The impact of metabolism on aging and cell size in single yeast cells
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Chapter 4

Calorie restriction does not elicit a robust extension of replicative lifespan in *Saccharomyces cerevisiae*

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Abstract

Calorie restriction (CR) is often described as the most robust manner to extend lifespan in a large variety of organisms. Hence, considerable research effort is directed toward understanding the mechanisms underlying CR, especially in the yeast *Saccharomyces cerevisiae*. However, the effect of CR on lifespan has never been systematically reviewed in this organism. Here, we performed a meta-analysis of replicative lifespan (RLS) data published in more than 40 different papers. Our analysis revealed that there is significant variation in the reported RLS data, which appears to be mainly due to the low number of cells analyzed per experiment. Furthermore, we found that the RLS measured at 2% (wt/vol) glucose in CR experiments is partly biased toward shorter lifespans compared with identical lifespan measurements from other studies. Excluding the 2% (wt/vol) glucose experiments from CR experiments, we determined that the average RLS of the yeast strains BY4741 and BY4742 is 25.9 buds at 2% (wt/vol) glucose and 30.2 buds under CR conditions. RLS measurements with a microfluidic dissection platform produced identical RLS data at 2% (wt/vol) glucose. However, CR conditions did not induce lifespan extension. As we excluded obvious methodological differences, such as temperature and medium, as causes, we conclude that subtle method-specific factors are crucial to induce lifespan extension under CR conditions in *S. cerevisiae*.

**Introduction**

In the last decades, a nutritious diet low in calories, commonly referred to as calorie restriction (CR), has been reported to extend the lifespan of a broad range of organisms, such as yeast, worms, fruit flies, mice, rats, and monkeys (1–6). This seemingly evolutionary conserved effect of CR on aging has sparked intense investigation into its underlying molecular mechanisms, especially in the budding yeast *Saccharomyces cerevisiae* (7–10). Some studies suggest that nutrient-responsive pathways, such as target of rapamycin (TOR), protein kinase A (PKA), and Sch9 (11), mediate CR-induced lifespan extension, whereas others hypothesize that CR increases the activity of sirtuin deacetylases (e.g. SIR2) extending lifespan by suppressing ribosomal DNA recombination (9, 12, 13).

In *S. cerevisiae*, lifespan can either be defined by the time cells remain viable in absence of nutrients, called chronological lifespan, or by the number of daughter cells a yeast cell produces during its life, which is referred to as replicative lifespan (RLS) (14). Although RLS can vary greatly between individual yeast cells, the average RLS (i.e. across a population of cells) is assumed to be a genotypic trait (15–19). However, besides CR, other environmental conditions, such as pH and carbon source, are known to have a strong impact on RLS (20, 21).

Here, through a systematic analysis of RLS data of *S. cerevisiae* using data from more than 27,000 cells, we obtained indication that small sample sizes might be the cause for the often observed significant variation between RLS data. Further, we identified that partly biased data exists. After removing such biased data, we determined the literature-wide average RLS of BY4741 and BY4742 at 2% (wt/vol) glucose and under CR conditions. As small sample sizes are inherent to the laborious microdissection technique most commonly used to measure RLS, we performed additional aging experiments with a recently developed microfluidic dissection platform that allows acquisition of data for large numbers of cells in a fully automated manner (22). Although identical RLSs were measured at 2% (wt/vol) glucose with the microfluidic dissection platform as with the classical microdissection method, CR failed to extend lifespan in the microfluidic dissection platform. After excluding factors, such as temperature and medium composition, we conclude that, in yeast, lifespan extension under CR conditions requires subtle method-specific factors.

**Materials and methods**

**Data acquisition from the literature**

Replicative lifespan curves from the literature were digitized using the GetData Graph Digitizer 2.25. In case there was no information provided about the number of cells used to generate the RLS curve, we inferred the cell number from the smallest step decrease in the fraction of viable cells.
Statistical analysis

Meta-analysis of lifespan data were performed using a linear mixed model (23). Survival curves were compared with each other using the log-rank test (36). For each condition the average replicative lifespan was given with the SE.

Strains, media and cultivation

Lifespan measurements were performed with BY4741 and YSBN6 [MATa FY3 ho::HphMX4 (29)], which is derived from S288c. YPD plates were prepared by adding 20 g/L agar to complete rich medium (50 g/L YPD; Formedium). Minimal medium [6.9 g/L yeast nitrogen base (YNB); Formedium] supplemented with filter-sterilized glucose (D-glucose monohydrate; Sigma) was used for liquid cultures. For BY4741, SC medium was used, which is minimal medium to which complete supplemental mixture was added (790 mg/L). A glucose concentration of 20 g/L [2% (wt/vol) glucose] was used for control experiments and 5 g/L [0.5% (wt/vol) glucose], 2.5 g/L [0.25% (wt/vol) glucose], 1 g/L [0.1% (wt/vol) glucose], and 0.5 g/L [0.05% (wt/vol) glucose] for calorie restriction experiments. One or 2 d before the experiment, cells were taken from the −80 °C freezer, streaked on YPD plates, and incubated overnight at 30 °C. A single colony was used to inoculate a 100-mL flask containing 10 mL SC medium with 2% (wt/vol) glucose. The flask was then incubated overnight at 30 °C with shaking at 300 rpm (Climo-Shaker ISF1-x, Kühner AG, Switzerland). The next morning, the culture was diluted (~30 μL) into a new 100-mL flask containing 10 mL medium with 2% or 0.5% (wt/vol), 0.25% (wt/vol), 0.1% (wt/vol), or 0.05% (wt/vol) glucose to yield an OD$_{600nm}$ of 0.3 after roughly 8 h. The culture was then diluted again and incubated overnight to yield an OD$_{600nm}$ of about 0.3 the next morning at the start of the aging experiment. This preculture procedure ensured that the cells were growing exponentially at the start of the RLS measurements. For 0.25% or 0.05% (wt/vol) glucose, the cultures were grown to an OD$_{600nm}$ of 0.05–0.1 to ensure that the cells were still growing exponentially at the start of the aging experiment.

RLS measurements with the microfluidic dissection platform

RLS was determined using a microfluidic dissection platform (22, 37) and an inverted fluorescence microscope (Eclipse Ti-E; Nikon Instruments). The microscope was placed in an incubator (Life Imaging Services) for cultivation at 30 °C. Brightfield images were taken every 10 min for ~100-120 h using a UV blocking filter and 60× objective (CFI Plan Apo; Nikon; NA = 1.4; working distance = 0.13 mm). Replicative lifespan was determined for each cell by counting the number of buds produced during its entire life. Experimental data obtained from cells that were washed out before death were incorporated into the lifespan curve as right-censored data using Kaplan-Meier analysis (38).
Calorie restriction does not elicit a robust extension of lifespan

RLS measurements with the microdissection technique

At the start of each experiment, cells were taken from a frozen −80 °C stock and streaked on a fresh YPD plate containing 2% (wt/vol) glucose. Cells were then grown for 2 days at 30 °C before being restreaked on a fresh YPD plate containing either 2% or 0.5% (wt/vol) glucose. The following day, the cells were streaked again on fresh YPD or SC plates containing either 2% or 0.5% (wt/vol) glucose and allowed to grow for another 12 h before being restreaked very thinly on a fresh plate. The cells were then incubated for 12 h at 30 °C. The very faint colonies present on the plate were then used to create small patches on each microdissection plate. In general, only one patch of cells was made per microdissection plate. The microdissection plates were then incubated for another 2–3 h before the start of the experiment.

Plates intended to be used for microdissection contained 50 mL of medium plus agar and were allowed to dry for 2 days on the laboratory bench to remove excess water before the start of the experiment. During the experiment, each individual microdissection plate was kept inside a plastic bag with a wet towel to prevent desiccation. All other plates used for initial plating before the microdissection experiment were parafilmed to prevent dessication. A SporePlay dissection microscope (Singer Instruments) equipped with a 50-μm–diameter dissection needle (Singer Instruments) was used to perform the microdissection experiments. Small groups of cells, which produced one to two buds in the 2- to 3-h incubation time before the start of the experiment, were gathered and rapidly moved to a fresh position on the agar plate for virgin daughter cell selection. It appeared crucial for overall cell viability to keep the amount of time that the cells spend in the microdissection needle to a bare minimum. Replicative lifespan measurements were then performed as described by Steffen et al. (39) with the exception that the fridge incubation steps were omitted, and cells were dissected 24 h/day in a 30 °C room during the entire lifespan measurement (roughly 5 days).

Results

RLS data harbor significant variation

We gathered RLS data for the yeast strains BY4741 and BY4742 because of the considerable amount of lifespan data that have been published for these relatively long-lived haploid yeast strains. In total, 220 lifespan measurements from 41 papers were collected with an average RLS between 19.4 and 41.1 buds (Figure 1A). Because the lifespan measurements were performed in various laboratories using different glucose percentages and strains with different mating types (Figure 1B), we determined how these variables individually affected RLS using a linear mixed model (23) (see SI Text, Tables S1 and S2).

The results of the linear mixed model showed that a reduction in glucose percentage from 2% (wt/vol) to 0.5% or 0.05% resulted in an increase in RLS (Figure 1C; linear mixed model: P < 0.001). However, the RLS determined at 0.5% and 0.05% glucose was identical (linear mixed model: P = 0.476), contradictory to earlier studies (6, 24). Although there was no effect of mating type (BY4741 or
BY4742) on lifespan (linear mixed model: $P = 0.1$; Figure S1), we found that the variable “laboratory” had a strong impact on RLS (Figure 1D; e.g. laboratory 1 vs. laboratory 8, t test: $P < 0.001$), suggesting that ambiguous differences between lifespan measurements (e.g. incubation times and preculture methods) may have a strong impact on RLS.

We observed that variation in RLS was not only present between laboratories, but to a varying degree also within laboratories (Figure S2). We hypothesized that the number of cells analyzed per experiment might have caused this variation. To understand the effect of sample size on experimental variation, we used a modified funnel plot, in which sample size is plotted against the measured RLS (Figure 2A). This plot shows an inverse relationship between sample size

![Figure 1. Replicative lifespan measurements harbor significant variation. (A) Overview of all lifespan curves at 2% (wt/vol) glucose gathered from 41 different papers for BY4741 and BY4742. (Inset) The RLS curve with the lowest and highest reported RLS for 2% (wt/vol) glucose (gray lines) and CR conditions (dashed lines). (B) Subdivision of the gathered lifespan data over variables, such as glucose percentage [2%, 0.5%, and 0.05% (wt/vol) glucose], strain (BY4741, BY4742, or an aggregate of BY4741 and BY4742), and laboratory. (C and D) Overview of the RLS published for different glucose percentages (C) and individual laboratories [D, only 2% (wt/vol) glucose]. Median RLS is indicated with a line inside the box plot. The box represents the first and third quartiles and the whiskers 1.5 times the interquartile range. Each RLS measurement is indicated by a dot.](image-url)
and the average RLS determined from all literature data. Measurements with a small sample size are scattered over a broad range of lifespans at the bottom of the graph, whereas measurements with a larger sample size converge toward the average RLS of all lifespan curves. Apparently, high numbers of cells are necessary to reliably estimate RLS. In fact, we determined using sample size estimation (25) that in order to reliably measure the effect of CR on replicative lifespan at least 100 cells need to be analyzed per lifespan curve. However, in most studies, only a limited number of cells are analyzed per experiment (median of 59 cells per experiment), which means that most studies are too small to reliably determine the effect of environmental and genetic perturbations on RLS.

**RLS measured at 2% glucose is shorter in CR studies compared with other studies.**

During our data analysis, we further noticed that the RLS reported at 2% (wt/vol) glucose in CR experiments was generally lower compared with those in other experiments in which the effect of CR on RLS was not measured (23.4 ± 0.3 vs. 25.9 ± 0.1 buds; P < 0.001; Figure 2B). Even within papers that studied CR, the average RLS at 2% (wt/vol) glucose was lower for controls of CR experiments.

![Figure 2. The average RLS at 2% (wt/vol) glucose in CR studies are biased toward shorter RLS. (A) Plot showing the measured RLS vs. the number of cells analyzed. Three outliers, which had a RLS of more than 10 buds longer than the average RLS determined for all 2% (wt/vol) glucose lifespan curves gathered in this study (25.8 ± 0.1 buds), were removed from the plot. As the sample size of the experiment increases, the reported RLS deviates less from the average RLS. (B) Density plot comparing the RLS measured under CR conditions (30.2 ± 0.3 buds) with the RLS measured at 2% (wt/vol) glucose in the accompanying CR control experiments (23.4 ± 0.3 buds) and in all other control experiments (25.9 ± 0.1 buds). (C) Overview of lifespan curves for BY4741 from Kruegel et al. (26). The lifespan curve obtained under CR conditions (curve 10, dashed black line) overlaps with most of the lifespan curves measured at 2% (wt/vol) glucose (gray lines). The CR control experiment at 2% (wt/vol) glucose (curve 9, black line) is exceptionally short-lived relative to all of the other lifespan curves. (Inset) Box-plot with the distribution of the median RLS reported for WT BY4741 in Kruegel et al. (26). Curve 9 is the single outlier.](image-url)
than for other controls, which were not associated with CR experiments (23.4 ± 0.3 vs. 25.8 ± 0.1 buds; P < 0.001; Figure S3). To illustrate this point, we focused on a study by Kruegel et al. (26) that reports an average RLS of 21.5 buds for 2% (wt/vol) glucose (Figure 2C, curve 9) and 27.1 buds under CR conditions (Figure 2C, curve 10). Although this comparison shows a substantial increase in lifespan under CR conditions (i.e. from 21.5 to 27.1 buds, P = 0.036), we discovered that the 2% (wt/vol) glucose control curve (curve 9) linked to the CR experiment (curve 10) had a significantly shorter RLS than all of the other 10 lifespan curves for 2% (wt/vol) glucose reported in the same paper (Figure 2C, Inset). Moreover, we found that the RLS under CR conditions (curve 10) is not significantly different from the majority of the RLS measured at 2% (wt/vol) glucose (Figure S4). Thus, the significant lifespan extension reported under CR conditions in this specific paper is solely caused by an unusually short-lived control experiment.

Because the average RLS reported for 2% (wt/vol) glucose controls in CR experiments was on average significantly shorter than the ones of other 2% (wt/vol) glucose measurements (Figure 2B), we removed all 2% (wt/vol) glucose data originating from CR control curves from our dataset (22 of 220 lifespan curves). On the basis of the remaining literature data, we then determined that the average RLS of BY4741 and BY4742 is 25.9 ± 0.1 buds under 2% (wt/vol) glucose conditions and that CR conditions extend the average RLS by 18% to 30.2 ± 0.3 buds (P < 0.001). When we performed microdissection experiments ourselves, we measured RLS for BY4741 that were almost identical to those determined from the consolidated literature data [25.5 ± 0.9 buds at 2% (wt/vol) glucose (n = 102 cells) vs. 30.4 ± 1.0 buds at 0.5% glucose (n = 102 cells); P < 0.001; Figure S5].

**CR does not extend replicative lifespan in a microfluidic dissection platform.**

As the classical microdissection method is a labor intensive technique to determine RLS, especially when larger sample sizes are required, we questioned whether the recently developed microfluidic dissection methods (22, 27, 28) could generate similar lifespan data. The advantage of using a microfluidic dissection method is that RLS can be measured in a semiautomated manner with a large number of cells under constant environmental conditions without incubation at low temperature during the night and with constant glucose concentration.

We performed a series of 10 independent RLS measurements at 2% and 0.5% (wt/vol) glucose with the microfluidic dissection platform developed by Lee et al. (22). Although at 2% (wt/vol) glucose the RLS measured with the microfluidic dissection platform was similar to the average RLS that we determined from the literature (26.5 ± 0.5 vs. 25.9 ± 0.1 buds; P = 0.8), we could not find any lifespan extending effect of CR with the microfluidic dissection platform. Instead, the RLS at 0.5% glucose was identical to the RLS measured at 2% (wt/vol) glucose (25.9 ± 0.7 vs. 26.5 ± 0.5 buds; Figure 3; P = 0.11). Also for the longer-lived prototrophic yeast strain YSBN6 (29), we could not observe any CR-induced lifespan extension with the microfluidic dissection platform (35.3 ± 0.9 vs. 35.3 ± 0.8 buds; P = 0.42; Figure S6).
Calorie restriction does not elicit a robust extension of lifespan

We hypothesized that a reduction in the glucose concentration of the medium may be sensed differently by the cells inside the microfluidic dissection platform compared with those on an agar plate. For instance, in the microfluidic setup, cells are completely surrounded by a flow of medium, while on an agar plate, a diffusion limitation could exist, which could generate a lower effective glucose concentration in CR experiments, leading to lifespan extension. We therefore performed additional RLS measurements with the microfluidic dissection platform with lower glucose concentrations, i.e. 0.25%, 0.1%, and 0.05% glucose. However, at these lower glucose concentrations, we obtained RLS values similar to those obtained at 2% (wt/vol) glucose [i.e. for 0.25% glucose, 25.5 ± 0.7; for 0.1% glucose, 27.9 ± 1.0; and for 0.05% glucose, 24.6 ± 0.8 vs. 26.5 ± 0.5 buds for 2% (wt/vol) glucose; Figure S7] and therefore no significant effect of CR on RLS.

CR-induced lifespan extension depends on method-specific factors.

As the experimental conditions with the microfluidic dissection platform and the classical microdissection technique are slightly different, e.g. in the microfluidic dissection platform temperature cycles are absent and synthetic complete (SC) medium is used instead of yeast peptone dextrose (YPD) medium, we wondered

![Figure 3. CR does not elicit a robust extension of replicative lifespan. With the microfluidic dissection platform, we measured an average RLS of 26.5 ± 0.5 buds (n = 2,806 cells of which 2,409 were washed out before death) at 2% (wt/vol) glucose and 25.9 ± 0.7 buds at 0.5% glucose (n = 2,055 cells of which 1,716 were washed out before death). Here, CR does not extend RLS. However, with the classical dissection method RLS is extended from 25.8 ± 0.9 buds at 2% (wt/vol) glucose (n = 122 cells) to 29.6 ± 0.9 buds at 0.5% glucose (n = 129 cells) The classical dissection and microfluidic experiments were both performed with YNB medium and at a constant incubation temperature of 30 °C.](image)
whether these factors played a role in eliciting a CR-induced lifespan extension. We therefore performed microdissection experiments with BY4741 on SC medium. Further, to avoid temperature cycles, we dissected cells continuously inside a 30 °C room. Under these conditions, we obtained an RLS of 25.8 ± 0.9 buds at 2% (wt/vol) glucose (n = 129 cells) and 29.6 ± 0.9 buds at 0.5% glucose (n = 122 cells), which both correspond well with the average RLS determined from our meta-analysis [for 2% (wt/vol) glucose, 25.8 ± 0.9 buds vs. 25.9 ± 0.1 buds; P = 0.9; for 0.5% glucose, 29.6 ± 0.9 buds vs. 30.3 ± 0.4 buds; P = 0.4; Figure 3]. Apparently, factors, such as the use of SC or YPD medium and the absence or presence of periods of lower temperature (4 °C), have no significant effect on the CR-induced extension of RLS. We therefore conclude that subtle method-specific factors, e.g. growth on a solid surface or handling with a needle, are crucial to elicit a CR-induced RLS extension.

Discussion

Although numerous studies reported lifespan extension in S. cerevisiae in response to CR, our meta-analysis uncovered several problems with the currently available RLS data, such as differences in the measured RLS between laboratories, short-lived 2% glucose CR controls, and small sample sizes. The significant variation that we observed between RLS measurements could explain several contradictions that exist about CR in literature, such as whether the histone deacetylase SIR2 (9, 30, 31) or the ability to respire are required for CR-induced lifespan extension (32, 33), whether CR is able to extend RLS in the yeast strain W303 (9, 13, 30), and whether the optimal glucose concentration for CR is at 0.5% or 0.05% glucose (24, 32). In fact, in our dataset, the RLS was identical in the presence of 0.05% and 0.5% glucose.

Based on our literature analysis of RLS data, we determined that the average RLS of the yeast strains BY4741 and BY4742 on 2% (wt/vol) glucose is 25.9 buds and 30.2 buds under CR conditions (0.5% and 0.05% glucose YPD medium). These average RLS values could serve as reference values in future yeast aging studies. In addition, we recommend that lifespan curves are generated with at least 100 cells to obtain more reliable RLS measurements. In this study, we obtained identical lifespans for BY4741 at 2% (wt/vol) glucose with the microfluidic dissection platform as with the classical dissection technique (26.5 ± 0.5 vs. 25.8 ± 0.9 buds; P = 0.3). However, we were unable to observe any CR-induced lifespan extension using the microfluidic dissection platform. This discrepancy between the two methods cannot be explained by differences in medium, the absence of temperature cycles or by a general inability of the microfluidic dissection platform to generate lifespan extension, because we previously demonstrated that the microfluidic platform generates identical RLS data for several longevity mutants as the classical dissection method [e.g. median RLS of 32 vs. 34 buds for fob1Δ and 13 vs. 13 buds for sir2Δ (22)]. Thus, apparently, CR-induced lifespan extension also depends on subtle, yet unknown, differences between the methods used to determine RLS.
In other organisms, methodology was also shown to be of key importance on the effect that CR has on lifespan. For example, although an initial study suggested that Rhesus monkeys live longer under CR conditions (3), a recent study found no evidence for CR-induced lifespan extension (34). The key difference between these studies appears to be the exact composition of the diet of the monkeys (34). Also in Drosophila (35), the effect of calorie restriction was not merely dependent on the amount of calories in the diet, but on the ratio of yeast and sugar in the diet. Overall, these recent developments suggest that lifespan extension by CR is not as robust as once believed.

Acknowledgments

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References


Supplementary Information

SI Text

Explanation of statistical analysis of the data using a linear mixed model

In our statistical analysis, we explain changes in average replicative lifespan (RLS) by means of three variables: glucose concentration in the medium [2%, 0.5%, and 0.05% (wt/vol)], strain (BY4741, BY4742, and BY4741/2), and laboratory. In our model, we consider lifespan data obtained for the yeast strain BY4741 at 2% (wt/vol) glucose as the reference condition (model intercept, I). Using the data obtained for BY4741 at 2% (wt/vol) glucose as a reference implies that the effects of glucose levels and strain types on average RLS are always determined relative to this reference (Tables S1 and S2). The variable “lab” has a special role because it is not expected to have any effect on the average RLS. In particular,
it reflects the random variation in the data between laboratories that cannot be explained by glucose concentration and strain in the model. Accordingly, we consider the laboratory as a random effect, whereas the differences between glucose levels and strains appear as fixed parameters in the model. The coefficients associated with the variable lab are Gaussian random variables, which means are assumed to be zero and variances are estimated from the data. In essence, the variable lab reflects the error term of linear models, which is typically Gaussian, and the variance (the so-called residual variance) is obtained from the data. We defined random effects per glucose level to account for the variability present in the data from individual laboratories at different glucose concentrations. Note, models that combine fixed (i.e. glucose concentration and strain) and random effects (i.e. laboratory) are called mixed models in the statistical literature. The formulation of the model is

\[
\text{RLS} = I + B_{BY4742}X_{BY4742} + B_{BY4741/2}X_{BY4741/2} + (B_{0.05} + R_{0.05})X_{0.05} + (B_{0.5} + R_{0.5})X_{0.5} + R_{1}X_{2} + E
\]  

(model 1)

where RLS is the average RLS of the cells; I is the model intercept (=average RLS of strain BY4741 at 2% (wt/vol) glucose); \(B_{BY4742}\), \(B_{BY4741/2}\), \(B_{0.05}\), and \(B_{0.5}\) are the parameters associated with the levels of the fixed effects of the model (glucose concentration and strain); \(R_{1}\), \(R_{0.5}\), and \(R_{0.05}\) are random effects of the model, which are added to account for the random variation produced by the laboratory within the three available glucose levels \(R_{1}\), \(R_{0.5}\), and \(R_{0.05}\) are Gaussian random variables with a mean of zero and variances \(s^{2}(\text{Intercept|laboratory})\), \(s^{2}(0.5|\text{laboratory})\) and \(s^{2}(0.05|\text{laboratory})\); E is the residual of the model, which is assumed to follow a Gaussian distribution with a mean of zero and variance \(s^{2}(\text{residual})\); \(X_{BY4742} = 1\) for strain BY4742; otherwise \(X_{BY4742} = 0\); \(X_{BY4741/2} = 1\) for strain BY4741/2; otherwise \(X_{BY4741/2} = 0\); \(X_{2} = 1\) when the glucose concentration is 2% (wt/vol); otherwise \(X_{2} = 0\); \(X_{0.5} = 1\) when the glucose concentration is 0.5%; otherwise \(X_{0.5} = 0\); and \(X_{0.05} = 1\) when the glucose concentration is 0.05%; otherwise \(X_{0.05} = 0\). Note that for strain BY4741 at 2% (wt/vol) glucose, model 1 reduces to \(\text{RLS} = I + R_{1} + E\); where the average RLS is I, with a random variation on top that is modeled by \(R_{1}\) [the random variation produced by the laboratories in 2% (wt/vol) glucose experiments] and E (the model errors). In a similar fashion, the model reduces to \(\text{RLS} = I + B_{BY4742}X_{BY4742} + (B_{0.5} + R_{0.5})X_{0.5} + E\) for strain BY4742 at 0.5% glucose. Initially, we used all of the available data to determine the parameters in model 1 (Table S1). Then, to validate that the obtained estimates are robust against outliers, we redetermined the parameters in model 1 using lifespan data that lies within the 95% CI for each glucose concentration (Table S2).


CALORIE RESTRICTION DOES NOT ELICIT A ROBUST EXTENSION OF LIFESPAN


**SI Figures**

**Figure S1.** Mating type does not affect RLS. Overview of RLS curves obtained at 2% (wt/vol) glucose presented by strain (black). For comparison all RLS curves obtained at 2% (wt/vol) glucose are plotted in the background in gray. (A) BY4741. (B) BY4742.
Figure S2. There is a significant variation in RLS curves measured between laboratories. Overview of RLS curves at 2% (wt/vol) glucose per laboratory (black). For comparison all RLS curves obtained at 2% (wt/vol) glucose are plotted in the background in gray. (A) Lab 1. (B) Lab 2. (C) Lab 3. (D) Lab 4. (E) Lab 5. (F) Lab 6. (G) Lab 7. (H) Lab 8.

Figure S3. The RLS of 2% (wt/vol) glucose calorie restriction (CR) control experiments is generally lower than that of other 2% (wt/vol) glucose controls. The average RLS of 2% (wt/vol) glucose CR controls reported by CR studies is 23.4 ± 0.3 buds, whereas the average RLS reported for other controls by CR studies is 25.9 ± 0.1 buds (P = 0.001).
Figure S4. (Left) The RLS curve used as a CR control is significantly shorter lived than the other 2% glucose controls. Overview of RLS data obtained from Kruegel et al. (1). The RLS curve measured under CR conditions is indicated with a dashed black line, whereas RLS curves measured at 2% (wt/vol) glucose are in black (used as CR control) and gray (used as other controls). One can observe that the RLS curve used as a CR control is shorter lived than the other 2% (wt/vol) glucose controls. (Right) Heatmap with pairwise comparisons showing the outcome of a pairwise comparison between all RLS curves within each paper using log-rank. P < 0.05 indicates that there is a significant difference.

Figure S5. CR extends the average RLS of BY4741 from 25.5 ± 0.9 buds at 2% (wt/vol) glucose (black; n = 102 cells) to 30.4 ± 1.0 buds at 0.5% glucose (gray; n = 102 cells; P < 0.001). RLS measurements were performed with the classical dissection method and yeast peptone dextrose (YPD) medium.
Figure S6. CR does not extend the RLS of YSBN6 measured with the microfluidic dissection platform. We measured an identical average RLS for YSBN6 of 35.3 ± 0.9 buds at 2% (wt/vol) glucose (gray; five replicates; n = 2,642 of which 2,295 cells were washed out before death) and 35.3 ± 0.8 buds at 0.5% glucose (black; seven replicates; n = 1,732 cells of which 1,419 cells were washed out before death). Experiments were performed with yeast nitrogen base (YNB) medium.

Figure S7. Low concentrations of glucose do not extend RLS in the microfluidic dissection platform. We measured an RLS of 26.5 ± 0.5 buds at 2% (wt/vol) glucose (n = 2,806 cells of which 2,409 were washed out before death), 25.9 ± 0.7 buds at 0.5% glucose (n = 2,055 cells of which 1,716 were washed out before death), 25.5 ± 0.7 buds at 0.25% glucose (n = 601 cells of which 450 were washed out before death), 27.9 ± 1.0 buds at 0.1% glucose (n = 586 cells of which 472 were washed out before death), and 24.6 ± 0.8 buds at 0.05% glucose (n = 651 cells of which 476 were washed out before death). A reduction in the glucose concentration of the medium does not elicit an extension of RLS in the microfluidic dissection platform.
Calorie restriction does not elicit a robust extension of lifespan

Table S1. Results of the mixed model analysis for the average RLS using glucose and strain as fixed factors and for laboratories, nested in glucose levels, as random factor

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<tr>
<td>I</td>
<td>25.650</td>
<td>0.659</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$B_{BY4742}$</td>
<td>-0.839</td>
<td>0.274</td>
<td>0.007</td>
</tr>
<tr>
<td>$B_{BY4741/2}$</td>
<td>-0.757</td>
<td>0.287</td>
<td>0.024</td>
</tr>
<tr>
<td>$B_{0.05}$</td>
<td>6.077</td>
<td>1.213</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$B_{0.5}$</td>
<td>7.385</td>
<td>1.081</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$S^2$ (intercept</td>
<td>laboratory)</td>
<td>7.505</td>
<td></td>
</tr>
<tr>
<td>$S^2$ (0.05</td>
<td>laboratory)</td>
<td>9.237</td>
<td></td>
</tr>
<tr>
<td>$S^2$ (0.5</td>
<td>laboratory)</td>
<td>6.164</td>
<td></td>
</tr>
<tr>
<td>$S^2$ (residual)</td>
<td>88.569</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The reference level (intercept, I) is defined by cells in 2% (wt/vol) glucose medium belonging to strain BY4741.

Table S2. Results of the mixed model analysis for the average RLS using glucose and strain as fixed factors and for laboratories, nested in glucose levels, as random factor

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Estimated value</th>
<th>SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>25.306</td>
<td>0.523</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$B_{BY4742}$</td>
<td>-0.565</td>
<td>0.275</td>
<td>0.095</td>
</tr>
<tr>
<td>$B_{BY4741/2}$</td>
<td>-0.527</td>
<td>0.287</td>
<td>0.146</td>
</tr>
<tr>
<td>$B_{0.05}$</td>
<td>5.872</td>
<td>0.959</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$B_{0.5}$</td>
<td>5.774</td>
<td>1.088</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>$S^2$ (intercept</td>
<td>laboratory)</td>
<td>3.720</td>
<td></td>
</tr>
<tr>
<td>$S^2$ (0.05</td>
<td>laboratory)</td>
<td>9.258</td>
<td></td>
</tr>
<tr>
<td>$S^2$ (0.5</td>
<td>laboratory)</td>
<td>6.610</td>
<td></td>
</tr>
<tr>
<td>$S^2$ (residual)</td>
<td>86.611</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In this case, we consider only data within the 95% CI at each glucose concentration. The reference level (intercept value, I) is defined by cells of strain type BY4741 cultured at 2% (wt/vol) glucose.