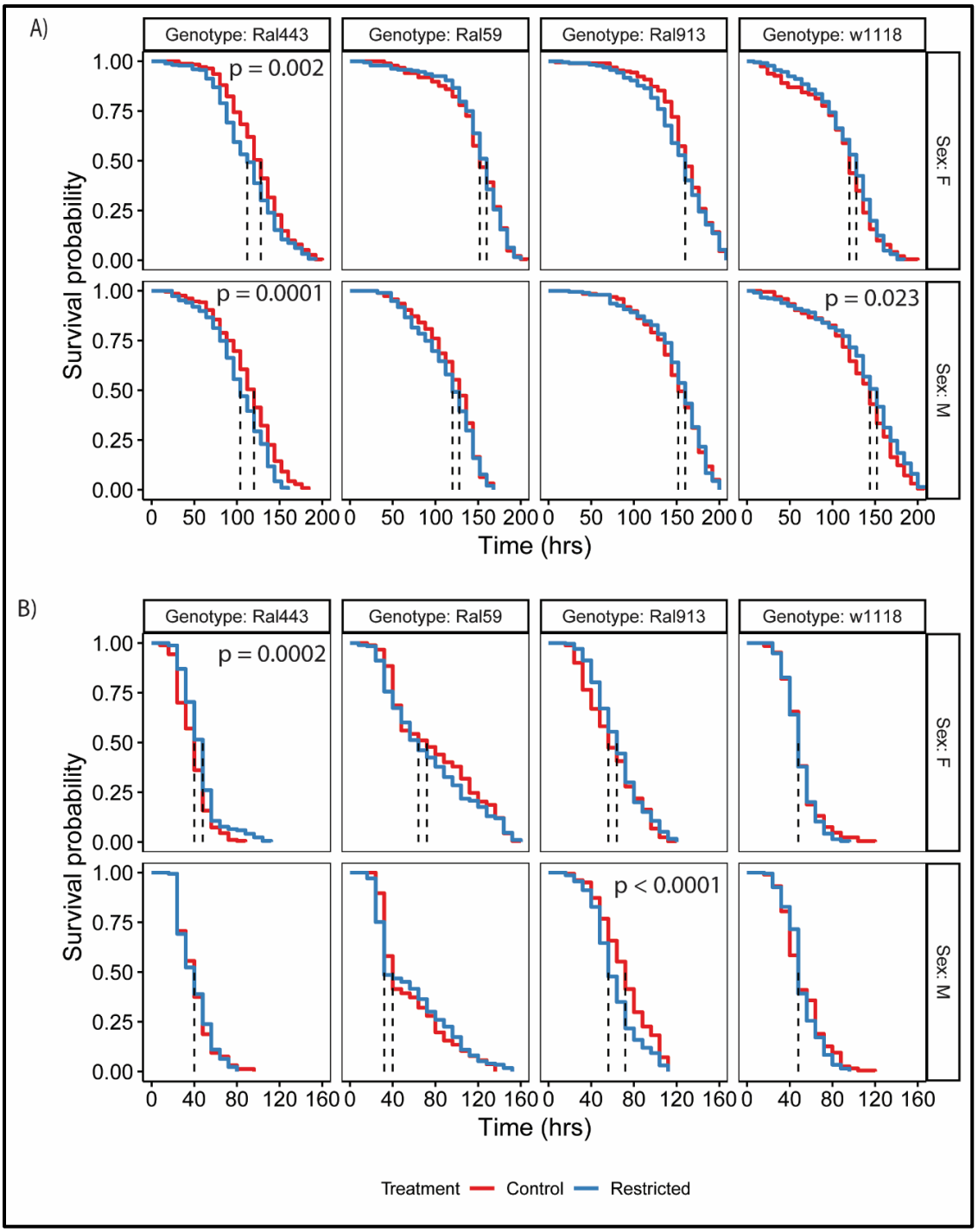

Figure 10. Oxidative stress resistance varies across sexes and genotypes. (A) Ral443 males (p = 0.002) and females (p = 0.0001), have reduced oxidative stress resistance at Day 5 of the treatment. *w1118* males see a small increase in stress resistance (p = 0.023) **(B) Oxidative stress resistance declines with age and is reduced in all strains at 20% mortality.** Ral443 females in the aged condition see a small increase in their oxidative stress resistance (p = 0.0002), while Ral913 males have reduced stress resistance (p < 0.0001). Significance based on log-rank test.

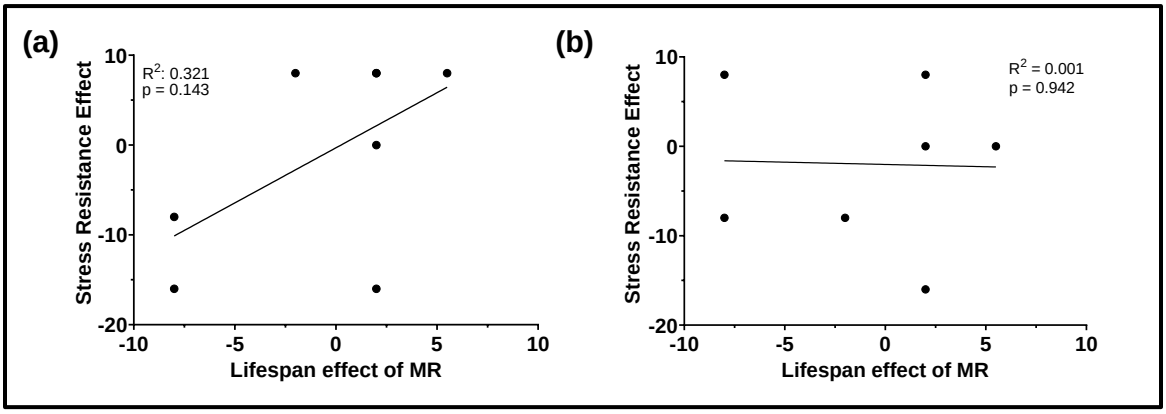

Figure 11. Effects of MR on median lifespan plotted against oxidative stress resistance in: (a) young (Day 5) and (b) aged (20% mortality) flies. There was no significant impact of MR on stress resistance in aged flies, but there was a weak correlation between the MR effect and stress resistance response in young flies.

SUMMARY AND CONCLUSIONS

The impact of dietary interventions on the aging process is complex and multifaceted. Restriction of individual amino acids, including the amino acid methionine, has been shown to alter lifespan and affect the health of model organisms from rats to flies (Grandison, Piper, & Partridge, 2009). However, the mechanism(s) affecting individual processes are largely unknown, and likely are dependent on a variety of other factors; sex, genetic background, age at which the intervention is implemented, and time of feeding foremost among them. To address this question, we tested a number of different fly genotypes to determine their response to a low-methionine diet and whether these phenotypes could be meaningfully linked to commonly proposed mechanisms.

Our results showed that the treatment diet produced significant changes in the survival response across genotypes in males and females. Several indicators of health

also saw a diversity of context-specific differences, including stress resistance, body weight, and climbing ability. This is in line with many previous studies suggesting the far-reaching effects of diet and nutrition on health and longevity (Kitada, Ogura, Monno, & Koya, 2019), and from similar studies in the DGRP and other model organisms such as the ILSXISS recombinant inbred strains that have shown that genetic variation can affect the outcomes of other dietary interventions (Wilson et al., 2020). Generally, these results suggest that the optimal ratio of methionine to other amino acids in the diet to promote healthy aging is different from the standard laboratory diet of Drosophila, and likely from other organisms under both lab and natural conditions as well. As we have previously stated, methionine has elsewhere been shown to be an essential component in reproduction and fecundity, suggesting antagonistic pleiotropic effects of methionine and possibly other amino acids in energy allocation between early-life reproductive ability and a pro-longevity paradigm in later life (Grandison, Piper, & Partridge, 2009, Zajitschek et al., 2013). This study provides evidence that the longevity effects of MR are likely regulated through different but overlapping mechanisms as other aging phenotypes.

The effect size of different diets on lifespan has been shown to vary widely both between and within species (Wilson et al., 2020). Prior studies have shown that survival response to MR varies but generally extends lifespan at certain concentrations (Lee et al., 2014). Here, we show small but significant increases in the lifespan of Ral913 males and females, as well as w1118 females. However, the Ral59 strain and Ral443 females actually see lifespan shortening compared to the control strain. While variation was expected, it is interesting to note that the direction of the variation with regard to survival is different from what might be expected based on previous studies of both MR and other

dietary interventions (Metaxakis & Partridge, 2013). In this study, more females than males see lifespan extension compared to controls, which is not the case in many published studies of caloric and amino acid restriction. Additionally, the short-lived strain Ral59 did not see significant benefits from the treatment, despite many studies indicating the shortest-lived strains often see the largest gains in *Drosophila*.

Likewise, we investigated oxidative stress resistance and found that the results are not fully aligned with the literature when viewed from the perspective of greater genetic variation and sex within our tested strains. Despite previous studies suggesting MR acts predominantly through reductions in oxidative stress, we found only a minimal association between increased oxidative stress resistance in either young or old flies and improved lifespan (Pang et al., 2023, Yang et al., 2019). Similarly, other measures of healthspan were not significantly increased by low-methionine status. Stress resistance and climbing ability declined with age as expected, but this was not ameliorated by MR in any sex or genotype. The lack of significant improvements in the health of treatment flies in this study may be due to cysteine levels, which are methionine dependent and altered by MR, or strain-specific microbiota differences, which is beyond the scope of this project, but a burgeoning and critical area of investigation for dietary interventions in the aging process.

All four strains and both sexes displayed unique responses to MR. Though no one sex or genotype saw improvements in each metric of health and longevity as might be expected from previous MR studies, some strains more variation in each assay than others. Particularly, the Ral443 strain saw significant decreases in oxidative stress resistance as well as lifespan, while the *w1118* strain males benefited from a small

improvement in stress resistance that was not associated with an increase in lifespan. This suggests that perhaps oxidative stress resistance, while not sufficient for lifespan extension on its own, is required in some contexts under MR to extend lifespan. The analysis of our results did not show a significant interaction between sex, genotype, and treatment with regards to oxygen consumption rate at either age. Genotype and sex were shown to have a stronger effect on lifespan and health in each parameter tested than did the MR diet. Future studies may investigate the impact of sex and genetic variation on the clinical applications of MR to different aging pathologies in human studies, where the translatability of dietary interventions requires further research. Because of the complex relationship between diet-responsive genetic mechanisms involved across strains and species and the aging process, this study demonstrates the importance of considering sex and genetics in applying MR and other dietary interventions generally as a strategy for life- and healthspan extension.

FUTURE STUDIES

Though many new aging studies have begun to shift the focus from whole calorie to the role of individual macronutrient restriction, there remains a dearth of knowledge with regard to the conditions under which MR is best implemented to increase lifespan or suppress aging-associated disease pathology. Additionally, while some studies support short-term health benefits from methionine-restricted diets (Olsen et al., 2020), the long-term feasibility of MR in humans to regulate life and healthspan is still unclear. The impact of MR on a wide variety of health parameters associated with aging has not been

researched fully, and some studies indicate that there may be undesirable tradeoffs between longevity and health.

While many of the health benefits of MR seem to be conserved across laboratory models (Olsen et al., 2020), and studies in many long-lived species have identified a low methionine diet as a potential contributor to lifespan, research into how these benefits translate into a clinical setting for humans is in its infancy and should be a focus of future studies (McCarty et al., 2009). Preliminary trials suggest that humans can tolerate a MR diet and experience beneficial metabolic changes in the short-term, but further understanding is limited by small sample size and the challenge of adherence to long-term dietary strictures (Olsen et al., 2020; Olsen et al., 2018; Richie et al., 2023). Plummer et al. suggest that an intermittent MR diet, similar to past studies of calorie restricted animals, can replicate many of the health benefits of lifelong MR and may have more practical applications for humans (Plummer & Johnson, 2022). Applying a MR diet in clinical or public health settings, potentially as a substitute for overall calorie restriction or fasting, is a feasible short-term goal that merits additional research.

Another issue is understanding the molecular mechanisms by which MR elicits geroprotective effects in animal models of cancer and neurodegenerative diseases. A MR diet has been shown to ameliorate neurodegeneration in a number of studies in animal models (Mladenovic et al., 2019; Naudi et al., 2007), and to improve cognition and neuronal health by relieving oxidative stress and improving insulin resistance (Feng et al., 2022). Although the methionine dependent nature of certain types of cancer has been known for years, further work is needed to determine its effectiveness in a clinical setting

as an intervention or supplement to existing therapies in cancer and possibly age-related neurodegenerative disease and how best to apply this intervention.

One area of study should be the relationship between MR and the microbiome. Dietary restriction naturally alters the gut microbiome, and MR has been shown to alter the content and health of gut microbiota and improve gut barrier function (G. Wu et al., 2020). Low-methionine diets have also been demonstrated to impact age-related diseases like cognitive impairment by improving the quantity and variety of beneficial organisms in the gut microbiota (Xu et al., 2022). One study by Wallis et al. demonstrates that MR can affect the microbiome in a sex-specific manner, suggesting possible sex-specific influence of the microbiome under MR on MR induced life and healthspan benefits (Wallis, Melnyk, & Miousse, 2020). As research into the effects of the microbiome on aging and age-related diseases has grown, the questions surrounding the role of diet and particularly MR on mediating these effects deserve further consideration.

Furthermore, future research should investigate the role of genetic and epigenetic variation on the response to MR, as many studies thus far have been in a limited number of commonly used genetic backgrounds, such as C57BL/6J mice (Lees et al., 2017) and w^{1118} flies (Lee et al., 2014). Research will by necessity focus on more accessible genetic means of inducing a MR state such as oral methioninase for clinical use. Future work could utilize genome wide association studies to uncover potential diet responsive genes that are involved in mediating the aging process and age-related diseases (Wilson et al., 2020). Investigation of potential roles for epigenetic regulation of the response of aging phenotypes to diet and nutrition, including low-methionine diets will also be important. This work can be expanded upon to greatly enhance current understanding of how

genetics impacts diet and aging, and to develop dietary therapies that are more broadly applicable.

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