CALORIC RESTRICTION IN *DROSOPHILA MELANOGASTER*

Thesis by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. Paul Kirchman , and has been approved by the members of her/his supervisory committee. It was submitted to the faculty of The Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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ABSTRACT

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Caloric restriction (CR), the reduction of nutrient intake short of malnutrition, extends the lifespan of various organisms and can improve measures of human health. Whether mechanisms of lifespan extension are conserved between humans and model organisms is unknown. In mammals, implementing CR is easily achieved by providing a restricted group with a fraction of the food consumed by an "ad libitum" fed group, which has unlimited food access. Due to the difficulty in directly controlling *Drosophila* food intake, caloric restriction, performed similarly to the mammalian paradigm, has never been tested in flies. Here, we demonstrate a system that allows measurement of food intake throughout life. This system will be used to measure fly lifespan under caloric restriction analogous to current mammalian studies. Our work will help tease apart the differences between the various caloric and dietary restriction paradigms in *Drosophila*, strengthening our understanding of how fly models relate to mammalian systems.

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Introduction

The world's population keeps increasing and is expected to reach 11 billion within 40 years. By 2030, there will be around 75 million older persons, twice the number of 2000. With an aging population comes the increase of age-related diseases. More than 80% of people 65 or older suffer from at least one chronic disease and 50% suffer from at least two diseases (1,2). At least 20% of persons aged 65 or older suffer from diabetes and as the population ages, the incidence of diabetes will increase from 1.2 million in 2000 to 4.4 million by 2050 (3).

Obesity is also a major health concern. In 2009, more than 30 % of Americans were considered overweight, and more than 35% suffered from obesity. In the last century, the increase in food intake led to reduced lifespan and higher incidences of age-related disease. Therapies carried out for patients to lose weight and reduce the incidences of diseases include caloric restriction (CR) and alternate day fasting (4). Hence, studying the mechanisms by which caloric restriction confers its benefits will prove to be valuable for the health community.

The reduction of nutrient intake short of malnutrition, CR, extends the lifespan of various organisms and can improve measures of human health by lowering inflammation rate, reducing the risk of cardiovascular disease, and maintaining blood sugar levels (5). McCay performed the first study of CR by placing rats on restricted or *ad libitum* diets. Although the 40% CR cohort showed extended lifespan, it also suffered from slower growth. Retarded growth, rather than the reduction of food intake, was thought to

be the reason of extended lifespan. Later studies linked CR with slower development of disease, leukemia, carcinogenesis (6,7), and reduced reproduction (8).

The physiological mechanisms underlying lifespan extension by CR have yet to be identified. Early in 1960, CR was thought to confer its benefits by reducing body fat content (9). This work challenged McCay's hypothesis since lower fat rather than retardation growth was thought to be the driving force behind CR. During the time, studies on invertebrates was established and in 1977, Klass found that reduced food intake extends the life of the nematode , *Caenorhabditis elegans* (10). Finding correlate with CR was of interest, and in 1991 long term CR was linked with high concentration of free plasma corticosterone (11). Corticosterone is known to be high when the organism reacts to a harmful environment suggesting that organism under CR are exposed to a stressful environment due to the lack of food or psychological depression.

McCarter findings suggest that the metabolic rate per unit of fat-free is similar for *ad libitum* or CR fed rats (12). Also, recent findings show a link between high insulin sensitivity and CR beneficial effects (13). Among many hypotheses, Warner developed the idea that CR confers its protective effect by increasing the rate of apoptosis (14) while Yu demonstrated that CR acts by attenuating oxidative damage (15). It is greatly possible that these modes of action interact when organisms are subjected to CR.

Although controlled experiments on the effect of CR on human lifespan have not been reported, observational studies in Japan on isolated populations show a correlation between extended lifespan and a low calorie diet (16). Scandinavians during war were

exposed to 20% CR and this has been linked with a reduced incidence of cardiovascular disease (17). Also, lower heart disease, diabetes and hypertension are observed in the population of northern Europe during the war (18). Evidence linking CR and subsequent beneficial effects are difficult to interpret since populations subjected to a CR diet are usually associated with poverty zones and starvation. Hence, mechanistic studies on CR require the use of model organisms.

An ongoing study, started over 20 years ago with Rhesus monkeys (Macaca mulatta), suggests that CR extends lifespan and reduces the risk of cancer and cardiovascular disease by more than 50% (19). Rhesus monkeys share high genetic and physiological similarities with humans and are commonly used in medical research. The monkey's size and long lifespan, however, make aging studies prohibitively expensive and tedious compared to other models. Invertebrates with short lifespan and ease of maintenance have been instrumental in furthering our knowledge of aging.

Several studies demonstrate that CR is not a universal phenomenon. To test whether the effect of CR was specific to some genotypes raised two different strains of mice *ad libitum* or 60% CR starting at 4 months of age. Overall, CR failed to increase lifespan in the lean mice. Switching from an *ad libitum* to a CR diet increased the mortality of 17 and 24 months old mice. The effect was more prominent for the lean mice (20). This demonstrates that CR does not confer its beneficial effects to all strains and decrease lifespan if carried out too late in life. CR was also tested in *Musca domestica* male houseflies and the *ad libitum* cohort show increase in lifespan compared to the 80% CR (21).

The fruit fly has long served as a model for human health, with over 2000 publications in the field of aging. The fly's short lifespan and well-documented genetics have made it an attractive model for studying human health, aging, and disease. The *Drosophila* genome has many homologs with humans, e.g., 60% of human disease genes are shared with the fruit fly (22). This genetic relationship between human and fly genetics facilitates the use of *Drosophila* to uncover mechanisms that improve human health and lifespan. Using *Drosophil*a to study aging mechanisms will inform future interventions of human health, including the reduction of food intake.

Unfortunately, while mammalian CR is easily achieved by providing a restricted cohort with a fraction of the food consumed by an *ad libitum* fed group, CR studies in invertebrates often require surrogate methods to limit nutrient intake. Fruit flies are typically housed in vials and are provided with a large excess of solid food. The difficulty in developing accurate techniques to restrict food intake in flies has led researchers to induce CR by manipulating nutrient concentration through medium dilution in solid food vials (23). However, since flies still have unlimited food access, current fly CR paradigms may be difficult to interpret due to compensatory feeding and dehydration stress. Indeed, flies compensate for dilution by eating more food (24). This leads to changes in water intake since the solid medium is the only source of food and water. Recent studies demonstrated that lifespan extension by food dilution can be mimicked by providing flies with water. This suggests that the effects of medium dilution are due to dehydration stress rather than to nutrient intake (25).

For my thesis research, I studied the affect of CR on lifespan in the fruit fly, *Drosophila melanogaster*. CR as implemented in the mammalian paradigm has never been tested in flies since its small size makes it very challenging to measure and control food intake. Here, we implement CR in *Drosophila* using a novel chamber system that facilitates the measurement of fly lifespan under different calorie-restricted and *ad libitum* fed conditions. This study will determine whether CR, as performed in the mammalian paradigm, extends lifespan in *Drosophila melanogaster*. Our preliminary results support findings in *Mediterranean* flies (26) and wild mice (27) where CR is detrimental early in life but shows late-life benefits.

Experimental Procedures

Materials

Fly chambers are made of three custom-cut acrylic pieces and are held together by stainless steel screws (Figure 1A). Each block contains four chambers, designed to house one fly each in a circular enclosure of approximately 10 cm^3. The bottom piece contains grooves to permit feeding cassettes to slide freely through each chamber. When assembled (Figure 1B), the chambers allow for food to be provided individually for each fly. Feeding cassettes have two basins (Figure 1C), the larger of which supplies the fly with water (1% agar) while the smaller cup provides food (liquid solutions of yeast and/or sugar). To re-supply food and water, fresh feeding cassettes are pushed in and the old cassettes are removed without allowing the fly to escape from the chamber.

Feeding Measurements

To determine the volume consumed by the *ad libitum* cohort, green food coloring is added to the medium. After 24 hours of feeding, volume of the remaining uneaten food is determined by pipette and concentration is measured by spectrophotometry to account for evaporation (Figure 2A). During a lifespan trial, feeding is generally measured once per week. Flies on CR usually consume all of the food that they are provided daily.

Medium Preparation

The medium consists of an aqueous mixture of sucrose and yeast extract with propionic and phosphoric acids to manage mold growth. A defined volume of ddH_2O is added to a

beaker and heated on a stirring hot plate. Upon boiling, sucrose and yeast extract are added. After ingredients are dissolved, the final volume is adjusted with ddH_2O. After cooling, propionic and phosphoric acids are added to final concentrations of 50 mM and 6 mM, respectively using a 100x stock solution. The 100x stock solution is made by mixing 4.15 mL of 85% phosphoric acid (14.61 M) and 41.8 mL of 100% propionic acid (13.36 M) and adding ddH_2O to 100 mL. The final solution is sterile filtered using a 0.22 micron syringe filter. Aliquots of the final solution are kept at 4 °C for daily use.

Lifespan measurements

Flies were raised in standard food bottles. Eclosed male flies were collected under carbon dioxide anesthesia and maintained in solid food vials or randomly sorted to CR chambers. The cassettes were kept in the incubator at 25 °C on a 12/12 hour light/dark cycle with humidity maintained. Chips were changed daily at a consistent time to provide flies with fresh food and account for death. For mid-life initiated CR, flies were maintained in solid food vials until needed.

Results and Discussion

We asked whether CR, performed similarly to the mammalian paradigm, would improve lifespan in *Drosophila melanogaster*. Previous systems, in an attempt to implement CR, provided *ad libitum* access to solid food and did not allow for direct measurement or manipulation of fly food intake. Our designed chamber system allows us to measure lifespan and feeding under different calorie-restricted and *ad libitum* conditions analogous to current mammalian CR studies. Flies are housed individually and are provided daily with a limited and measurable amount of fresh liquid food and water.

To measure *ad libitum* feeding, flies are provided with dye-labeled liquid food for 24 hours. Using an independent assay to measure feeding (28), we find that the dye does not affect fly food consumption (data not shown). Feeding is generally constant when performing consecutive measurements (Figure 2B). Weekly measurements are made throughout life and the average *ad libitum* consumption is used to calculate a CR diet.

To implement a CR diet, flies were fed 80% or 60% CR diets which consist of reducing food intake by 20% and 40%, respectively. The 60% CR cohort, showed a significant decrease in average and median lifespan, suggesting that reducing calories by 40% is a harsh treatment (Figure 3A). The 80% CR cohort showed a more modest decrease in lifespan compared to the *ad libitum* cohort. However, we also observed a slight lifespan extension later in life, suggesting that CR might be beneficial only after a certain age. To test whether 80% CR initiated later in life would exhibit the same effect, we maintained flies in solid food vials and switched them to the chambers on day 40 (Figure 3B). Although median lifespan is not extended, a slight improvement in late life

is again observed. Hence, these studies converge towards the idea that caloric restriction in *Drosophila* is beneficial late in life. One interpretation is that the fly might need time to adapt physiologically to a CR diet before exhibiting beneficial results. Alternatively, as the fly ages, its energy allocation toward reproduction diminishes which may allow a better tolerance to support CR. Hence, trade-offs between reproduction and longevity might explain the positive impact of CR late in life.

Interestingly, our results are supported by two similar findings in Mediterranean fruit flies (26) and wild mice (27). In both of these studies, a CR diet is implemented throughout life and, although CR is detrimental early in life, lifespan extension is observed in late life. These studies, together with our results, support the idea of a conserved mechanism of CR between species. However, our results also challenge the idea that CR broadly extends lifespan since we demonstrate that beneficial effects are only observed late in life.

The alleged universality of CR has been questioned for many years. In 1976, a study suggested that CR is only beneficial for mice that gained weight during early adulthood (29). Similarly, a recent study demonstrated that an obese mouse strain benefited from CR but not a lean strain (30). This suggests that the benefits of CR are not broadly conferred, but rather are strain-specific. Since inbred laboratory strains may have lost their ability to exhibit natural behavior, using wild caught lines may prove to be essential in mimicking relevant physiological conditions. Also, the beneficial effects of CR might differ according to gender. A study on rats demonstrated that females under CR adapt differently than male (31). Hence, conducting studies on both genders will help

dissect the different mechanisms by which CR operate. Other factors, such as the diet composition, play an important part in modeling the effects of CR. Flies have a lower lifespan on a high fat diet (32), and larger beneficial effects of CR may be more prominent on deleterious diets. Finally, the use of long-lived fly mutants will help determine if CR operates through similar mechanisms as known longevity genes. By using our novel CR chambers on these various parameters, we will continue to develop and improve the fly CR model and dissect the mechanisms by which it operates.

Figures

Figure 1. (A) Disassembled fly chambers showing screws, acrylic cover and solid white bottom piece containing grey grooves to allow feeding cassettes to slide freely through each chamber. (B) Assembled apparatus. (C) Close up of a feeding cassette with two bassins.

Figure 2. Formula to calculate volume consumed. A = absorbance (λ_{max} of the dye = 630 nm), V = volume. (B) Feeding data for two consecutive days starting with 32 days old flies. Flies were in the CR chambers for two day before measurement.

Figure 3. (A) Lifespan of early life-initiated CR. Male Dahomey adults (3-4 d old) were transferred to the CR chambers on day 0. Consumption of liquid food (2% yeast extract + 5% sucrose) by the *ad libitum* fed group was measured weekly and used to calculate 60% and 80% CR diets for the restricted groups (CR started on day 6). *Ad libitum* consumption was fairly constant throughout life, and restricted cohorts generally consumed their entire daily food aliquot. N = 17 (*ad libitum*), 16 (80% CR), 16 (60% CR). (B) Lifespan of mid-life initiated CR. Groups of 20 Dahomey males were maintained in solid food vials (2% yeast extract + 5% sucrose + 1% agar) before being transferred to the CR chamber on day 41 . Feeding measurements of the *ad libitum* group were used to calculate an 80% CR diet, which began on day 44. N = 27 (*ad libitum*), 20 (80% CR).

Figure 4. Wild mice and Mediterranean fly lifespan curves, derived from Harper et al. (10) and Carey et al. respectively (13). Although detrimental early in life, CR extends lifespan at late ages.

A **B** **C**

Figure 1

A

$$V_{consumed} = V_{initial} - V_{leftover}\left(\frac{A_{leftover}}{A_{initial}}\right)$$

B

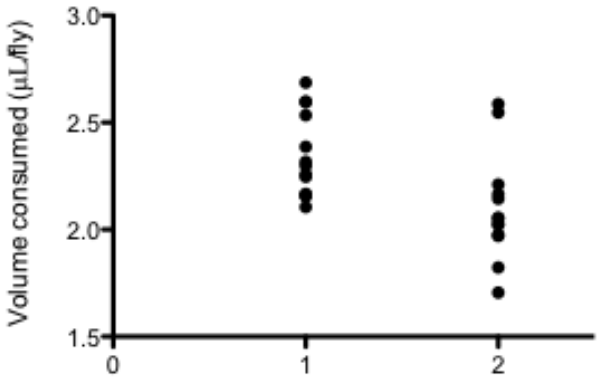
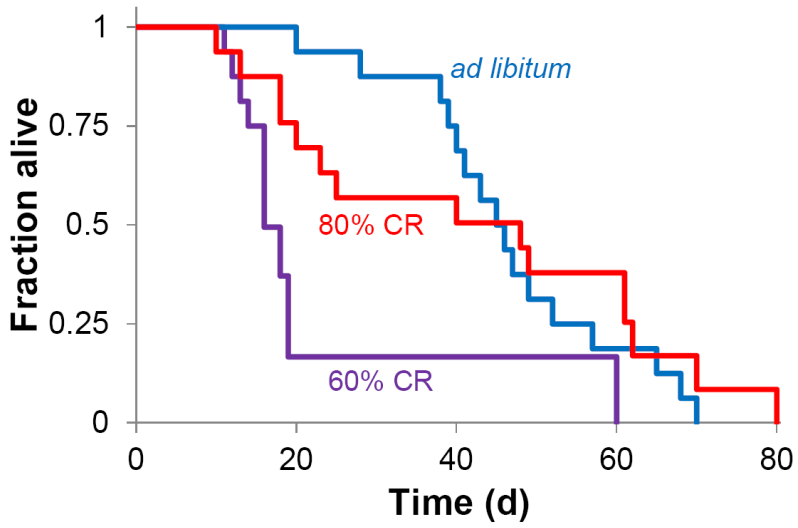


Figure 2

A


Fraction alive
1
0.75
0.5
0.25
0
0
20
40
60
80
Time (d)
ad libitum
80% CR
60% CR

B

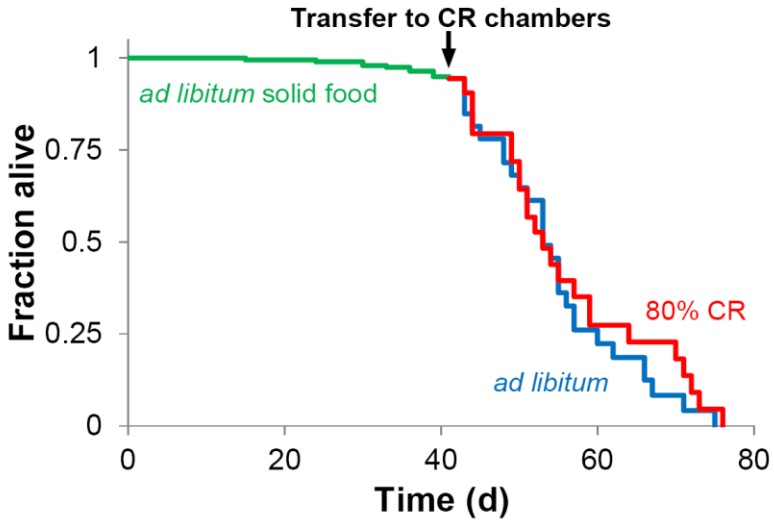
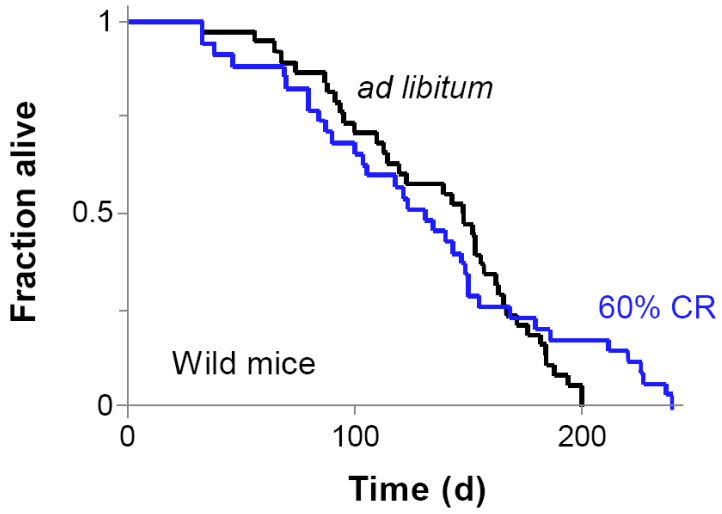

Transfer to CR chambers
Fraction alive
1
0.75
0.5
0.25
0
0
20
40
60
80
Time (d)
ad libitum solid food
80% CR
ad libitum

Figure 3

A


1
0.5
0
Fraction alive
ad libitum
60% CR
Wild mice
0
100
200
Time (d)

B

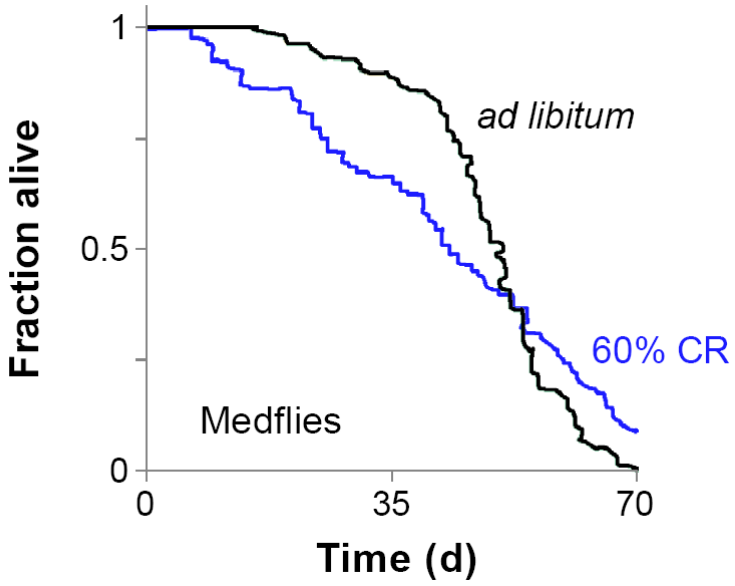

1
0.5
0
Fraction alive
ad libitum
60% CR
Medflies
0
35
70
Time (d)

Figure 4

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