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Moderate dietary restriction delays the onset of age-associated sarcopenia in *Caenorhabditis elegans* due to reduced myosin UNC-54 degradation.

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Abstract

Sarcopenia, a gradual decrease in skeletal muscle mass and strength, is a major component of frailty in the elderly, with age, (lack of) exercise and diet found to be the major risk factors. The nematode *Caenorhabditis elegans* is an important model of sarcopenia. Although many studies describe loss of muscle function in ageing *C. elegans*, surprisingly few report on the loss of muscle mass. Here, in order to quantify loss of muscle mass under various dietary restriction (DR) conditions, we used an internal GFP standard to determine levels of the major body wall muscle myosin (UNC-54) in transgenic *unc-54::gfp* worms over their lifespan. Myosin density linearly increased during the first week of adulthood and there was no significant effect of DR. In contrast, an exponential decrease in myosin density was seen during the second week of adulthood, with reduced rates of myosin loss for mild and medium DR compared to control. UNC-54 turnover rates, previously determined using pulse-labelling methods, correspond well with the $t_{1/2}$ value found here for UNC-54-GFP using fluorescence (control $t_{1/2}$ = 12.0 days), independently validating our approach. These data indicate that sarcopenia is delayed in worms under mild and medium DR due to a reduced rate of myosin UNC-54 degradation, thereby maintaining protein homeostasis.

Keywords – Aging; sarcopenia; *C. elegans*; healthspan; myosin UNC-54

Introduction

One of the key changes associated with ageing is sarcopenia, the gradual loss of skeletal muscle mass and strength, which leads to loss of functional mobility and independence and increasing risk of falls and fractures (Cruz-Jentoft and Sayer, 2019). Multiple factors have been implicated in the development and progression of sarcopenia, such as hormonal changes, loss of neurons and satellite cells, altered proteostasis, inflammation and oxidative stress, but the molecular mechanisms involving sarcopenia remains unclear (Dao et al., 2020; Wiedmer et al., 2021). Currently there is no effective treatment to reverse sarcopenia, although dietary intervention and exercise can slow the rate of muscle loss (Kim et al., 2022; Domingues-Faria et al., 2016; Phu et al., 2015). With a growing ageing population, it is essential to understand the molecular mechanisms behind sarcopenia in order to develop effective therapies and improve healthspan (Han et al., 2018; Kwak and Kwon, 2019).

The nematode *Caenorhabditis elegans* has proven to be a valuable model for ageing research, because of its short lifespan (18-21 days), genetic tractability and relatively simple physiology (Christian and Benian, 2020; Son et al., 2019). Similar to what is seen for human sarcopenia, ageing *C. elegans* undergo loss of sarcomere integrity and mobility (Park et al., 2017) (Ibáñez-Ventoso et al., 2016). However, quantifying the extend of sarcopenia in *C. elegans* has proven to be problematic, with most studies using visual assessment of myofilament organization, combined with motility assays that rely on manual scoring, which can be subjective (Herndon et al., 2002). One study recently reported an automated approach to analyze sarcopenia in *C. elegans*, using various descriptors of muscle integrity and organization, for example counting of thick filaments, which allows prediction of biological age and health span (Dhondt et al., 2021). Although this automated approach allows morphological staging of sarcopenia based on muscle architecture, it does not report on the rate of muscle loss, one of the key features of sarcopenia.

The current study addresses this gap using a molecular approach to quantify sarcopenia and the rate of muscle loss. To validate this method, sarcopenia rates are evaluated in *C. elegans* that are known to experience a delay in sarcopenia onset because of dietary restriction (DR) and compared to a control group. Reducing calorie intake by 20-40%, has proven to be effective in extending lifespan and improving health span, attenuating and delaying the onset of sarcopenia in a wide range of organisms, including *C. elegans* (Hwangbo et al., 2020; Xie et al., 2020). Although this type of DR does not prevent the loss of muscle mass, it can slow down the process significantly, as demonstrated in both rats and rhesus monkeys (McKiernan et al., 2004) (Rhoads et al., 2020) respectively. In *C. elegans*, using longevity mutants, Bansal *et al.* demonstrated that lifespan-extending mutations (*daf-2, eat-2, ife-2* and *clk-1*) also extend the period of frailty with overall reduced mobility, but did not report specific loss of muscle related to sarcopenia (Bansal et al., 2015).

This study reports in detail the myosin density of *C. elegans* body wall muscle during its entire lifespan to establish the potential effect of various DR regimes on the rate of muscle loss. Instead of a genetic model of DR in *C. elegans* (*e. g.*, *eat-2* mutant worms*)*, our work uses the solid DR method (sDR) and a transgenic *C. elegans* strain (*unc-54::GFP)*, to quantify myosin density in *C. elegans* using fluorescence spectroscopy. This strain has one of its myosin heavy chain isoforms (UNC-54) labelled with GFP (Várkuti et al., 2012). The *unc-54* gene codes for the muscle myosin heavy chain B (MHC B), a major component of the body-wall muscle in *C. elegans* and is essential for locomotion and maintaining structural stability in sarcomeres (Várkuti *et al*., 2012). To our knowledge, this is the first study documenting the detailed rate of the loss of the major muscle myosin isoform UNC54 in *C. elegans*, exposed to different severities of DR (Greer at al., 2007), over the whole course of its lifespan. Our results show that the onset and rate of sarcopenia is delayed in worms under mild and medium DR, compared to the control, with corresponding slower rates of myosin UNC-54 loss for mild/medium DR groups. This

suggests that slower myosin UNC-54 turnover rates contribute to the delay in sarcopenia progression seen in these groups.

Materials and Methods

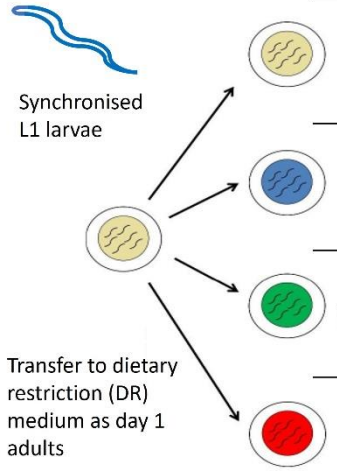
C. elegans strain and Maintenance

Worms expressing UNC-54::GFP were a generous gift from Dr Andras Malnasi-Csizmadia (Eötvös Loránd University, Budapest, Hungary) and were used in all DR assays. This strain was generated by transforming *unc-54(e1092)* mutants with punc-54::UNC-54::GFP by microparticle bombardment and the subsequent selection of homozygous worms from integrated lines (Várkuti et al., 2012). All populations were grown initially on standard nematode growth medium (NGM) at 20ºC with *Escherichia coli* OP50 as the food source (Stiernagle, 2006). Unless otherwise stated, *E. coli* OP50 cultures used for seeding plates were inoculated from a single bacterial colony in Luria broth (LB) and allowed to grow overnight at 37°C, before seeding on NGM plates. Synchronous populations of worms were generated by treating gravid adult worms with sodium hypochlorite (bleaching) and the eggs were maintained at 20°C without food for 24 hours. After 24 hours, plates with L1 worms were seeded with OP50 and let to develop for three days to adulthood. The adult worms were then transferred to the experimental plates designed for the assay (Stiernagle, 2006).

Dietary restriction assays

The solid DR (sDR) method was adapted from Greer *et al*. (2007) and bacterial concentration was estimated using the method described by (Ching and Hsu, 2011) and summarized in Figure S1. Briefly, OP50 bacteria was grown to saturation, overnight at 37 °C. The concentration of the overnight bacterial culture was calculated by plating a serial dilution and subsequent counting of colony forming units (cfu). The bacteria stock was then diluted to achieve concentrations of $8x10^{10}$ (mild DR-mDR), $8x10^{8}$ (medium DR-mdDR) and $8x10^{6}$ (severe DR-sDR) cfu/ml. 100 µl of these diluted bacterial cultures were

seeded on 55mm nutrient growth media (NGM) plates. The NGM plates were modified by excluding peptone to prevent bacterial growth (Stastna et al., 2015). The *ad libitum* (control) was $8x10^{10}$ cfu/ml and 100µl of this was seeded on standard NGM plates. 15 adult worms were placed on each plate starting at day 1 of adulthood, with 4 plates per treatment, giving a total of 60 individuals per treatment. Plates were randomised and blind coded. Worms were moved to fresh plates every other day to prevent starvation and maintain the experimental conditions (Greer et al., 2007). 5-fluoro-2'deoxyuridine (FUdR) was added on top of the bacterial lawn 24 hours before worms were introduced to the plates on the first day of adulthood to prevent reproduction (Hosono, *et al.*, 1989). Worms were transferred off the FUdR-containing plates once reproduction has ceased (7-10 days). A scheme that summarizes the experimental setup and the frequency of the measurements being taken is included below:



	Lifespan*	Motility*	Fluorescence*
Control (no DR)	Daily	Daily	Every 2 days
Mild DR	Daily	Daily	Every 2 days
Medium DR	Daily	Daily	Every 2 days
Severe DR	Daily	Daily	Every 2 days

* Recordings started on day 1 of adulthood and ended when all members of a group had died

Scheme 1: summary of experimental setup and frequency of measurements. Recordings started of day 1 of adulthood (L4, young adult) and ended when all individuals of a group had died, typically 3-4 weeks later.

Lifespan assays

All lifespan assays on *C. elegans* were performed at 20°C and started on day 1 of adulthood (L4 stage, young adult), immediately after transfer of the synchronised adult worms to their respective treatment plates. For each lifespan assay, 4 plates with 15 adult worms each were used per treatment, giving a total of 60 individuals per treatment. Lifespan was measured daily until the end of the population's lifespan, typically 3-4 weeks. by counting the number of dead and alive worms in each population, and recording it as a percentage of the day's population (Lee et al., 2006). Worms were considered dead when they no longer responded in any way to the stimulus of contact with the worm pick, and were removed from the plates (Lee et al., 2006). Worms were censored if they crawled off the plate or died from vulvul bursting. Data from the lifespan assay were analyzed using Kaplan-Meier Survival curves with the statistical significance determined by log-rank analysis in Minitab Statistical Software. Differences were considered statistically significant at $p<0.05$.

Motility Assays

The effect of DR on motility of *C. elegans* was determined by a motility assay (Herndon *et al.,* 2002*).* Worms were treated as per the lifespan assay described above and started on day 1 of adulthood (L4 stage, young adult), immediately after transfer of the synchronized adult worms to their respective treatment plates. For each motility assay, 4 plates with 15 adult worms each were used per treatment, giving a total of 60 individuals per treatment. Motility was recorded every day for the different treatment groups until the end of the population's lifespan, typically 3-4 weeks. Briefly, motility was assessed daily by subjecting the worms to gentle stroking with a worm pick and their response to the stimulus was grouped into one of three classes and then the percentage of the total living population on the observation day in each class was recorded The classes were defined as follows: Class 1 shows continuous smooth movement and fast movement when simulated, Class 2 individuals display slow halting movement and smooth movement when stimulated whereas Class 3 shows small movement of head or tail and very slow

movement when stimulated (Golden et al., 2008). Non-linear regression analysis (Prism software) was used to determine the average number of days for 50% of the initial population to move from motility class 1 to class 2 (parameter $C_{1\text{-}2}$), and from class II to class III (parameter $C_{2\text{-}3}$). The average time spend in class II (parameter ΔC_2) is defined as the difference between parameters $C_{2\text{-}3} - C_{1\text{-}2}$. Statistical significance of motility parameters was determined using One-way ANOVA with Fisher's LSD post hoc test (GraphPad Prism 9).

Fluorescence microscopy

Fluorescence intensity images of *C. elegans* were measured by taking a random sample of 5 specimens from each DR population. Measurements started on day 1 of adulthood (L4 stage, young adult), immediately after transfer of the synchronised adult worms to their respective treatment plates. After day 2 of adulthood, fluorescence measurements were done every other day (e.g., day 4, 6, 8, 10, 12, 14, 16….) until the end of the population's lifespan, typically 3-4 weeks. The worms were immobilized on slides with 10µl of 1:4 ethanol to M9 buffer solution, and whole-body fluorescence images were taken in the presence of an internal standard, In-Speck Green (505/515 nm) calibration beads, using an Olympus IX83 fluorescence microscope. A standard calibration curve was constructed by plotting the average fluorescence of 10 individual beads for each fluorescence concentration (0.1-100%) (Figure S3). Fluorescence images were corrected for background fluorescence (Patterson et al., 1997) and analyzed using ImageJ (Schneider *et al*., 2012). Statistical analysis of the fluorescence data was performed by using two-way ANOVA with GraphPad Prism (Version 8.0). Fluorescence differences were considered statistically significant at $p<0.05$. Fluorescence images were also used to determine average body length and width using Image J software.

Results

Mild and medium DR increases lifespan of *C. elegans unc54::GFP*

In order to validate our DR approach and use of unc54::GFP strain the effect of various dietary restriction regimes on the lifespan of *C. elegans* (*unc54::GFP*), is depicted in Figure 1 and summarized in Table 1. The extension of mean lifespan was most effective under mild DR conditions with an increase of 28% whereas the medium DR conditions resulted in a moderate increase of 18%. In contrast, the severe DR groups showed a reduction of the mean life span by 16% compared to the control. These findings are consistent with previous studies in which exposure of worms to moderate DR had a beneficial impact on lifespan while severe DR resulted in detrimental effects on lifespan (Greer et al., 2007; Greer and Brunet, 2009; Mair and Dillin, 2008; Piper and Partridge, 2007). The median life span of the control group reported here for *C. elegans* (*unc-54::GFP*) agrees with previous studies that used wildtype (N2) worms (Bolanowski et al., 1981; Croll et al., 1977; De Cuyper and Vanfleteran, 1982; Hosono et al., 1980), validating using the *unc54::GFP* strain.

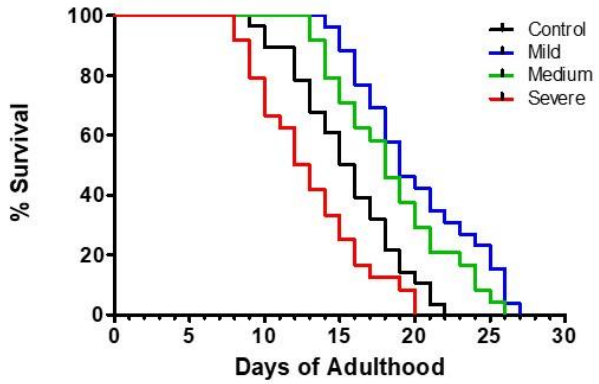


Figure 1: Effect of different DR regimes on *C. elegans* lifespan. Lifespan assays were done at 20°C. Survival plots were drawn by Kaplan-Meier survival assay followed by Log-rank analysis for statistical significance. The figure shows the result of one experiment and is representative of two independently completed experiments.

Table 1. Effect of different DR regimes on the mean lifespan of *C. elegans*. Shown are the mean and maximum lifespan and the % change compared with control. Negative values indicate a significant decrease in mean lifespan. Each diet group was compared to control by the log rank test to determine statistical significance.

Diet	Mean lifespan (days)	% Increase in Mean Lifespan	Maximum lifespan (days)	% Increase in Maximum Lifespan
Control	15.5±0.7		22	
Mild	19.9±0.8[1]	28.4%	27	22.7%
Medium	18.3±0.8[2]	18.1%	26	18.2%
Severe	13±0.8[3]	-16.1%	20	-9.1%

[1]Statistically different compared to Control, $p < 0.001$

[2]Statistically different compared to Control, $p = 0.011$

[3] Statistically different compared to Control, $p = 0.026$

Mild and medium DR increase health span of *C. elegans*

Individuals in each DR group were allocated to one of three distinct motility classes (I, II and III) and health span in *C. elegans* defined as the period where ≥50% of the worm population is classified as motility class I (J. H. Hahm et al., 2015). The results of the motility assay are shown in Figure 2 and Figure S2 and summarised in Table 2. In line with previous findings (Herndon et al. 2002; and Greer et al., 2007) differences in onset and progression of motility decline was observed across all groups. During the early stages of adulthood (day 1-3), 100% of the worm population exhibited spontaneous smooth movement (Class I) (Figure S2A), but the differences became more pronounced as the worms aged, as can be seen for instance on day 6, 10 and 14 (Figure 2).

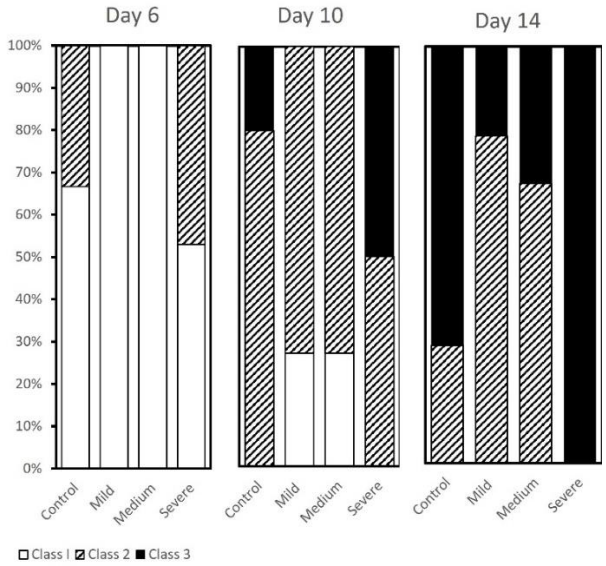


Figure 2: Effect of dietary restriction on *C. elegans* motility on day 6, 10 and 14. Classification of movement according to Herndon et al., 2002. (A) Class I – Continuous smooth movement, fast movement when stimulated. (B) Class II – Slow halting movement, smooth movement when stimulated. (C) Class III – Small movement of head or tail, very slow movement when stimulated.

Table 2. Summary of age-related changes in motility. Average number of days for 50% of initial population to move from motility class 1 to class 2 (parameter C_{1-2}) or from class 2 to class 3 (parameter C_{2-3}, based on maximum % in class 2). The difference between the two mid-points, represented by parameter ΔC_2, is the average time spent in class 2. Averages are based on data from two independent experiments and statistical significance was determined using One-way ANOVA ($p<0.05$).

	C_{1-2} (days)	C_{2-3} (days)	ΔC_2 (days)	Mean life span(days)
Control	7.3 ±0.4	11.7 ±0.7	4.4±1.1	15.5±0.7
Mild	10.0 ±1.4[1]	15.4 ±0.1[1]	5.4±1.4	19.9±0.8
Medium	9.3 ±0.8[1]	15.2 ±0.9[1]	5.9±0.1	18.3±0.8
Severe	6.9 ±0.5	10.7 ±0.7	3.9±0.3	13±0.8

[1]($p<0.05$).

Non-linear regression analysis of motility data (Figure S2) was used to compare the average number of days for 50% of the initial population to move from motility class 1 to class 2 (Table 2, parameter $C_{1\text{-}2}$). This revealed that worms under mild and medium DR spend significantly longer in class I ($C_{1\text{-}2}$ = 10.9 and 9.3 days respectively) compared to control and severe DR ($C_{1\text{-}2}$ = 7.3 and 6.9 days respectively). Similar analysis showed that transition from class II to class III was also significantly delayed for worms under mild and medium DR ($C_{2\text{-}3}$ = 15.4 and 15.2 days respectively) compared to control and severe DR (Table 2, parameter $C_{2\text{-}3}$ = 11.7 and 10.7 days). The average time spend in class II increased as well for the mild and medium DR groups (ΔC_2 = 5.4 and 5.9 days respectively) compared to the control and severe DR populations (ΔC_2 = 4.4 and 3.9 days) although this increase was statistically not significant (compared to control).

Health span in *C. elegans* is defined as the period where ≥50% of the worm population is classified as motility class I, while gerospan is defined as the period when ≥50% of the worm population has lost its class I motility (Bansal *et al.,* 2015). Using the motility data (parameter $C_{1\text{-}2}$, Table 2), the health span period for control and severe DR was just under seven days, whereas for mild and medium DR the healthspan was extended to 8.5 days (Figure 3A). Altogether, these findings show that mild and medium DR-treated worms retained movement capability longer and thus had a longer health span compared to control and severe DR, although the resulting normalized healthspan to gerospan ratios were similar among the various DR groups (Figure 3B). Thus, although the healthspan period is extended for mild/medium DR, for these groups the gerospan period was prolonged as well. This suggests that mild/medium DR not induce prolonged healthspan overall, but rather delay the onset of gerospan.

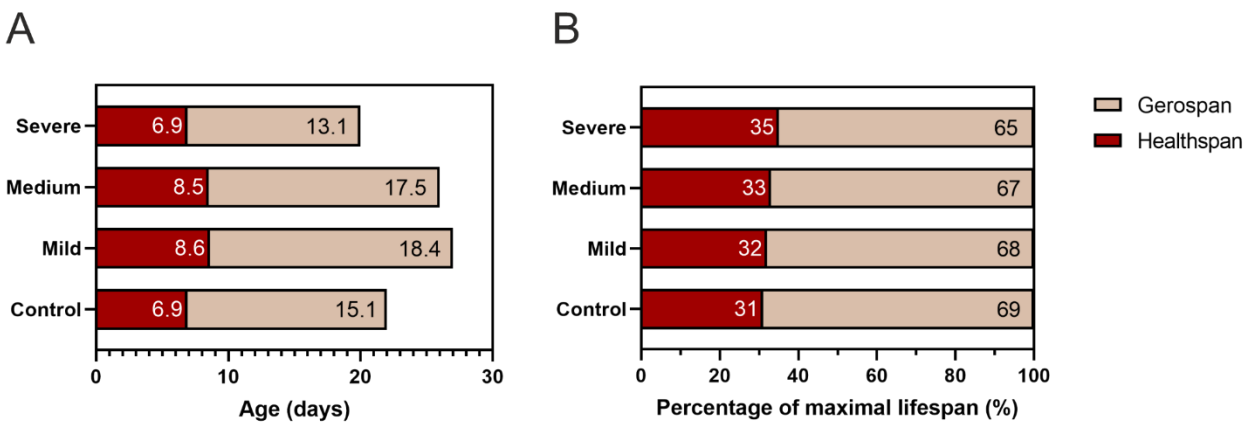


Figure 3: Comparison of healthspan and gerospan in control and DR groups. A) Healthspan was defined as the period when > 50% of the initial nematode population show type-I motility. Gerospan was defined as the period when < 50% of the initial nematode population show < 50% of type-I motility. B) The ratio of healthspan to gerospan was normalized to their maximal lifespan in control and DR worms (J. H. Hahm et al., 2015).

Age-dependent decline in myosin levels is delayed under mild and medium DR conditions.

To quantify the loss of muscle mass under various DR conditions, we used fluorescence microscopy to visualize the body wall muscles of UNC54-GFP transgenic worms over the entire lifespan of *C. elegans.* The calibration curve of the GFP-standards confirmed linearity (R^2 = 0.999) with a limit of detection (LOD) = 4028 (0.17%) and a limit of quantitation (LOQ) = 4621 (0.51%) as shown in Figure S3. Figure 4A shows representative fluorescence images for all four DR groups at different time points (day 4, 10, and 14), illustrating fluorescence intensity increased and decreased in an age-related manner (for a full data set of fluorescence intensity over lifespan see Figure S4). Boxplots of fluorescence intensity start to show significant differences in fluorescence for mild DR and medium DR groups compared to control from day 10 onwards (Figure 4B). The fluorescence data in this study do not address heterogeneity in the fluorescence of individual worms. as the individuals used for fluorescence measurements were randomly

selected, based on age and diet, and their motility was not considered. Therefore, at certain timepoints, one could have individuals from different motility classes and hence a wider data spread in fluorescence intensity (Figure 4B).

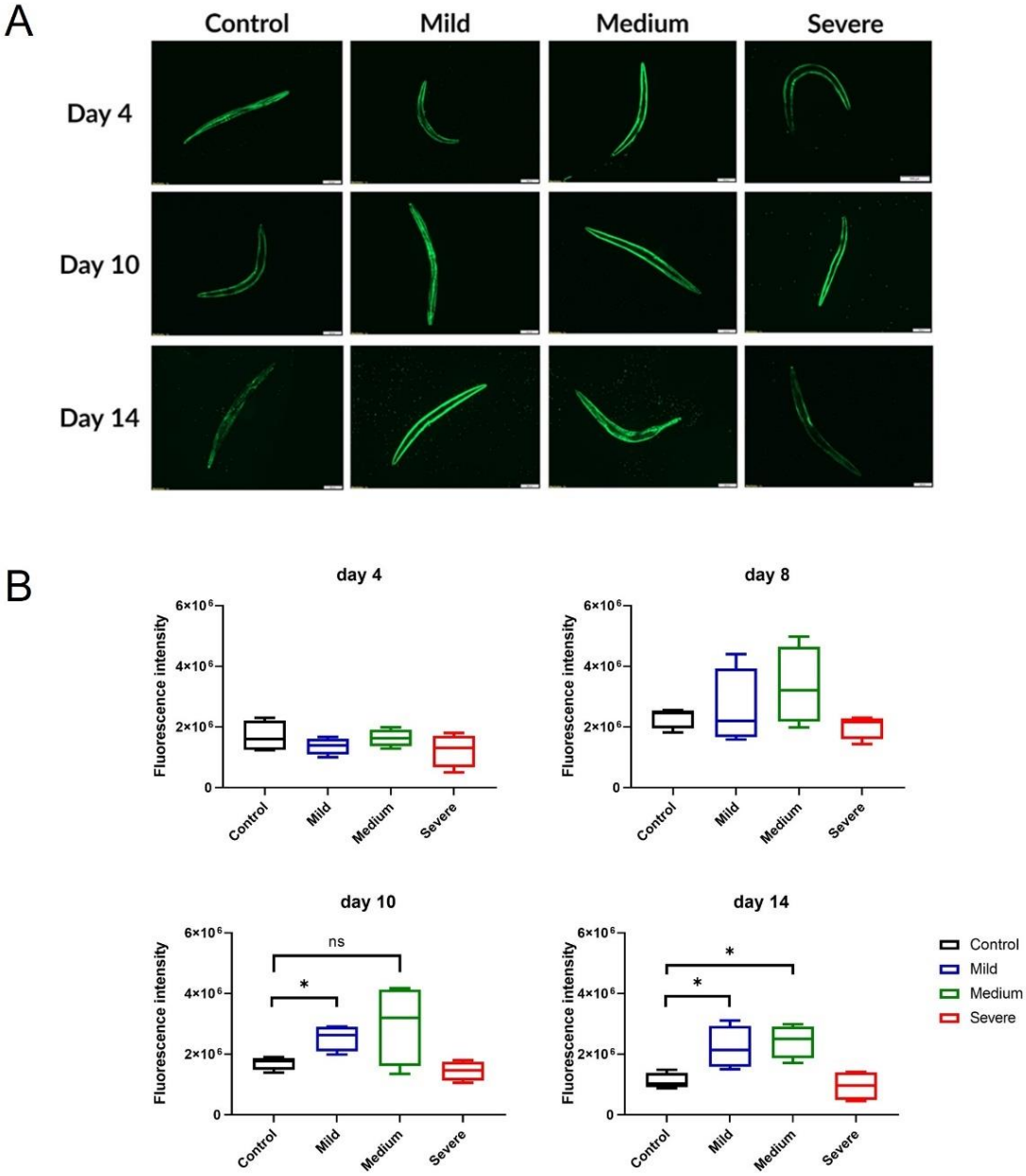


Figure 4: Visualisation of muscle density over C. elegans lifespan using fluorescently labelled myosin (UNC54-GFP). (A) Representative fluorescence microscopy images of C. elegans grown under various DR conditions (mild, medium, severe) compared with control (no restriction) at day 4, 10, and 14. (B) Myosin UNC-54 density of body-wall muscle at different time points for various DR conditions (mild, medium, severe) compared to control (no restriction). * Indicates significant differences between the control and DR groups (*P < 0.05) using 2-way ANOVA.

The increase in fluorescence seen during the first eight days of adulthood is indicative of the rate of muscle growth. The medium DR group on average shows the largest increase in myosin density, followed by mild DR and control, whereas severe DR consistently showed the lowest myosin density increase although statistically not significantly different (Figure 5A). A decrease in fluorescence is seen after day 8 (Figure 5B) with the amount of GFP-labelled myosin (UNC54-GFP) reducing for all groups. Single exponential fits of the average fluorescence intensity show that mild and medium DR groups lost 50% of their maximum UNC54-GFP myosin at 15.3 days and 14.8 days respectively ($t_{1/2}$ in Table 3), compared to control and severe DR groups which lost 50% of peak UNC54-GFP myosin at 12 days and 13.1 days, respectively. The rate at which UNC54-GFP levels decrease, represented by the rate of fluorescence loss (k_F in Table 3), is slower for mild and medium DR compared to control and severe DR, suggesting that the onset and rate of sarcopenia is delayed in worms under mild and medium DR, compared to the control and severe DR groups. No significant differences in average body length and width were found for the mild DR and medium DR groups, compared to control, whereas the severe DR group did show lower average body length and width compared to control (Figure 6).

Table 3. Summary of age-related changes in fluorescence and expression of myosin UNC54-GFP.

	Fluorescence increase[1] (10^5 day^{-1})	Fluorescence loss[2] k_F (day^{-1})	$t_{1/2}$[3] (days)	$T_{0.5}$ UNC54-GFP[4] (day)
Control	3.4±0.6	0.14±0.03	5.0	13.0
Mild	3.5±0.3	0.052±0.005	13.4	21.4
Medium	4.9±0.3	0.066±0.005	10.6	18.6
Severe	2.6±0.4	0.14±0.03	5.0	13.0

[1]Measured from day 1- 8.
[2]Determined from day 8 (maximum fluorescence) until day 24.
[3]Determined using ln (2)/k_F
[4] Age at which 50% of maximum UNC-54-GFP myosin is lost.

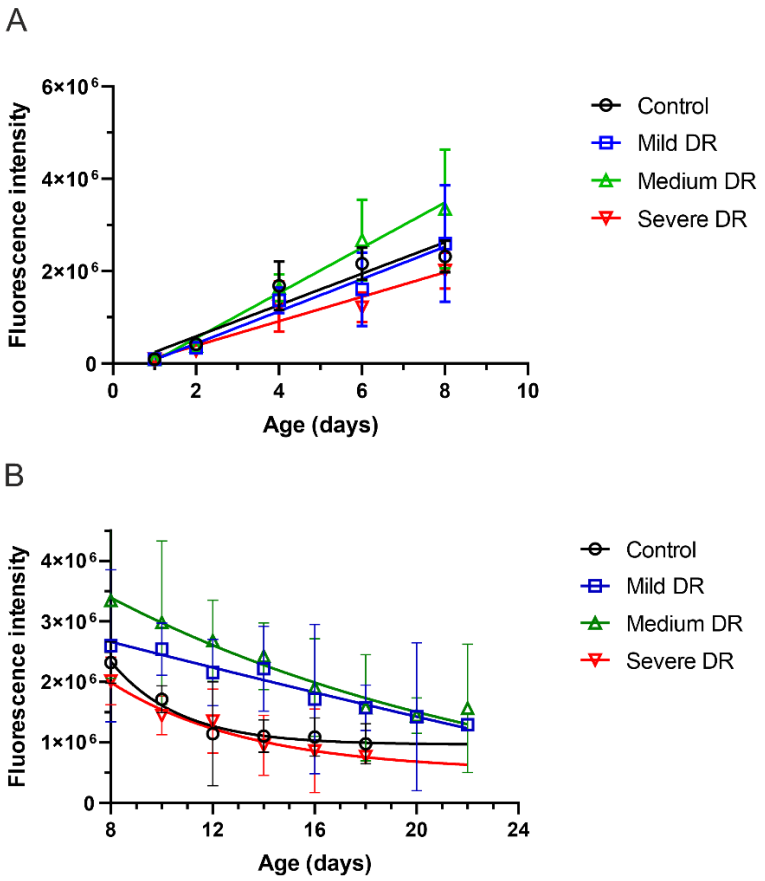


Figure 5: Expression of myosin UNC54-GFP as a function of age. Average fluorescence intensity with standard deviation is shown (n=5). (A) Average fluorescence increases during day 1-8 for the various DR groups. Linear regression resulted in values for mild and medium DR with slope = $3.5\pm0.3\text{x}10^5$ and $4.9\pm0.3\text{x}10^5$ respectively (corresponding R^2-values of 0.97 and 0.99). Control and severe DR groups yielded slopes of $3.4\pm0.6\text{x}10^5$ and $2.6\pm0.4\text{x}10^5$ respectively (R^2-values of 0.91 and 0.93). (B) Average fluorescence decreases from day 8 towards end of lifespan for control, mild DR, medium DR and severe DR. Single exponential fits were used to calculate $t_{1/2}$ values of 13.0 days (control), 18.4 days (mild), 15.6 days (medium) and 12.9 days (severe). a rate of fluorescence loss k_F of 0.14 $days^{-1}$ (control), 0.052 $days^{-1}$ (mild), 0.066 $days^{-1}$ (medium) and 0.14 $days^{-1}$ (severe). (R^2-values = 0.86 (control), 0.95 (mild), 0.97 (medium) and 0.89 (severe).

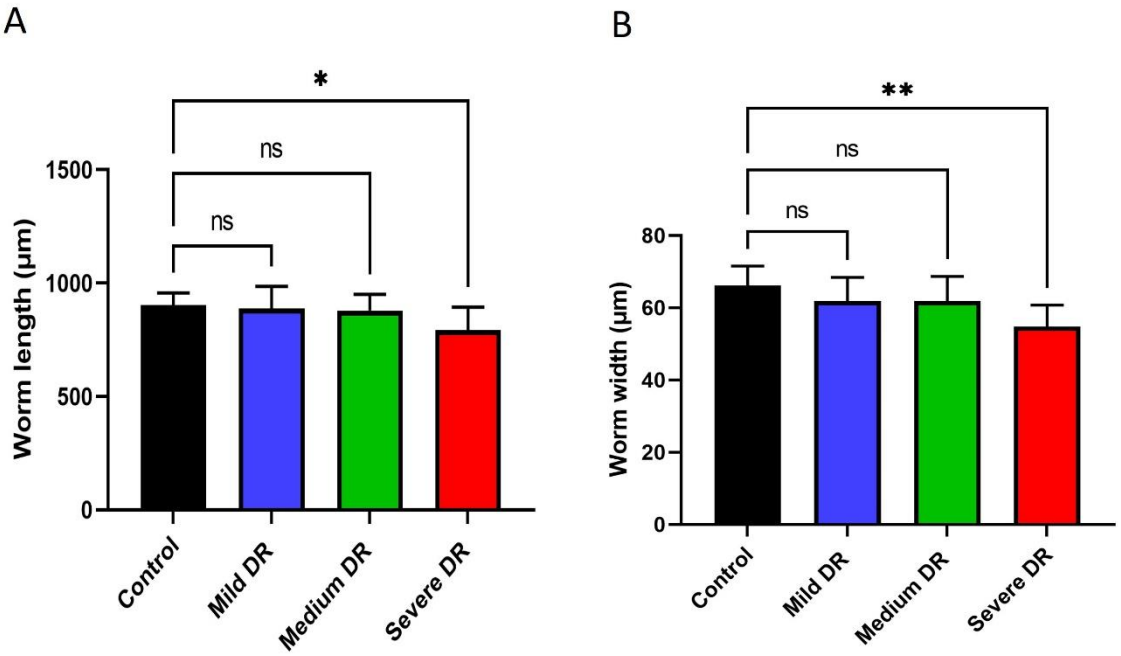


Fig. 6. Body length and body width of C. elegans (*unc-54::gfp*) exposed to various dietary treatments. (A) Average body length of *unc-54::gfp* worms exposed to various DR regimes. (B) Average body width of *unc-54::gfp* worms exposed to various DR regimes. Statistical analysis was one-way ANOVA, followed by Dunnett's post hoc analysis (N=27 control, N= 33 mild DR, N=33 medium DR, N=28 severe DR). (* $p< 0.05$; ** $p< 0.005$).

Discussion

In order to quantify sarcopenia rates, this work used the sDR method and a transgenic *C. elegans* strain (*unc-54::GFP)*, to determine myosin density in *C. elegans* body wall muscle during its complete life span. Based on the average rate of fluorescence decrease (k_F), loss of UNC54 myosin is about two-fold slower for the mild and medium sDR groups compared to control and severe sDR. The timepoint by which the average fluorescence intensity is 50% of the maximum fluorescence ($t_{1/2}$ value) and the corresponding rate of fluorescence loss (k_F) correlate well with the time point in the motility assay when moving from class I to class II (C_{1-2}), which agrees with previous studies that reported a good

correlation between maximum velocity of *C. elegans* and health span (J.-H. Hahm et al., 2015). Thus, the average rate of UNC-54 myosin loss, representing sarcopenia rates and reported here for the first time, is a good predictor of health span ($C_{1\text{-}2}$) in *C. elegans*, but not of lifespan (Figure 7A/B). The timepoint in the motility assay when moving from class II to class III ($C_{2\text{-}3}$) is a better predictor of lifespan (Figure 7C).

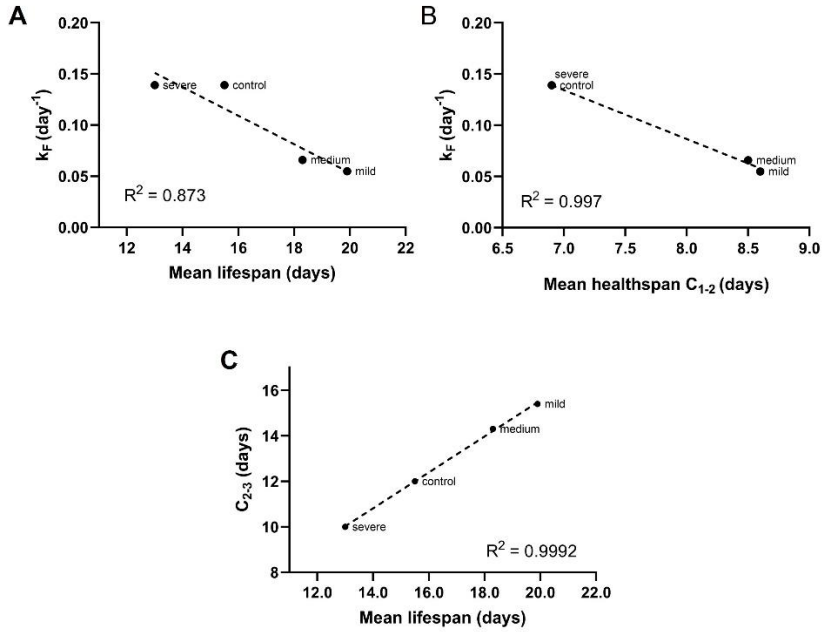


Figure 7: Rate of myosin loss (k_F) correlates with healthspan. (A) Correlation of rate of myosin loss (k_F) with mean lifespan (B) Correlation of rate of myosin loss (k_F) with mean healthspan, from motility parameter $C_{1\text{-}2}$ (C) Correlation of motility parameter $C_{2\text{-}3}$ and mean lifespan.

Protein levels vary over time due to changes in protein synthesis, protein degradation or both (Ross et al., 2021). The change in protein abundance over time (d[P]/dt) is defined by rate of protein synthesis (k_{syn}) and protein degradation (k_{deg}) according to:

$$d[P]/dt = k_{syn} - k_{deg}[P]$$

The overall increase in UNC-54 abundance, seen during the first week of adulthood for all groups, is attributed to UNC-54 synthesis (k_{syn}). The rate of protein degradation (k_{deg}) is

presumed zero during the first week of adulthood as UNC-54 protein has been reported to be extremely stable with very slow turnover (Dhondt et al., 2016). The overall fluorescence decrease, seen during the second week of adulthood (Figure 5B), represents overall UNC-54 degradation (first-order process) which follows an exponential decay with degradation rate constant k_{deg}. Reduced myosin UNC-54-GFP degradation rates are found for the mild-DR and medium DR treatment groups compared to control -DR. As there is no significant difference in body length/width between the mild-DR and medium-DR groups, compared to control-DR, this excludes the possibility that changes in myosin UNC-54-GFP levels are caused by the worms being different in size. Protein turnover studies in *C. elegans*, using pulse-labelling methods, reported minimal turnover of proteins of the muscle contractile apparatus. For myosin isoform UNC-54, turnover rates with an average half-life of 298 hr (=12.4 days) have been reported (Dhondt et al., 2016), which is very similar to the $t_{1/2}$ value found here for UNC-54-GFP using fluorescence (control $t_{1/2}$ = 13.0 days). Considering the extremely slow turnover of muscle proteins (Dhondt et al., 2016), the $t_{1/2}$ values measured here for UNC-54-GFP abundance, represent UNC-54 protein turnover. Therefore, we predict, based on the $t_{1/2}$ values measured here, that exposure to mild and medium DR results in reduced UNC-54 degradation compared to control.

Our results align with previous studies that report a decrease in myosin UNC-54 levels in ageing *C. elegans*. Under ad libitum conditions, unc-54 mRNA expression was found to be downregulated for *C. elegans* (Matheny et al., 2022); (Mergoud dit Lamarche et al., 2018) (Adamla and Ignatova, 2015) with an overall decline in UNC-54 protein levels seen from day 8 of adulthood (Matheny et al., 2022). Under DR conditions there is conflicting data, as some studies report upregulation of unc-54 mRNA (Rollins et al., 2019) whereas another study reports unc-54 mRNA levels unchanged as a result of DR (Depuydt et al., 2013). Changes in mRNA levels are not always a good predictor for changes in protein abundance (Kamkina et al., 2016) and therefore direct measurements of UNC-54 protein

levels could be more informative. It was reported previously that muscle preservation in *C. elegans* exposed to DR, is not due to increased mRNA expression, and therefore other mechanisms, like selective inhibition of muscle protein degradation, must be involved in maintaining increased UNC-54 levels under DR conditions compared to control (Depuydt et al., 2013). Our results align with those findings, although with the caveat that the DR methods between the two studies differ (solid DR versus liquid DR). As different DR methods can induce different signaling pathways (Greer and Brunet, 2009) future studies using liquid DR, are needed to address this in more detail.

Using this UNC-54-GFP method allows quantification of subtle changes in sarcopenia rates in *C. elegans* which can be used as a starting point to develop a screening method to determine sarcopenia rates in response to various interventions. Although the results here demonstrate the potential of this GFP-based technique in *C. elegans*, further studies are needed to optimize the methodology, considering the heterogeneity in the individual worms, and address the autofluorescence, caused by lipofuscin that accumulates in the intestine with age (Pincus et al., 2016). Although the present study was able to reduce the autofluorescence effect by selecting the body wall muscle region, and not the intestine, a triple band GFP filter is needed to validate this in more detail (Teuscher & Ewald, 2018).

Numerous studies have demonstrated that, in *C. elegans*, the life-extending effect of DR is controlled by a number of overlapping and independent signaling pathways (Chamoli et al., 2014; Cypser et al., 2013; Greer and Brunet, 2009; Walker et al., 2005). In addition, many studies have shown a decrease in skeletal muscle metabolism with aging, mostly due to a decrease in mitochondria activity (Figueiredo et al., 2009; Pugh et al., 2013; Short et al., 2005; Yamada et al., 2013). Conversely, there is growing evidence indicating that metabolic reprogramming that occurs in response to DR, may underlie the beneficial effects of DR (Anderson and Weindruch, 2010; Barger et al., 2015; Feng et al., 2016). Given this plethora of signalling pathways, it is likely that the DR employed in this study

could act on several distinct pathways to extend lifespan and motility in *C. elegans*. Moreover, the considerable difference in lifespan and motility between the different DR regimens, supports the idea that DR affects lifespan and motility through distinct pathways. More recently, Rhoads et al., demonstrated that the preservation of muscle mass and physical function, frequently seen with dietary-restricted rhesus monkeys are linked with metabolic pathways (Rhoads et al., 2020).

In conclusion, to quantify sarcopenia rates in *C. elegans* we have measured myosin UNC-54-GFP density over its entire lifespan, using an internal GFP-standard, As UNC-54 proteins demonstrate extreme slow turnover, this method allowed us to determine UNC-54 synthesis and degradation rates at various DR treatments. Our results showed reduced UNC-54 degradation rates for moderate DR compared to control, which correlated well with improved motility parameters and lifespan. This suggests that moderate DR allow *C. elegans* to maintain UNC-54 levels and protein homeostasis for longer periods and thereby delaying the onset of sarcopenia.

Competing interest

The authors declare no competing interests.

Author contributions

MB and SH provided study design. All authors acquired, analysed, or interpreted data. ST and MB drafted the manuscript with the help of all co-authors. MB supervised the study.

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Supplementary Material:

Table S1: Effect of different DR regimes on the mean lifespan of *C. elegans*. The table shows mean lifespan and % increase in mean lifespan for each DR group as determined from two independent experiments. Negative values indicate a significant decrease in mean lifespan. Statistical significance in mean lifespan compared to controls was calculated using the Log-rank test (Graphpad Prism 9). In bold, series shown in the survival plot of Figure 1.

	Replicate 1*			Replicate 2*				
Diet	Mean lifespan (days)	% Increase in Mean Lifespan	DR vs Control (p-value)	Mean lifespan (days)	% Increase in Mean Lifespan	DR vs Control (p-value)	Mean Lifespan[1]	% Increase in Mean Lifespan[2]
Control	12.3			**15.5**			13.9±1.6	
Mild	17.3	40.7	0.0052	**18.2**	17.4	<0.0001	17.8±0.5	29.1
Medium	16.7	35.9	0.0365	**17.6**	13.6	0.0081	17.2±0.5	24.8
Severe	11.5	-6.8	0.0249	**12.9**	-16.8	0.0261	12.2±0.7	-11.8

*Each replicate used 240 worms, with 60 worms per diet group (4x15 individuals).

Table S2: Summary of age-related changes in motility under DR from two independent experiments. The table shows the average number of days for 50% of the initial population to move from motility class I to class II, represented by $C_{1\text{-}2,}$ and average number of days for 50% of the class II population (based on maximum % in class II) to move to class III (represented by $C_{2\text{-}3}$). The difference between the two mid-points ($C_{1\text{-}2}$ and $C_{2\text{-}3}$) was used to calculate the average time spent in class 2, represented by parameter $\Delta C_{2.}$ Statistical significance compared to control was calculated using the One-way ANOVA, Graphpad Prism 9.

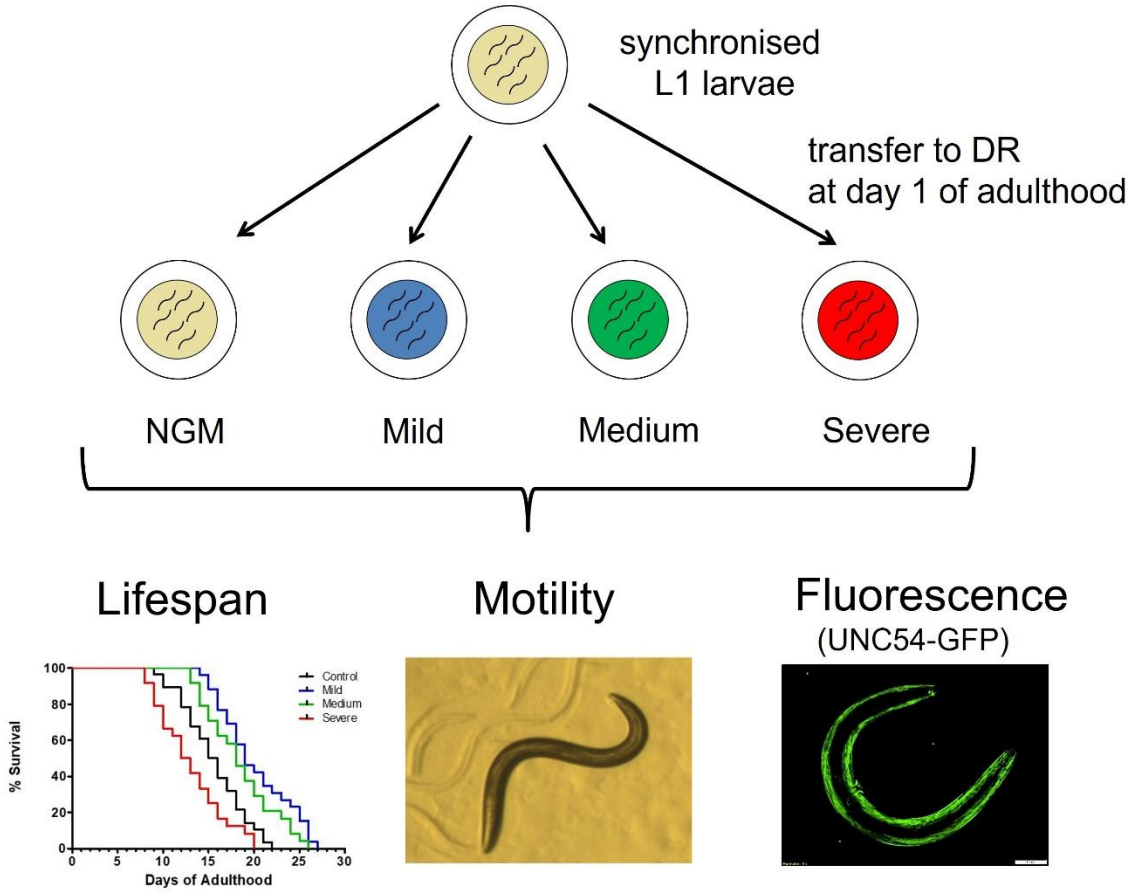
	Replicate 1*			Replicate 2*		
Diet	$C_{1\text{-}2}$ (days)	$C_{2\text{-}3}$ (days)	ΔC_2 (days)	$C_{1\text{-}2}$ (days)	$C_{2\text{-}3}$ (days)	ΔC_2 (days)
Control	7.7	11.0	3.3	6.9	12.4	5.5
Mild	11.3	15.3	4.0	8.6	15.4	6.8
Medium	10.1	16.0	5.9	8.5	14.3	5.8
Severe	7.3	11.4	4.1	6.4	10.0	3.6
Averages						
Diet	$C_{1\text{-}2}$ (days)[1]	DR vs Control (p-value)	$C_{2\text{-}3}$ (days)[2]	DR vs Control (p-value)	ΔC_2 (days)[3]	DR vs Control (p-value)
Control	**7.3±0.4**		**11.7±0.7**		**4.4±1.1**	
Mild	**10.0±1.4**	**0.023**	**15.4±0.1**	**0.030**	**5.4±1.4**	**0.453**
Medium	**9.3±0.8**	**0.048**	**15.2±0.9**	**0.034**	**5.9±0.1**	**0.301**
Severe	**6.9±0.5**	**0.520**	**10.7±0.7**	**0.416**	**3.9±0.3**	**0.668**

*Each replicate used 240 worms, with 60 worms per diet group (4x15 individuals).

[1]Average motility parameter $C_{1\text{-}2}$ was calculated from individual $C_{1\text{-}2}$ values.

[2]Average motility parameter $C_{2\text{-}3}$ was calculated from individual $C_{2\text{-}3}$ values.

[3]Average time spent in class 2: average $C_{2\text{-}3}$ – average $C_{1\text{-}2}$



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688 **Figure S1: Schematic representation of the DR assays using C. elegans**

689 **expressing GFP-tagged unc-54 (*unc54::GFP*).**

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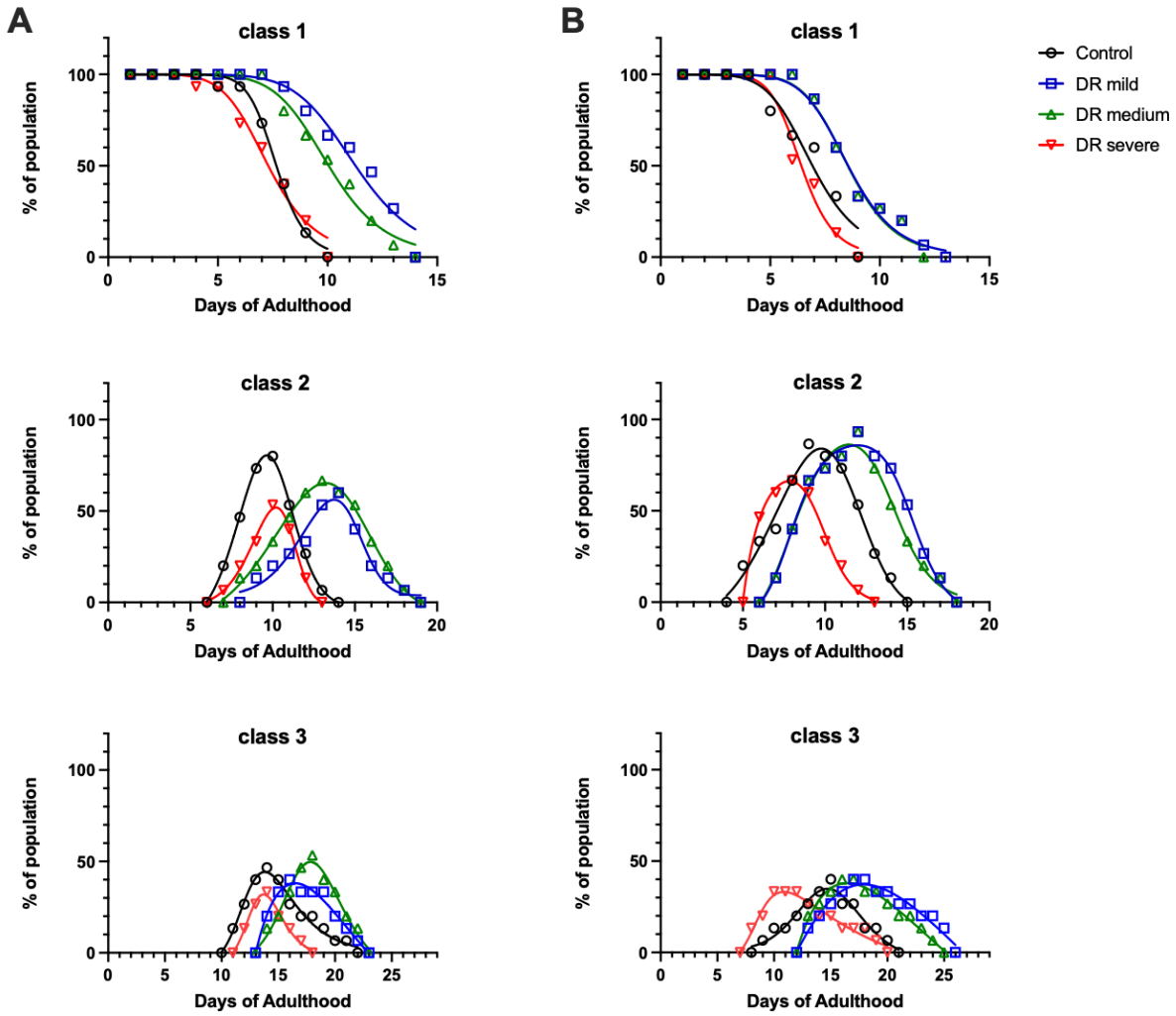

Figure S2. Age-related changes in motility of DR worms. Classification of movement according to Herndon et al., 2002. (A) Class I – Continuous smooth movement, fast movement when stimulated. (B) Class II – Slow halting movement, smooth movement when stimulated. (C) Class III – Small movement of head or tail, very slow movement when stimulated. Data is normalised with maximum number of worms at the start of the assay set at 100%. (A) and (B) are two independent experiments.

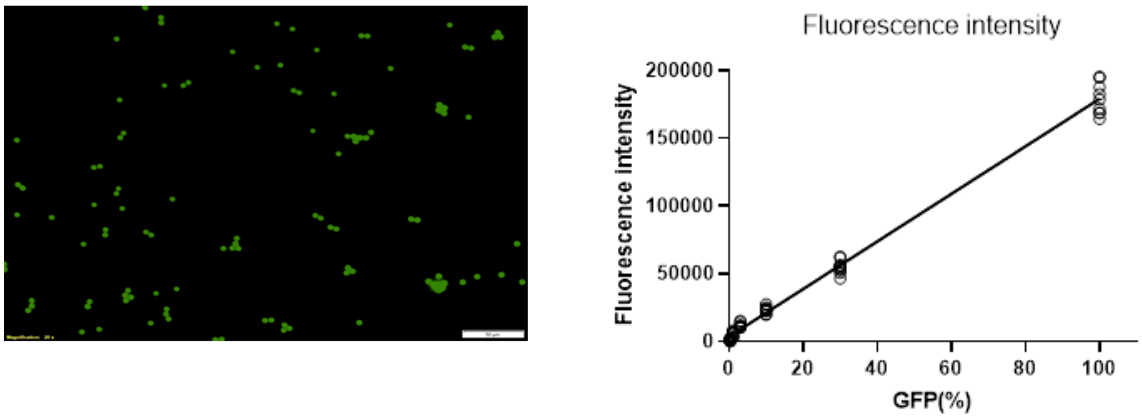

Figure S3: GFP-labelled microbeads were used as an internal fluorescence intensity standard. A) Left panel: Fluorescence microscope image of GFP-labelled microbeads (3%) in M9 buffer. B) Right panel: Calibration curve of microsphere concentration versus average fluorescence intensity shows a good correlation between the different relative microsphere concentrations (R2 = 0.9986). All replicates are shown here, indicating the data spread.

GFP-calibration curve detailed analysis:

GFP-beads and Image J analysis gave following intensities:

GFP (%)	1	2	3	4	5	6	7	8	9	10
0.3	1624	1624	439	346	405	2352	1033	829	1975	351
1	5580	6263	7034	8064	6932	6139	2757	3103	3273	3037
3	12290	11310	10846	10825	10212	10792	10748	10379	14377	15442
10	19886	19775	22181	24049	23431	25231	27520	22920	23793	23602
30	62655	56385	62166	55692	52422	55846	53999	46417	50542	54140
100	171918	187816	194615	178633	195478	168711	164197	182644	168640	

After background correction, non-linear regression using Prism gives the following:
Best fit model: Y = Y(intercept) + slope*X
Equation found by Prism: Y = 3736 + 1753*X
Standard error of slope: 28.01
Standard error of intercept: 1200
R^2 = 0.999 (confirms linearity)
From standard error to standard deviation: SD = SE * √(n) (n=10);
SD slope = 28.01 * √10 = 88.58
SD intercept = 1200 * √10 = 3794
Limit of detection (LOD) defined as:
Y_{LOD} = 3.3* SD (slope) + intercept = (3.3 * 88.58) + 3736 = **4028**
Corresponding concentration LOD (C_{LOD}):
C_{LOD} = (Y_{LOD} – intercept) / slope = (4028 – 3736)/ 1753 = **0.17 %**
Limit of quantitation (LOQ):
Y_{LOQ} = 10* SD (slope) + intercept = (10* 88.58) +3736 = **4621**

Corresponding concentration LOQ (CLOQ):
CLOQ = (Y_{LOQ} – intercept) / slope = (4621 – 3736)/1753 = **0.51%**

Measured fluorescence intensities < 4621 cannot be accurately quantified and relative GFP concentrations < 0.5% can also not be determined accurately as these are below the LOQ.

To reduce the effect of autofluorescence, known to increase as C. elegans ages, only the body wall muscle area of *C. elegans* was selected for fluorescence analysis, and not the whole worm body.

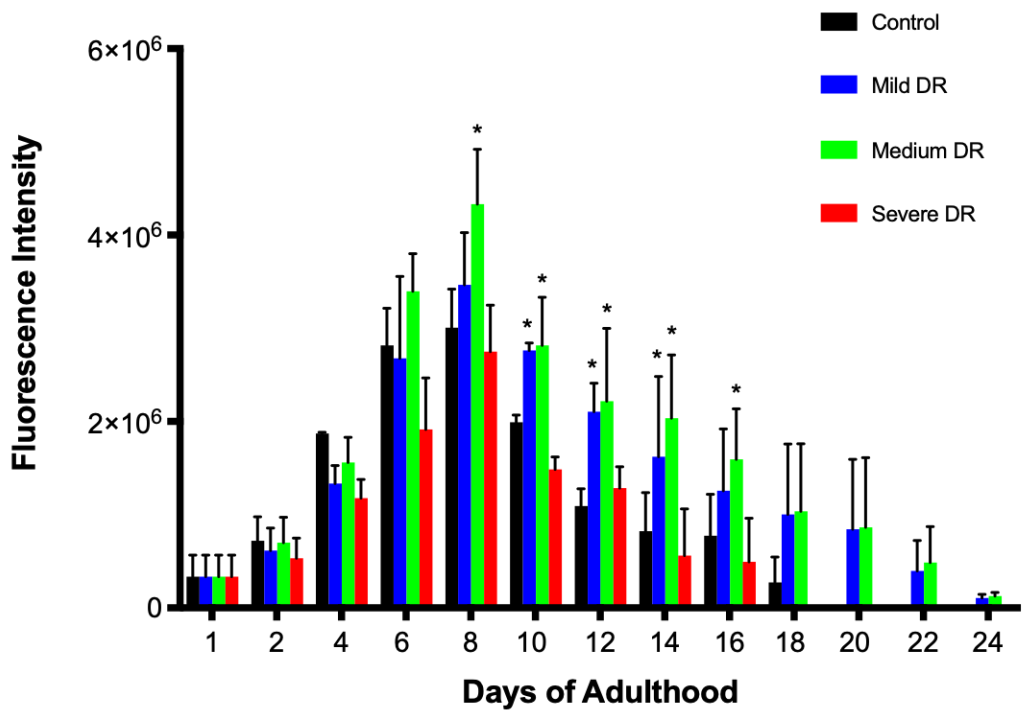

Figure S4: Average fluorescence intensity as a function of age under control and various DR conditions, expressing UNC54: GFP. Fluorescence intensity represents myosin density of body-wall muscle at different time points for various DR conditions (mild, medium, severe) compared to control (no restriction). (Two-way ANOVA, *P < 0.05). Shown are averages from two independent experiments.