Calorie Restriction Prolongs Maximal Lifespan in Genetically Diverse Mice

1 Introduction

Since early history, longevity has been a topic that has fascinated; even in modern times, what mediates longevity and why remains a key question in ageing research. Many hypotheses currently exist to explain the variation in longevity seen across different members of the same species, some relating to the small combined effect of many genetic polymorphisms, while others focus on age-associated pathology such as reduced DNA repair capacity, increased reactive oxygen species production, and aberrant proteostasis (Aunan *et al.*, 2016; Viña *et al.*, 2007).

However, one other key hypothesis is the contribution of nutrition, particularly that of dietary restriction, towards longevity (Green *et al.*, 2022). Dietary and caloric restriction have been established as modulators of longevity across a range of organisms since the early 20th century, first demonstrated in 1935 in rats where caloric restriction resulted in an extended lifespan, relative to *ad libitum*-fed counterparts (Zainabadi, 2018; McCay *et al.*, 1939).

Further studies in other organisms such as mice (*Mus musculus*), *Caenorhabditis elegans*, and *Drosophila Melanogaster* have recapitulated this link between caloric restriction and longevity, strengthening the idea that nutrition may be a modulator of health- and lifespan across a wide range of eukaryotes (Lakowski and Hekimi, 1998; Taormina and Mirisola, 2014; Tu and Tatar, 2003).

Most recently, a study published by Francesca *et al.* (2024), investigated the relationship between caloric restriction and lifespan in a genetically heterogeneous strain of outbred female mice. The aforementioned mice were allowed to reach 6 months of age before the respective dietary regimen began, either ad libitum feeding (AL), 1-day fasting (1D), 2-day fasting (2D), 20% caloric restriction (20), or

40% caloric restriction (40). The data gathered during this study period forms the basis of the statistical analysis reported here.

This report aims to determine the significance of the relationship between diet, body, weight, and their interaction upon variation in lifespan of genetically heterogeneous mice. This report employs model selection to arrive at a 1-way ANOVA analysis of dietary regimen as the covariate explanatory variable and lifespan as the response variable. In addition, a separate one-way ANOVA analysis was conducted, treating diet and mean lifetime bodyweight as explanatory and response variables, respectively.

2 Methods

2.1 Data Manipulation and Analyses

Raw data for analyses were obtained from the supplementary data in the published material. All subsequent analyses were performed in R ver. 4.4.0 (2024). Data were accessed from two datasets: bodyweight.csv and survival.csv. Mice that died before the initiation of the restriction regime at 6 months were filtered from the survival dataset using dplyr filter for mice that died prior to day 179 of the study. Data was then processed to find summary statistics for lifespan per dietary regimen while mean lifetime body weight was extracted from the body weight dataset and was calculated per mouse from multiple body weight measurements over the lifespan of the individual mice. Data frames containing summary statistics for diet groups and mean body weight for each mouse were then merged by the unique Mouse ID in order to perform modelling and visualisation.

Linear models were generated with lifespan as a response variable, and a combination of explanatory variables: either dietary regimen or body weight as the sole explanatory variable, both dietary regimen and body weight as explanatory variables, or both dietary regimen and body weight and their interaction as explanatory variables. Post-hoc Tukey tests were conducted on the final generated models to assess pairwise differences in mean lifespan across the different dietary regimens

2.2 Model Selection and Evaluation

Model selection was undertaken by employing likelihood ratio tests (LRTs) and stepwise comparison of the most complex model (model 1) to the simpler

model (model 2) and then this was again compared to two simpler models with a single explanatory variable each (model 3 and 4). Models were evaluated by comparative scoring of the log-likelihood of the two models via calculation of 2Δ(Log-Likelihood) and subsequent χ^2 test to assess significance (Table 1). In this instance, the null hypothesis is that the more complex and simpler model are equivalently fitted to the data. A significant result from the χ^2 test indicates that the null hypothesis should be rejected and the complex model adopted as opposed to the simple model due to better fit.

Model	**Model Structure**	**Log-Likelihood**	**df**
Model 1	Lifespan $\sim$ Diet * Bodyweight	-6577.173	11
Model 2	Lifespan $\sim$ Diet + Bodyweight	-6578.477	7
Model 3	Lifespan $\sim$ Diet	-6578.968	6
Model 4	Lifespan $\sim$ Bodyweight	-6607.546	3

Table 1: Model Comparison

Model evaluation illustrated than the combined additive and interaction model 1 was not significantly better at explaining data as compared to the additive model 2 (χ^2 = 2.608, df = 4, p = 0.625) and thus the simpler, additive model was adopted over the complex model. Moreover, when model 2 was compared to model 3 and 4, consisting of either single explanatory variable respectively, the additive model was significantly better than the model 4 (χ^2 = 58.138, df = 4, p = 7.138 x 10^{-12}) but not significantly better compared to the diet model (χ^2 = 0.982, df = 1, p = 0.321). On the basis of this, the simpler model, accounting for only diet as an explanation for variation in lifespan was adopted for further statistical analyses. Subsequent diagnostic analysis revealed no violations of assumptions that precludes the use of this model (Figure 1)

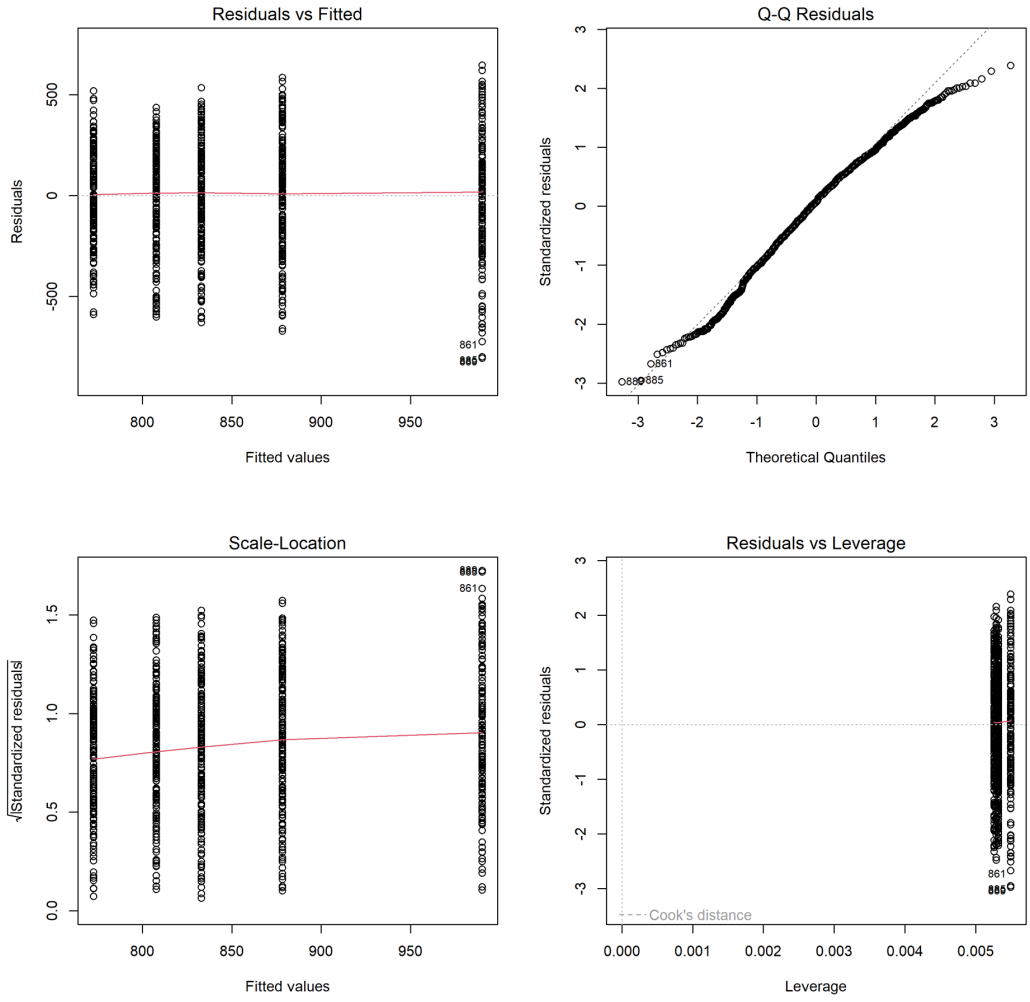

Figure 1: **Diagnostic plots for the model: Lifespan ~ Diet**

2.3 Model Structure

The algebraic structure of the two general linear models employed in further statistical analyses were thus:

Model: Survival ~ Diet

$$f = \begin{bmatrix} a_{AL}(0) \\ a_{1D} \\ a_{2D} \\ a_{20} \\ a_{40} \end{bmatrix} + c$$

Model: Mean Lifetime Body Weight $\sim$ Diet

$$f = \begin{bmatrix} a_{AL}(0) \\ a_{1D} \\ a_{2D} \\ a_{20} \\ a_{40} \end{bmatrix} + c$$

3 Results

3.1 Statistical Analysis

The average mouse lifespan across all dietary regimens (*n* = 937) was 855 days while the median lifesapn was 870. The maximal lifespan observed was 1638 days while the minimum was 183 days. By diet, the highest average lifespan was recorded in the 40% CR cohort (*n* = 182), with a mean and median lifespan of 990 (95% CI = 943.4, 1036.7) and 1033 days respectively (Figure 2A, B) . Meanwhile, the lowest average lifespan was observed in the ad libitum-fed mice (*n* = 188), with a mean and median lifespan of 773 (95% CI = 740.5, 804.6) and 763 days, respectively. Intermediate dietary regimens, 1-day fasting (*n* = 188), 2-day fasting (*n* = 190), and 20% caloric restriction (*n* = 189) increased mean lifespan relative to the AL regimen in that order.

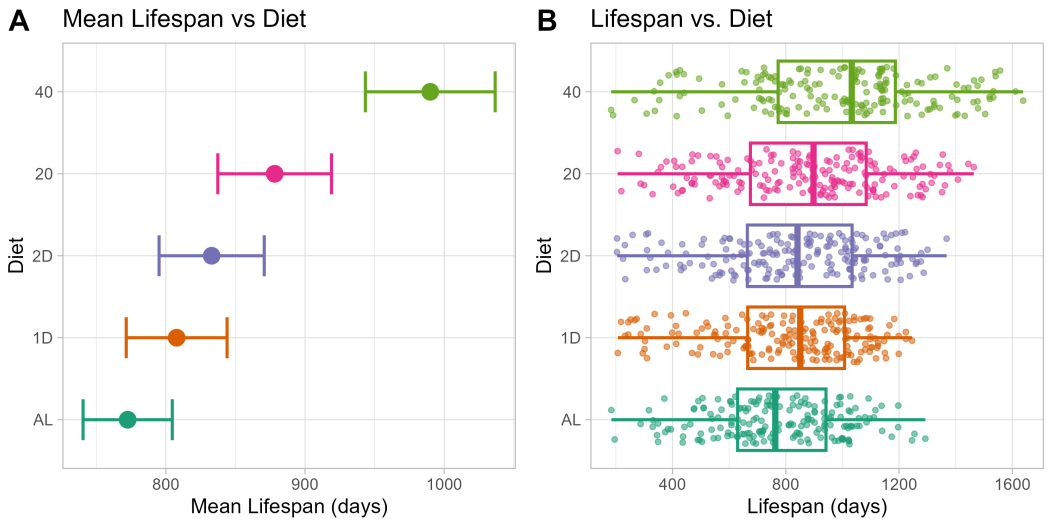

Figure 2: **Mean lifespan per diet and a boxplot of lifetime body weight per diet:** (A). Mean lifetime body weight per dietary regimen: AL (*n* = 188), 1D (*n* = 188), 2D (*n* = 190), *n* = 189), 20% CR (*n = 189*), 40% CR (*n = 182*). Data represent calculated means and error bars represent upper and lower 95% confidence intervals. (B) boxplot of all lifespan measurements, showing distribution of mean lifespan across each dietary regimen (box highlighting median (centre) and lower and upper quartiles, with whiskers representing 1.5 times the interquartile range)

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3.2 Effect of Dietary Regimen upon Lifespan

An ANOVA analysis of different dietary regimens upon lifespan revealed a significant effect of diet upon lifespan, with increasing modes of caloric restriction being associated with lifespan extension ($F_{4,932}$ = 17.63, p = 5.81 x 10^{-14}). Moreover, dietary group explained a modest but only partial proportion of lifespan variation (adjusted R^2 = 0.066). Further post-hoc analysis via a Tukey test confirmed a significant difference in mean lifespan between the AL and 40% CR group (difference = 217.5, $p < 0.001$), and between AL and 20% CR (differnece = 105.6, $p = 0.0016$). Moreover, pairwise comparison of mean lifespan in the 40% and 20% CR groups indicated a significant difference in mean lifespan (difference = 34.69, $p = 0.0007$). In contrast, a significant difference was not observed between the AL regimen and either the 1-day ($p = 0.718$) or 2-day fast regimens ($p = 0.196$).

3.3 Effect of Dietary Regimen upon Mean Lifetime Body Weight

While mean lifetime body weight was excluded from the linear model of mice lifespan, a separate ANOVA model of dietary regimen upon mean lifetime body weight highlighted a highly significant effect, with increasing dietary restriction and mean lifetime body weight being negatively correlated ($F_{4,932}$ = 86.13, p < 2.2 x 10^{-16}) (Figure 3A, B). Additionally, a substantial proportion of variation of mean lifetime body weight could be explained by different dietary regimens (adjusted R^2 = 0.066).

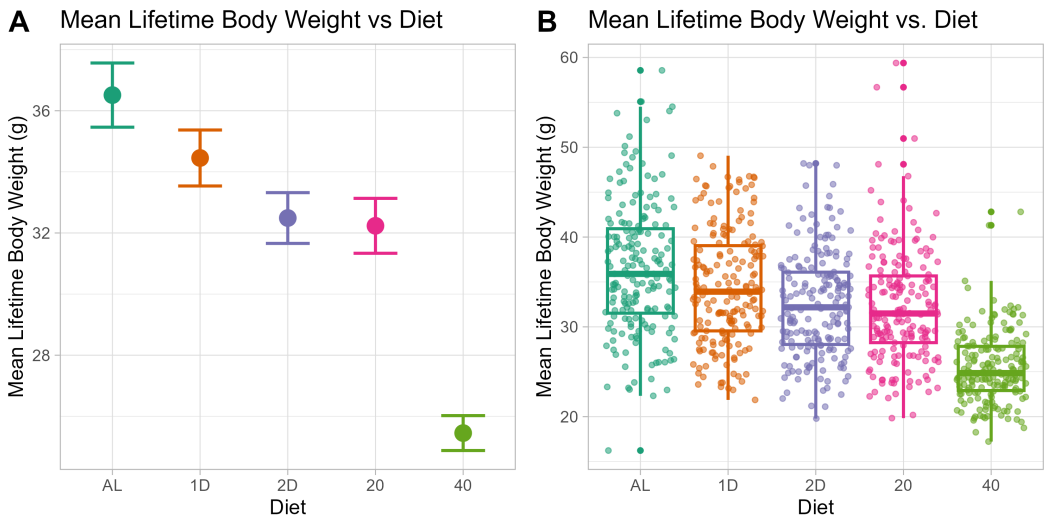

Figure 3: **Mean lifetime body weight per diet and a boxplot of lifetime body weight per diet:** (A). Mean lifetime body weight per dietary regimen: AL (*n* = 188), 1D (*n* = 188), 2D (*n* = 190), *n* = 189), 20% CR (*n = 189*), 40% CR (*n = 182*). Data represent calculated means and error bars represent upper and lower 95% confidence intervals. (B) boxplot of all lifetime body weight measurements, showing distribution of mean lifetime body weight across each dietary regimen (box highlighting median (centre) and lower and upper quartiles, with whiskers representing 1.5 times the interquartile range)

Notably, post-hoc Tukey tests illustrated that all levels of dietary regimen displayed a highly significant decrease in mean body weight relative to the AL regimen.

The Pearson correlation was calculated to assess the relationship of mean life time body weight and lifespan and revealed a weak but nonetheless significant negative correlation of mean lifetime body weight and lifespan (r = -0.11, t_{935} = -3.354, p = 0.00083, 95% CI = -0.17, -0.045). Thus, decreases in mean lifetime body weight likely

follow as a consequence of increasing calorie restriction, in contrast to mediating the extension in lifespan itself (Figure 4A, B).

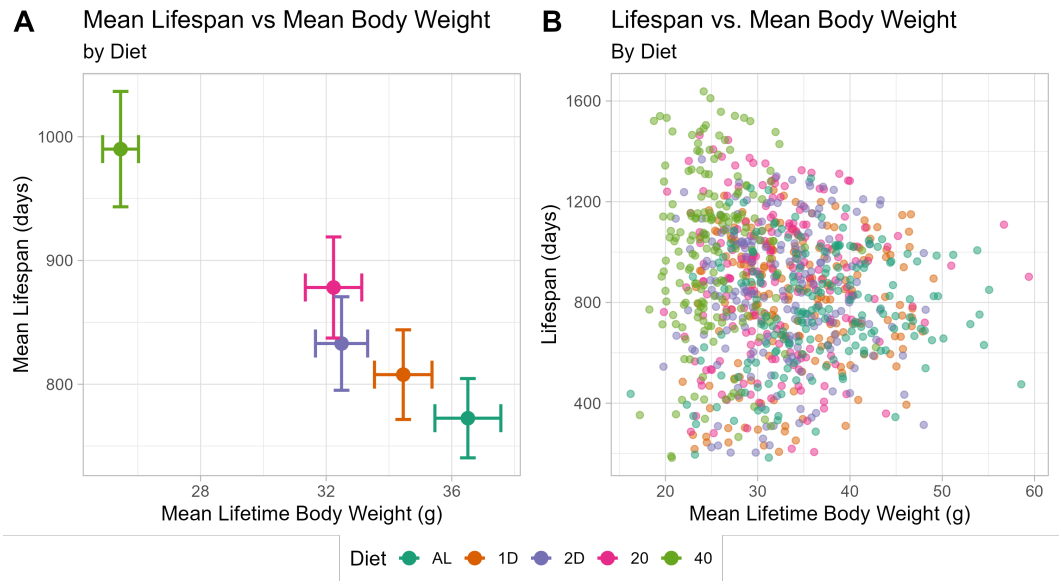

Figure 4: **Scatterplot of mean lifetime body weight by mean lifespan per diet and a scatterplot of all individual lifespans by all individual mean lifetime body weights:** (A). Mean lifespan by mean lifetime body weight per dietary regimen: AL (n = 188), 1D (n = 188), 2D (n = 190), 20% CR (n = 189), 40% CR (n = 182). Data represent calculated means and error bars represent upper and lower 95% confidence intervals. (B) Scatterplot of all observations of lifespan by mean lifetime body weight.

4 Discussion

The statistical analyses taken together demonstrate a significant positive effect of increasing degrees of caloric restriction upon lifespan, in accordance with previous research suggesting dietary, and particularly caloric, restriction associates with lifespan extension in not only mice but other eukaryotes such as *D. melanogaster* and *C. elegans* (Taormina and Mirisola, 2014).

Interestingly, although the highest lifespan was observed in 40% calorically restricted mice, the lowest lifespan, 183 days, was also recorded in this group. Moreover, variation in lifespan was greatest within the 40% calorie restriction group (SE = 23.79) while the least variation was observed within the AL-fed group (SE = 16.35). This illustrates that although significant in explaining variation in lifespan, there are other factors not investigated here that are additionally

mediating the lifespan extension seen in mice, as noted by the original authors (Francesco *et al.*, 2024) in addition to other studies of the effect size of dietary restriction upon longevity (Sleiman *et al.*, 2022).

Subsequent post-hoc analyses only demonstrated statistical significance of caloric restriction upon lifespan of the 40% and 20% calorie restriction groups relative to AL-fed groups, while the two fasting regimens were non-significant in this respect. Despite significant decreases in mean lifetime body weight within the fasting regimens relative to the AL regimen, overall calorie restriction was comparable with the AL-fed mice, resulting from an increased calorie consumption outside of the fasting period that equalises the total value. As such, this finding indicates that lifespan extension likely arises from the dietary restriction itself, coinciding with decreases in mean body weight but not mediated by it.

A weak but significant correlation was noted between lifespan and mean bodyweight, irrespective of dietary regimen. However, taking into account the model selection, an additive model of mean body weight and dietary regimen as explanatory variables for variation in lifespan, the response variable, was not deemed as well-fitted as the model incorporating only dietary regimen. Thus, it can be surmised that although there is indeed a significant, negative relationship of lifespan and mean lifetime body weight, decrease in body weight is a consequence of increasing modes of dietary restriction that coincides with lifespan extension.

The data here are limited in that the body weight metric is calculated as the mean body weight across the entire lifespan of each mouse, and as increased body weight is associated with ageing, this may obfuscate the true relationship of the two (Aunan *et al.*, 2016). Moreover, calorie restriction on its own does not account for the different contributions and effects of particular macronutrients on health outcomes (Piper, Mair, and Partridge, 2005). Additionally, only female mice were employed in the study, omitting the differences in lifespan that may arise between sexes (Sleiman *et al.*, 2022).

Previous research has highlighted that although calorie restriction consistently extends lifespan, restriction of particular macronutrients, such as specific amino acids like isoleucine, may play a underappreciated role in mediating longevity (Green *et al.*, 2023). In order to assess this, additional dietary regimens comprising a protein, fat, and carbohydrate restricted diet may elucidate the individual contributions of each macronutrient in restriction-mediated longevity.

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