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Research Article

Caloric restriction and methionine and their effects on longevity in *Drosophila melanogaster*

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Abstract: Caloric restriction (CR) has shown to increase lifespan in several model organisms including *Drosophila melanogaster*. Several pathways have been studied in order to better understand the biochemical mechanisms for this increase in longevity. Methionine has been shown to play a role in promoting antioxidant responsiveness to oxidative stress and other forms of cellular degeneracy. A reduction of dietary methionine has shown to increase longevity. The experiments run in this study were designed to bolster these findings. If an increase in dietary methionine limited lifespan, a corollary could be drawn to methionine restriction studies. This experiment dealt with the effects of dietary methionine supplementation in conjunction with caloric restriction, and how these effects are linked to longevity in *D. melanogaster*. The results of these studies were consistent with previous caloric restriction studies. However, the effects of methionine on CR proved to be inconclusive.

Introduction

For nearly 70 years, caloric restriction has been known to extend lifespan in rodents and more recently in many other taxonomic groups. There are many classic effects of CR, including changes in mammalian metabolism and the neuroendocrine system. These effects have lent themselves to several hypotheses as to how CR affects the aging process. *D. melanogaster* has proven to be an excellent model organism in studying caloric restriction through the use of variant dietary media. Variation in longevity among the sexes has also been shown. In all, five physiological mechanisms have been pinpointed that may affect longevity within this model organism. Methionine restriction has recently been studied as contributing to these mechanisms. Studies described here were designed to test the role of dietary methionine by supplementing experimental lines of *D. melanogaster* with excessive amounts of dietary methionine.

Caloric restriction has been defined as a dietary regimen low in calories without causing undernutrition (Koubova et al., 2003). Restricting calories from carbohydrates, fats, and proteins by 25%–60% brings about CR. Extension of lifespan has been documented in many organisms. These include: yeast, spiders, worms, fish, mice, rats, and nonhuman primates. Besides

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lifespan, CR also delays several diseases. Kidney disease, diabetes, and neoplasias are just a few examples. CR has also been shown to prevent decline in psychomotor and spatial memory tasks.

Even with these findings, the exact physiological and biochemical mechanisms altered by CR have not been explained. Several models of CR have been theorized. One of the most prominent of classical theories deals with oxidative damage created in the body by reactive oxygen species (ROS). These species are a result of cellular respiration and may cause oxidative damage to DNA, RNA, proteins, and lipids. They may damage any part of the cellular machinery and thereby limit lifespan. The body naturally counters the effects of ROS via an enzyme called superoxide dismutase (SOD). It has been observed that CR causes greater genetic expression of SOD, leading to reduced levels of cellular ROS and increased lifespan (Koubova et al., 2003).

A second view of CR suggests that constraints on longevity may be caused by a buildup of aberrant proteins within the body. As fat reserves are exhausted during CR, the body begins to degrade these built up oxidized proteins. This leads to a greater turnover of aberrant proteins within the body. Insulin and blood glucose levels are also lowered during CR. Because of lowered glucose levels, fewer proteins can be covalently modified through glucose derivatives. This results in less accumulation of advanced glycation end products, which have also been linked to age-related pathologies (Koubova et al., 2003). Since longer healthier lives in humans is a possible outcome of CR research, studies in other mammalian models have been beneficial in correlating experimental results. *D. melanogaster* has been used as a model organism during CR research for several reasons. First, it is an obligate aerobe just like humans. This makes *D. melanogaster* a good model for studying oxidative stress and damage that could occur in mammalian organisms. It can easily be studied through the use of demography and mortality rates. Differences conferred on males and females can easily be observed (Partridge et al., 2005).

D. melanogaster populations can be maintained on agar gel media, typically containing sugar, yeast, and some type of corn meal as a base. Nutrients may be added or removed from the medium to study their effects on the organism. In CR studies, dilution of the food medium is an accurate and simple way to induce and monitor CR in *D. melanogaster*. Median and maximum lifespan have been extended using these types of media dilutions. Peak lifespan has been shown to occur with intermediate food concentrations, with graded decline on either side of the extension (Partridge et al., 2005).

Female *Drosophila* have also been shown to exhibit a greater response to CR than males. The reasons for this difference among the sexes is still not well understood. One possible theory is based on differences in nutritional needs for the separate sexes. Females show greater anabolic activity due to the production of eggs. Under CR conditions, females also reduce egg production. This reduction could result in a more drastic change in metabolism once CR is induced (Partridge et al., 2005). Since males have no means of egg production, changes in fertility could play a more limited role in determining lifespan, as less nutritional constraints are placed on males due to reproduction.

Restriction of the essential amino acid methionine has been shown to increase lifespan in several species including rodents and *Drosophila* (Richie et al., 1994). Methionine is a precursor of glutathione (GSH). GSH is a tripeptide that plays a critical role as an antioxidant and has been found in high cellular concentrations in nearly all living cells (Richie et al., 1994). Its documented roles include: detoxification of free radicals and lipid peroxides, protein maintenance, maintenance of protein synthesis and degradation, and maintenance of immune function. Low cellular levels of GSH have been associated with cell damage and toxin susceptibility. Conversely, increased levels of GSH have lead to prolonged lifespan. How then could low methionine levels increase lifespan? It has been hypothesized that by limiting methionine intake, the organism may demonstrate adaptive changes in sulfur amino acid metabolism as a means of maintaining GSH

levels in peripheral tissues. While these results have not been fully proven, it has been shown that GSH must be maintained for effective longevity even if methionine is restricted (Richie et al., 1994). It also shows the adaptive ability of the body during caloric restriction.

The purpose of this study was to observe changes in lifespan in *D. melanogaster* based on changes in caloric intake and dietary methionine supplementation. A calorically restricted diet should increase lifespan in *D. melanogaster* relative to flies fed a normal diet rich in calories. Additionally, a CR diet supplemented with methionine should decrease lifespan in *D. melanogaster*. In this study, I found that CR did lead to an increase in lifespan as expected. The effects of supplemental methionine were inconclusive and warrant further investigation.

1% Yeast Medium	**4% Yeast Medium**
20% Sucrose	20% Sucrose
8.0% Cornmeal	8.0% Cornmeal
0.5% Bacto-agar	0.5% Bacto-agar
1.0% Propionic acid	1.0% Propionic acid
1.0% Bacto-yeast extract	4.0% Bacto-yeast extract
1% Yeast Medium with Added Methionine	**4% Yeast Medium with Added Methionine**
20% Sucrose	20% Sucrose
8.0% Cornmeal	8.0% Cornmeal
0.5% Bacto-agar	0.5% Bacto-agar
1.0% Propionic acid	1.0% Propionic acid
1.0% Bacto-yeast extract	1.0% Bacto-yeast extract
10 µg L-Methionine	10 µg L-Methionine

Figure 1. Components used in all four experimental media. 1% and 4% yeast media were used as a control for caloric restriction. Yeast media with added methionine were used to determine the effects of methionine independently from caloric restriction.

Materials and Methods

Ten separate recombinant inbred lines of *D. melanogaster* raised in the laboratory were used to monitor lifespan in this study. These lines included: RW 4 sensitive, RW 16 sensitive, RW 39 resistant, RW 71 resistant, RW 87 resistant, RW 95 sensitive, RW 114 resistant, RW 120 sensitive, RW 133 sensitive, and RW 141 resistant. Resistant lines were observed to have shown resistance to increased nutrition. Sensitive lines were observed to die earlier when placed on an enriched diet or on normal media. These lines were expected to exhibit longevity only on a calorically restricted diet. Each line was monitored in four separate cages, each cage containing 20 males and 20 females. These were freshly emerged flies of the same age. They were sexed and counted under ether before being placed in their respective cages. Each cage received a different diet. One cage received a 1% yeast diet while another received a 4% yeast diet. The media used are shown in Figure 1. The remaining two cages were fed the same diet mentioned above. However, 10 micrograms (µg) of L-Methionine were added per liter of media. Tegosept was also added to all media as an antifungal agent. The yeast medium recipes were courtesy of the Pankaj Kapahi lab. Cages were stored at constant humidity and room temperature. Media containing additional methionine were to be used to test for the effects of increased methionine in the diet. Cages without additional methionine acted as a control, providing only a basal amount of methionine to the organisms. One percent yeast was used as a control for caloric intake and represented CR conditions. Four percent yeast allowed for increased caloric intake to test against the methionine. In this way, a decrease in lifespan could better be attributed to either solely increased methionine or to a change in caloric intake as well.

Dead flies were removed from the cages every four days and sexed. Mortality rates for both males and females were recorded for each cage. Food concentrations were kept static, but fresh media was presented to the organisms every four days at the time of counting. Fresh media kept eggs laid by female flies from hatching in the cages and being introduced into the experimental population. This also insured that an optimum diet was being presented at all times. The four-day cycle was continued until a 100% mortality rate had been reached for *Drosophila* in all cages. Resistant fly lines were expected to survive equally well on both CR and normal diets, while sensitive lines were expected to show normal lifespan only on the one percent yeast medium. Lifespan in these lines was to be limited under normal caloric conditions. Both lines were

expected to show decreased lifespan when additional methionine was placed in the media. The purpose of this experiment was to show that lifespan would decrease in *Drosophila* both resistant and sensitive to caloric intake when methionine was supplemented in their diet.

Results

Mean survival times were calculated for males and females on all four types of media. This was calculated by multiplying the number of dead flies by the number of days on which they were collected. These products were summed for all 64 days and then divided by the total number of flies in the cage. These results are summarized in Figures 2 through 5. These results were not used in statistical analysis, but are presented to show the trends in the four types of media. Mean survival times are used to represent lifespan and longevity among the male and female lines.

The 1% yeast medium did well as a model for caloric restriction, increasing lifespan in all lines of flies. Comparatively, overall longevity and mean survival time were limited when flies were grown on 4% yeast medium. The 4% yeast medium showed the effect of supplemental media. Males and females survived longer on 1% yeast medium with added methionine than on 4% yeast medium with added methionine. Methionine also caused a greater variation in lifespan among different recombinant inbred lines when added to the respective medium. However, no specific trends exist based solely on the addition of methionine.

Discussion

Trends were somewhat variable among flies grown on different media. Caloric intake caused a shift in lifespan for both males and females. The effect of methionine added to the diet was not as straightforward.

Fecundity has been well documented as placing limits on lifespan. Mean survival time for males raised on a regular 1% yeast medium was longer than females consuming the same 1% yeast medium. While male mean survival time decreased by only a few days on a 4% yeast medium, female survival time decreased to a greater degree. This could be attributed to the enhanced nutritional needs of females while egg laying. Males may have had less nutritional demand during the experiment, as egg laying wasn't a factor. This trend could be seen on other media types as well to varying degrees. Since both sexes were

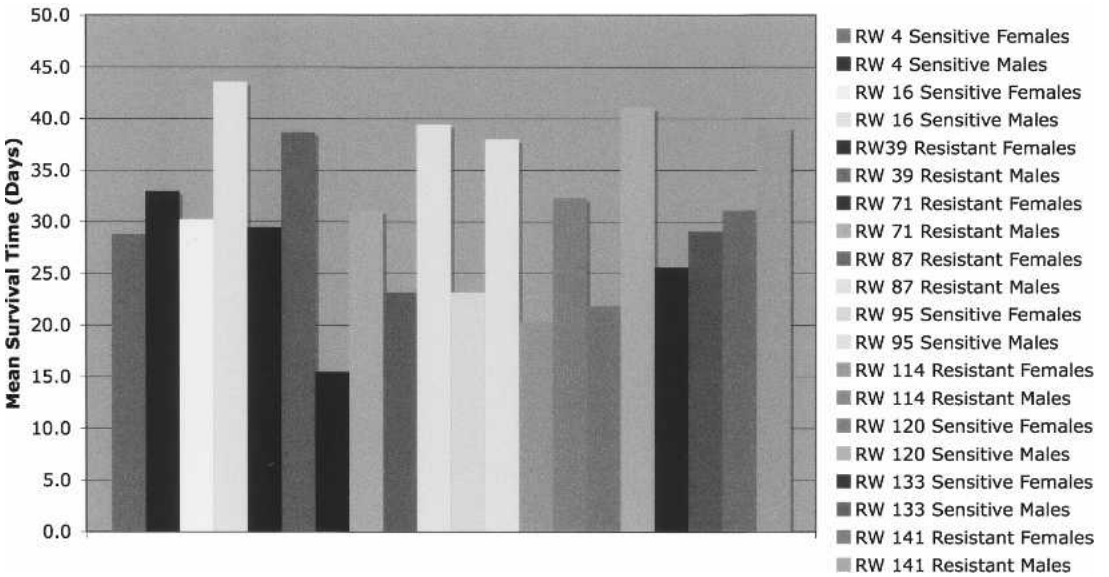

Figure 2. Mean survival times for male and female lines raised on 1% yeast media.

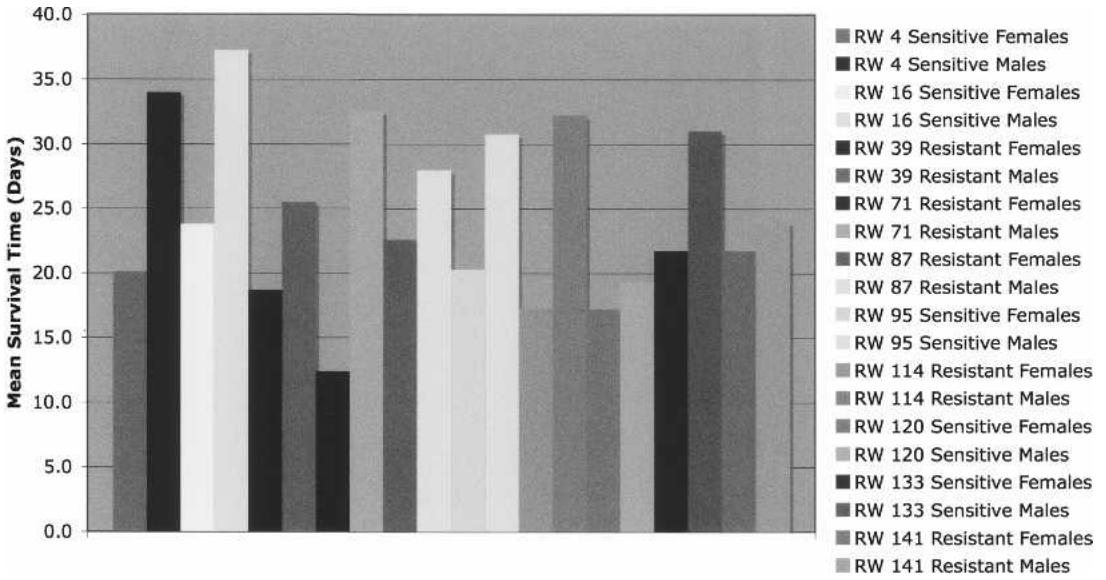

Figure 3. Mean survival times for male and female lines raised on 4% yeast media.

monitored on the same media, the difference in nutrition was the only variable leading to differences in lifespan. Although this comparison aids in giving consistency to the study, it does little in terms of defining the role of methionine and caloric intake on lifespan. It acts as a control in that it positively correlates experimental data with previously documented fecundity trials.

Aside from fecundity, it is better to study the results solely within one sex. Female average lifespan decreased on 4% yeast media compared to 1% yeast media. These results concur with previous caloric restriction studies. Since CR causes an increase in longevity, increased caloric intake should then cause a loss of longevity. Males also show this trend on basic 1% and 4%

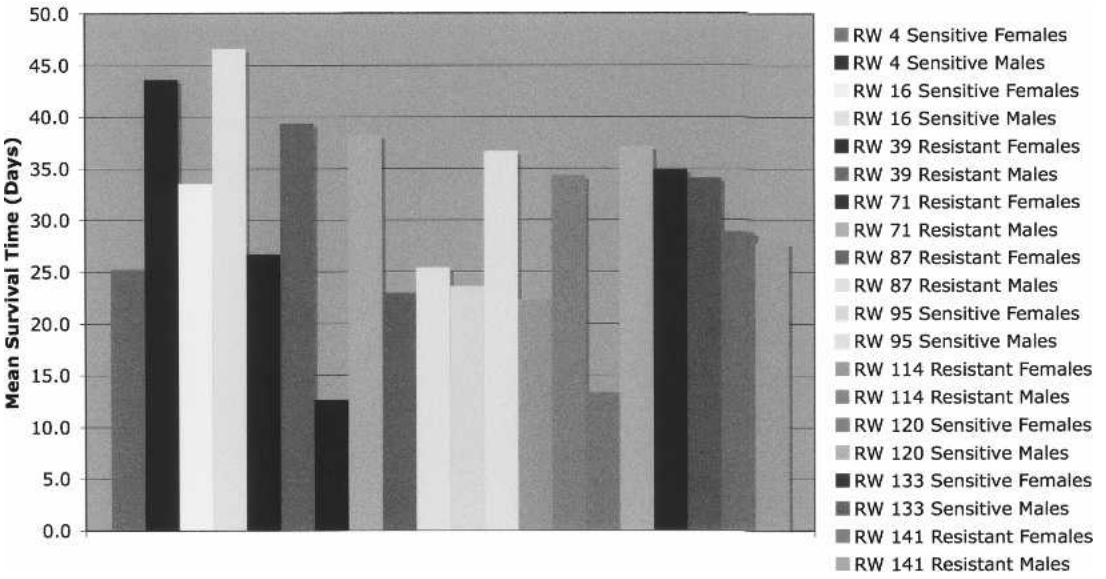

Figure 4. Mean survival times for male and female lines grown on 1% yeast media with additional methionine.

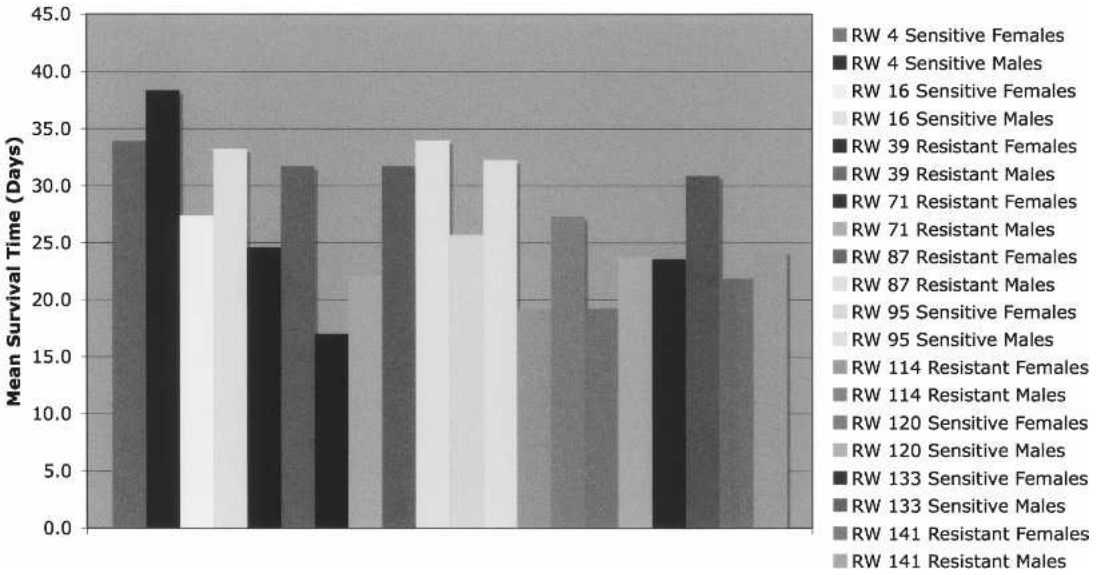

Figure 5. Mean survival times for male and female lines grown on 4% yeast media with additional methionine.

yeast media. A change in caloric intake (4% yeast medium) caused decreases in longevity. This gives no evidence of a CR mechanism, but could support integrated pathway theories like TOR and ILS signaling (Kapahi et al., 2004a; 2004b). A change in caloric intake seems to mediate lifespan regardless of whether or not this is a positive or negative change. Receptor-mediated response could be one way by which a positive and negative change could be physiologically monitored by the body.

While the data seem to support previous CR studies, the effect of methionine is not clear. Shifts in longevity seemed to be negligible when methionine was added to the medium. The most noticeable shift occurred in males on 4% yeast media with added methionine. Even then, a decrease of only a few days was seen. Some females on 4% yeast media with added methionine may have actually lived longer than those raised solely on a 4% yeast medium. If methionine metabolism is mediated during CR, an increase in methionine seemed to have little or no effect. If metabolic processes are already maximally using amino acids on a normal diet, an increase in amino acid intake would have no effect on the output of these processes. Oxidative free radicals and other harmful cellular products would already be causing their maximum cellular impact. However, if methionine is removed from the diet, these metabolic processes would be involved in less production of metabolites. Because of this, nutrient mediated pathways can't be ruled out. Amino acid metabolism could still have an impact during CR without decreasing longevity when boosted. Even so, the study does little to show that methionine metabolism plays a critical role in longevity.

The most prominent problem with this experiment was the isolation of methionine's effects. The use of two concentrations of media was useful in displaying the effects of caloric restriction on the model organism. It also served as a necessary control and confirmed previous caloric restriction studies. However, an increase in caloric intake may have masked the results of pure methionine effect on lifespan. The effects of methionine enrichment in diet were not clearly definable and did little to confirm the effects of amino acid limitations on longevity. Perhaps a better control would allow future studies to validate the necessity or limitations that methionine provides in longevity. The amount of additional methionine seems to be relatively small when compared to the amount of food made available in the cages. Flies may have been able to process this

extra methionine effectively. Especially under CR conditions, the organism's metabolism may have been able to utilize the amino acid to sustain necessary cellular respiration pathways. A greater amount of methionine should be used in future studies so that its effects on longevity could be seen more prominently and observed independently from the effects of CR.

This experiment was a good starting point in analyzing the effects of methionine and amino acid synthesis in longevity. The addition of methionine rather than restriction was also a novel approach to studying longevity that should be furthered in order to correlate more data with previous studies. The results from this study soundly supported previous caloric restriction studies. Future experiments will be necessary to further isolate the role of methionine in longevity, as this study was marginal in that area. Results do not negate the possibility of signaling pathway mediation of lifespan. Further research in this area could be very useful in determining the cellular mechanisms involved in lifespan extension and longevity.

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